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Purification and characterization of a novel trimeric and thermotolerant laccase produced from the ascomycete *Scytalidium thermophilum* strain

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ABSTRACT

A novel laccase from the thermophilic ascomycete fungus *Scytalidium thermophilum* strain was purified and their biochemical properties were determined. The laccase was purified 8-fold with a specific activity of 139.4 U/mg. This laccase was found to be a homotrimeric protein (subunit molecular-weight of about 28 kDa) with 82 kDa as a total molecular-weight. Its optimum activity pH varied and was substrate dependent. Indeed, it was 5.0 for ABTS and DMP and 6.0 for guaiacol and hydroquinone. Its optimum temperature was 80 °C using DMP as substrate. The enzyme retained 50% of its activity after 120 min of incubation at 70 °C. Under standard assay conditions, laccase K_m values were 0.36 mM and 0.26 mM towards 2,6-DMP and ABTS, respectively. It has shown a degrading activity towards a variety of phenolic compounds. The laccase was inhibited by NaN_3, DTT, SDS and *p*-coumarate but not by EDTA, L-Cys, NaF and NaBr. Furthermore, this laccase was stable in the presence of some metal ions.

1. Introduction

Although oxidation reactions are essential in several industries, most of the conventional oxidation technologies have the following drawbacks: non-specific or undesirable side-reactions and use of environmentally hazardous chemicals. This has impelled the search for new oxidation technologies based on biological systems such as enzymatic oxidation. These systems show the following advantages over chemical oxidation: enzymes are specific and biodegradable catalysts and enzymatic reactions are carried out under mild conditions [1].

Enzymatic oxidation techniques have potential within a great variety of industrial fields including the pulp and paper [2], textile [3–5] and food industries [6]. Enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones. Thus, laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) is a particularly promising enzyme for the above-mentioned purposes [7]. The laccase molecule is a monomeric, dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites [7]. They are widespread in nature, being produced by a wide variety of plants, fungi and also bacteria [8]. The functions of the enzyme differ from one organism to another and typify the diversity of laccases in nature [9,10]. This enzyme catalyses the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions coupled to the reduction of molecular dioxygen to water [11]. The catalytic ability of laccases has, not surprisingly, led to diverse biotechnological applications of this enzyme [11,12].

Laccases have received much attention from researchers during the last decades due to their ability to oxidize both phenolic and nonphenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for the application within several biotechnological processes [1]. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, use as a tool for medical diagnostics and as a bioremediation agent to clean up soils contaminated with herbicides, pesticides and certain explosives [1]. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics [1]. In addition, they are a useful tool for bioremediation purposes owing to their capacity to remove xenobiotic substances and produce polymeric products.

In this paper, a fungal strain belonging to the ascomycete phylum and identified as a *Scytalidium thermophilum* strain was studied with respect to its laccase production. *S. thermophilum* has been discovered previously by means of a developed screening (unpublished data). We isolated and characterized the laccase produced by this strain, the single ligninolytic enzyme secreted by this fungus in the studied conditions. The aim of this work was to contribute to a better understanding of the *S. thermophilum* biochemical

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properties. The high levels of the atypical laccase secreted by the *S. thermophilum* strain as well as its stability suggest that it could be a useful tool for environmental and biotechnological applications.

2. Materials and methods

2.1. Fungus and culture conditions

S. thermophilum strain (accession number: FJ560721) was isolated from locally prepared compost in the north of Tunisia. Strain **ST26** was identified using the universal ITS1 and ITS4 primers according to [13]. *S. thermophilum* produces laccase and protease activities under optimized conditions (unpublished data). The isolate was maintained on 2% (w/v) malt extract agar at 4 °C. The fungal inoculum was cultivated on the same media at 45 °C. For laccase production and induction studies, the M7-medium was used as described by [14]. The pH of the solution was adjusted to 5.5. The mycelia were homogenized using a Waring blender for 5 min at 20,000 rpm. The homogenized culture was used to inoculate 250 ml Erlenmeyer flasks containing 100 ml of M7-medium. The cultures were incubated at 45 °C in a rotary shaker at 200 rpm and samples were taken periodically.

2.2. Improvement of laccase production

In order to improve laccase production by *S. thermophilum* several inducers such as copper, ethanol, veratrate, vanillate, ferulic acid, coumaric acid, veratrylic alcohol, caffeic acid, 3,4-dihydroxybenzoic acid and vanillin were examined to determine their effects on extracellular laccase production. All inducers were used at 200 μM as a final concentration. Copper which yielded the highest laccase production was tested at increasing concentrations from 0 to 1000 μM. The test of carbon source was carried out with monomeric (sorbose, inositol, succinic acid, D-xylose, mannitol, glucose), dimeric (lactose, cellobiose, saccharose) and polymeric (cellulose, starch, pectin, gruel, malt extract) carbon sources added to the culture medium at 10 g/l in order to study its inductive effect on laccase production. The production of laccase was followed during 7 days in the presence of 500 μM of copper (optimum copper concentration for laccase production) to induce laccase activity. Glucose which yielded the highest laccase production was tested at increasing concentrations from 5 to 30 g/l. To determine the nitrogen source which could yield a better laccase production, various substrates were also tested such as soya, meat and casein peptone, meat and yeast extract, plant, wheat, and gelatine soya at a concentration of 5 g/l. Soya peptone which yielded the highest laccase production was tested at increasing concentrations from 1 to 12.5 g/l. The cultures were incubated at 45 °C and 200 rpm in rotary shaker. In addition, the effect of temperature was studied by incubating cultures at 25, 30, 37, 42, 45, 50 and 55 °C.

2.3. Laccase purification

S. thermophilum laccase was purified from 5 day old liquid culture induced with 500 μM of $CuSO_4$ The liquid medium was separated from mycelia by filtration on whatman paper. Proteins were precipitated from 180 ml by the addition of 2 volumes of acetone, precipitated 30 min at −80 °C and centrifuged at 9000 rpm for 30 min. The precipitate was resuspended in 50 mM sodium phosphate buffer pH 6.6 and extensively dialyzed against the same buffer. The enzyme solution (16.5 ml) was applied to a size exclusion chromatography (Bio-Rad) column (Biogel S200, 2.5 by 120 cm), equilibrated with 50 mM Tris–HCl buffer (pH 8.0) at a flow rate of 0.3 ml/min. Fractions containing laccase activity were pooled, concentrated, and dialyzed against 50 mM borate buffer pH 9.0. The samples were applied to a Mono-Q anion-exchange column (Bio-Rad, 1 by 5 cm) equilibrated with 50 mM borate buffer pH 9.0 at a flow rate of 0.5 ml/min. The retained proteins were eluted over 80 min using the following salt gradient: the concentration of NaCl was increased by 100 mM each 8 min until 1000 mM. Fractions with laccase activity were pooled, desalted against 50 mM borate buffer pH 9.0 and concentrated. Samples of 1.8 ml were applied to a second Mono-Q anion-exchange column ResourceQ (Amersham-Pharmacia HR, volume of 6 ml) equilibrated with the same buffer at a flow rate of 1 ml/min. Retained proteins were eluted with the following NaCl gradient: from 0 to 500 mM NaCl over 60 min and from 500 to 1000 mM NaCl over 17 min. The laccase peak was pooled, concentrated, and dialyzed against 50 mM sodium phosphate buffer pH 6.6. Samples of 1 ml were applied to a second size exclusion chromatography column Superdex 200 (1.6 by 60 cm) equilibrated with the same buffer at a flow rate of 1 ml/min. Fractions with laccase activity were pooled, concentrated, and stored at −20 °C for later applications.

2.4. Laccase activity and enzyme kinetics

Laccase activity was determined spectrophotometrically at 469 nm by following the oxidation of 2,6-dimethoxyphenol (2,6-DMP; Sigma) at pH 5.0 in 100 mM citrate buffer. The pH optima for oxidation of ABTS (2,2′-azino-bis(3-ethylbenzathiazoline-6-sulfonate), Sigma), 2,6-DMP, guaiacol (2-methoxyphenol, Sigma) and hydroquinone (Fluka) were determined in a 100 mM citrate buffer with a pH range from 2.0 to 10.0. The oxidation of 2,6-DMP, ABTS, guaiacol and hydroquinone was followed spectrophotometrically at 469, 420, 436, and 248 nm, respectively. The molar extinction coefficients for 2,6-DMP, ABTS, guaiacol and hydroquinone were $\varepsilon_{469} = 27{,}500\ M^{-1}\ cm^{-1}$, $\varepsilon_{420} = 36{,}000\ M^{-1}\ cm^{-1}$, $\varepsilon_{436} = 6400\ M^{-1}\ cm^{-1}$ and $\varepsilon_{248} = 17{,}542\ M^{-1}\ cm^{-1}$, respectively. UV-vis spectrophotometer (Shimadzu UV160U, Japan) was used in all measurements. 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 one μmol of substrate per minute. Extracellular proteins were determined by the Bradford method [15], using Bio-Rad protein assay and bovine serum albumin as standard [14].

2.5. Characterization of laccase

Apparent molecular weights were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) on 12% polyacrylamide gels performed in a Bio-Rad Mini-PROTEAN®3 chamber. Low-molecular-weight standards (BioRad) were used. For protein gel staining, coomassie dye G-250 was used. For zymograms, DMP, ABTS and guaiacol were used at a concentration of 5 mM.

Molecular weight of native protein was also estimated by gel filtration. It was carried out by HPLC on analytic Bio-Sil®SEC column (Bio-Rad), calibrated with thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (13.5 kDa).

The UV–visible (200–700 nm) absorption spectrum of the purified laccase from the ascomycete *S. thermophilum* was recorded in pure water at 1.23 mg/l as a protein concentration using a UV-visible spectrophotometer (Shimadzu).

Thermotolerance of the isolated laccase was determined over the temperature range from 40 °C to 80 °C by incubating the enzymes in 100 μl aliquots for 1–120 min. After the incubation, the tubes were chilled on ice and the residual laccase activity was measured with 2,6-DMP as described above. Laccase activity at 25 °C without thermal treatment was marked as 100%. The effect of temperature on laccase stability was determined by incubating the

laccase solution at various temperatures ranging from 4 to 75 °C and then determining the residual laccase activity with the 2,6-DMP assay method. The laccase was incubated in acetate buffer (100 mM, pH 4). To determine the optimum temperature for the enzyme, activity was measured at temperature ranging between 20 and 95 °C.

The pH optima of the laccase were estimated by measuring the oxidation of 2,6-DMP, ABTS, guaiacol, and hydroquinone. The pH in the reactions was adjusted with citrate buffer (100 mM) with pH 5.0 for 2,6-DMP and ABTS, and with phosphate buffer (100 mM) pH 6.0 for guaiacol and hydroquinone. The pH dependence of the purified laccase was determined with 2,6-DMP (5 mM) as a substrate in 200 mM sodium citrate buffers (pH 2.0–3.0), 200 mM sodium acetate buffers (pH 4.0–5.0), 200 mM sodium phosphate buffers (pH 6.0–8.0) and 200 mM sodium borate buffer (pH 10.0).

2.6. Substrate specificity and effect of inhibitors

The oxidation of substrates by *S. thermophilum* laccase was determined spectrophotometrically at the wavelength of maximum absorption of each substrate in a citrate buffer (100 mM, pH 5.0). The laccase concentration used for the oxidation of each substrate was the same (0.8 U/ml). These substrates are guaiacol, pyrogallol, catechol, ferulate, 2,6-DMP, ABTS and hydroquinone. The substrates were used at 5 mM.

The effect of potential inhibitors on laccase activity was determined using 2,6-DMP (5 mM) as a substrate in a sodium acetate buffer (100 mM, pH 4.0) in the presence of inhibitors. These were NaN_3, NaF, DTT, NaBr, L-Cysteine, *p*-coumarate, EDTA and SDS.

2.7. Effect of heavy metals on laccase activity and stability

The activity of laccase was tested in the presence of several metal ions including Cu^{2+}, Co^{2+}, Ca^{2+}, Cr^{3+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, F^-, Zn^{2+}, Fe^{2+}, Cd^{2+} and Li^+. The residual activities were determined using the 2,6-DMP assay. Laccase was incubated with the salts of the above-mentioned ions at concentrations of 10 and 100 mM at 4 °C. Samples were collected after 24 h incubation and residual activities were measured.

2.8. Steady-state kinetic measurements

The laccase steady-state kinetic parameters (K_m and k_{cat}) were determined using 2,6-DMP and ABTS as reducing substrates. Reactions were initiated by the addition of 0.078–20 mM of 2,6-DMP or ABTS in citrate buffer (100 mM) at 25 °C. Initial rates of product formation were obtained from the first linear phase of the progress curves [14]. Duplicate experiments were carried out with each substrate. The K_m and V_{max} values were calculated based on Lineweaver–Burk plots and the k_{cat} values were calculated as V_{max}/Lac protein concentration.

2.9. Statistical analysis

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

3. Results and discussion

3.1. Enhancement of S. thermophilum laccase production

The time course production of *S. thermophilum* was studied in the presence of various inducers, carbon and nitrogen sources. For laccase induction, some putative laccase inducers commonly used for fungal cultures including copper, ethanol, veratrate, vanillate, ferulic acid, coumaric acid, veratrylic alcohol, caffeic acid, 3,4-dihydroxybenzoic acid and vanillin were supplemented to the media after 2 days of cultivation. Low laccase activity was detected in the absence of inducers which suggests that the fungus produced a basal level of enzyme. However, in the presence of these laccase inducers, higher levels of laccase were recorded in all cultures. The highest induction was obtained in the presence of copper. Similarly to the most laccase-producing fungi, copper sulfate proved to be a promising inducer for laccase production by this strain [7,14,16]. Aromatic inducers are either toxic or expensive. The choice of the non-toxic and cheaper inducers plays a key role in the applicability of the enzyme. *S. thermophilum* laccase production was studied in the presence of different Cu^{2+} concentrations. Copper has been reported to regulate the expression of fungal laccases at a transcription level [14]. The addition of $CuSO_4$ to basal medium enhanced the production of laccase produced by the strain *S. thermophilum* isolated in Tunisia. Fig. 1a shows that the laccase production increased with copper concentration up to 500 μM resulting in an increasing laccase activity of nearly 3-fold. Whereas higher concentrations decreased fungal biomass and laccase activity, which may probably be due to a toxic effect on the fungal culture [14].

To further improve laccase production, various carbon sources were tested such as sorbose, cellulose, starch, lactose, pectin, inositol, succinic acid, cellobiose, D-xylose, gruel, saccharose, mannitol, malt extract and glucose. Laccase activity was measured daily for each experiment, in the presence of 500 μM of copper during a period of 7 days. There was no growth on pectin, inositol, succinic acid and mannitol. Glucose was the carbon source which yielded the highest laccase production, with a maximum laccase production being reached after 5 days of cultivation. The production of laccase was maximal when 10 g/l of glucose were used. At higher glucose concentrations, the laccase production was decreased. The absence of glucose in the culture medium could be beneficial by avoiding catabolic repression which is often observed in the case of extracellular enzymes production [17].

Another essential factor for efficient laccase production which are the nitrogen sources used for fungi cultivation was also investigated. Laccase production was affected distinctly by nitrogen sources. Among all tested nitrogen sources, Soya peptone, at a concentration of 5 g/l, yielded the highest laccase production. No growth of *S. thermophilum* was observed using ammonium sulfate, urea and ammonium tartrate. Thus, the importance of the nature of the nitrogen source, not only to fungal growth but also for laccase production [18] is further confirmed. Laccase activity was high when using organic nitrogen sources compared to inorganic ones. Lignin modifying enzymes are produced by white-rot fungi during their secondary metabolism. The synthesis and secretion of these enzymes is often induced by limited carbon or nitrogen levels [19].

The optimal temperature of laccase production has been reported to differ greatly from one strain to another [7]. Among the eukaryotic organisms, several species of fungi have the ability to thrive at temperatures between 45 and 55 °C. The thermophilic fungi have a minimum growth temperature at or above 20 °C and a maximum growth temperature at or above 50 °C [20]. In this study, there were significant differences in fungus mycelial biomass yield and extracellular laccase activity for all of the cultural conditions studied. Growth of the fungus was initiated after 1 and 3 days of inoculation for cultures incubated at temperatures up to 42 °C. Results showed that fungal mycelial biomass yield and laccase activity increased with incubation temperature. At 42 °C, laccase activity and fungal mycelial biomass yield were the highest resulting in 5.9 U/ml and 119 mg/ml, respectively (Fig. 1b). Temperature within the range 42–55 °C linearly reduced both laccase activity and biomass yields. It was reported that temperatures higher than 30 °C reduce the activity of mesophile ligninolytic enzymes [21]. This may be attributed to the alteration of the three dimensional structure of the enzymes [21].

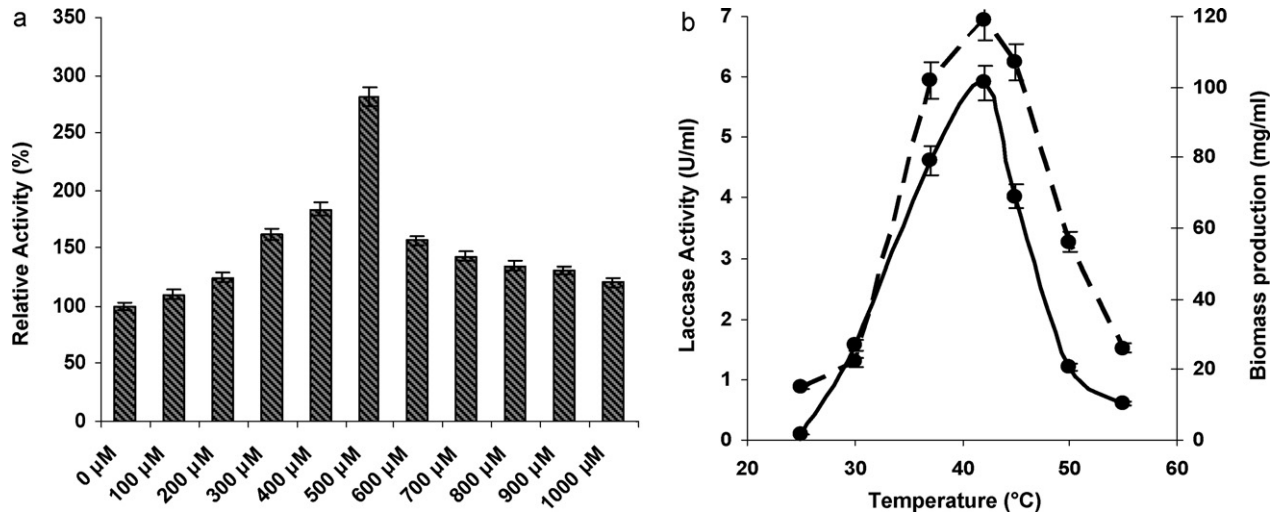

Fig. 1. (a) Effect of varying concentrations of copper (0–1000 μM) on laccase production by *Scytalidium thermophilum* when grown in shaken flasks at 45 °C. Values shown are the means of triplicate cultivation experiments. (b) Production of *Scytalidium thermophilum* laccase (—) and biomass (– – ·) at different incubation temperatures.

The onset of laccase activity in *S. thermophilum* occurred on day 2 and reached its maximum on day 5 (5.9 U/ml) (Fig. 1b). Then, laccase activity dropped sharply for 6-day-old culture. A similar profile was observed in the cultures of *Trichoderma harzianum* with a maximum laccase activity reached on day 4 [16]. Compared to other mesophilic fungal species for which a longer production time (12–30 days) is required [14], this property is of great interest from the industrial point of view. The addition of Cu^{2+} (500 μM) produced the strongest laccase induction. It is also noteworthy that the presence of copper in culture medium is required for the production of laccase [14,16]. It was reported that the presence of ethanol in the cultures can increase laccase production in some basidiomycetes [14]. However, in our case, the addition of ethanol to the cultures had an inhibitory effect and this behaviour was noticed with some other fungi [14].

3.2. Purification of laccase

Various steps were used for the purification process to separate laccase from other extracellular proteins secreted in Cu^{2+} induced culture medium (Table 1). After precipitation with acetone, the concentrated protein precipitate was suspended in 50 mM phosphate buffer (pH 6.0) and subjected to dialysis (at 4 °C). During the first chromatographic step (gel filtration on Biogel S200), the laccase activity was separated from most impurities. One of these impurities is a brown pigment which absorbs strongly at 280 nm. During the second anion exchange chromatography (Mono-Q), laccase activity was detected as a symmetric peak separated from other contaminant proteins (data not shown). The third step was also an anion exchange chromatography, on a Resource Q^{TM} column (Pharmacia) with high resolution (Fig. 2a). This step permitted to separate laccase from the other proteins present in the fraction, removed almost all brown-colored substances secreted during the secondary metabolism and yielded a single fraction with high laccase activity. This fraction, being blue in color, was further purified to homogeneity with a gel filtration chromatography (Superdex 200) (Fig. 2b) which was the last step that yielded one laccase activity peak. Increase in the specific activity of the laccase was observed and a single homogenous protein was obtained with a purification yield of 30%. The procedure adopted for laccase purification allowed to concentrate the enzymatic preparation by 8-folds.

3.3. Properties of the laccase secreted by S. thermophilum

The laccase molecular weight was 82 kDa as estimated by gel filtration chromatography and 28 kDa as determined by SDS-PAGE (Fig. 3). The estimated molecular weight of the laccase from *S. thermophilum* (82 kDa) was similar with most fungal laccases (50–80 kDa) [7,9,14,16]. However, this enzyme is an atypical homotrimeric laccase with a sub-unit molecular weight of 28 kDa. To our knowledge, this is the first report describing the production of such laccase by the ascomycetous *S. thermophilum.* In fact, it was reported that *Chaetomium thermophilum* laccase was a homotrimeric phenoloxidase with a sub-unit molecular weight of 36 kDa [22].

The *S. thermophilum* laccase was purified and characterized. The enzyme was a typical blue laccase as demonstrated by its UV–visible spectrum which shows an intensive peak around 597 nm, corresponding to the type-1 copper site, which is responsible for the deep blue color of the enzyme. It has a shoulder peak at 327 nm, corresponding to the presence of the type-3 copper site of a typical blue laccase. In addition, this laccase has some properties similar to or in the range of most known mesophilic and some thermophilic fungal blue laccases [14,16].

The effect of temperature on laccase activity was tested between 4 and 95 °C. Fig. 4a shows that *S. thermophilum* laccase was active

Table 1
Scheme of purification of *Scytalidium thermophilum* laccase.

Purification step	Volume (ml)	Activity (U/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Culture filtrate	180	4.9	50.6	882	17.43	1	100
Precipitation (acetone)	10	82	42.1	820	19.5	1.1	93
Filtration							
Gel S 200	3	191.6	25.5	575	22.5	1.3	65
MonoQ	1.8	256.14	7.43	461	62	3.5	52
MonoQ HR	1.8	159.59	2.52	287.27	115	6.5	32.5
Superdex 200 HR	1	265	1.9	265	139.4	7.9	30

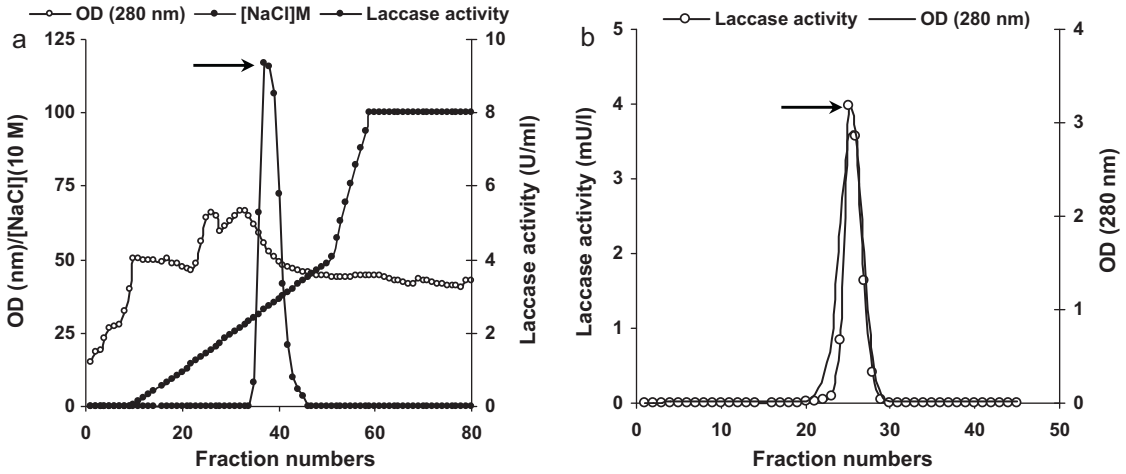

Fig. 2. Purification of *Scytalidium thermophilum* laccase from 500 μM Cu^{2+}-induced culture: (a) by anion-exchange chromatography on a 6 ml Resource Q column and (b) by gel filtration chromatography on a Superdex 200 column. Laccase peaks are indicated with arrows.

in the temperature range 65–85 °C, with a maximum activity at 80 °C. Whereas, other authors showed that a monomeric laccase from *S. thermophilum* had an optimum activity temperature at 65 °C [23]. Temperature profiles of *S. thermophilum* laccase activity did not differ from other laccases with optimum temperature between 50 and 80 °C [7] and stability at temperature below 50 °C [7,14].

The temperature range where the enzyme is active is remarkably wide. The laccase purified from *S. thermophilum* had greater thermal stability (Fig. 4b) compared to laccases from *Pycnoporus cinnabarinus*, *Pycnoporus sanguineus*, and *Pycnoporus coccineus* [24]. The half lifes at 65 °C (120 min) and at 70 °C (90 min) were superior to the thermostable laccase from *Peniophora* species [23]. This laccase presented an important thermal stability even when compared with those produced by thermophilic fungi as *Chaetomium thermophilium*, *Melanocarpus albomyces* [16] and *S. thermophilum* [23].

At 4 °C and in borate buffer (10 mM) laccase was more stable at alkaline pH values (9.0–10.0) than at acidic values of pH during 5 days. Four substrates were used to determine the effect of pH on laccase activity as laccase enzymes tend to react differently to

Table 2
Activity of purified *Scytalidium thermophilum* laccase towards different substrates.

Substrate	Laccase activity (U/ml)
ABTS	0.393
DMP	0.549
Pyrogallol	0.049
Guaiacol	0.276
Hydroquinone	0.326
Catechol	0.058
Ferulate	0

pH with diverse substrates [14]. The pH optimum obtained for *S. thermophilum* laccase was in the acidic region (Fig. 4c) and representative of typical laccases. However, this optimum was found to depend on the substrate. In fact, the laccase exhibited an optimum pH at 5.0 for ABTS and DMP and 6.0 for guaiacol and hydroquinone as substrates, like many other fungal laccases [14,16]. The difference in pH optima for substrates is typical for laccases and it reflects the difference in oxidation mechanism towards different substrates [14,16]. *S. thermophilum* laccase showed an excellent stability at 4 °C and at alkaline pH values; over 76% and 83% of the activity

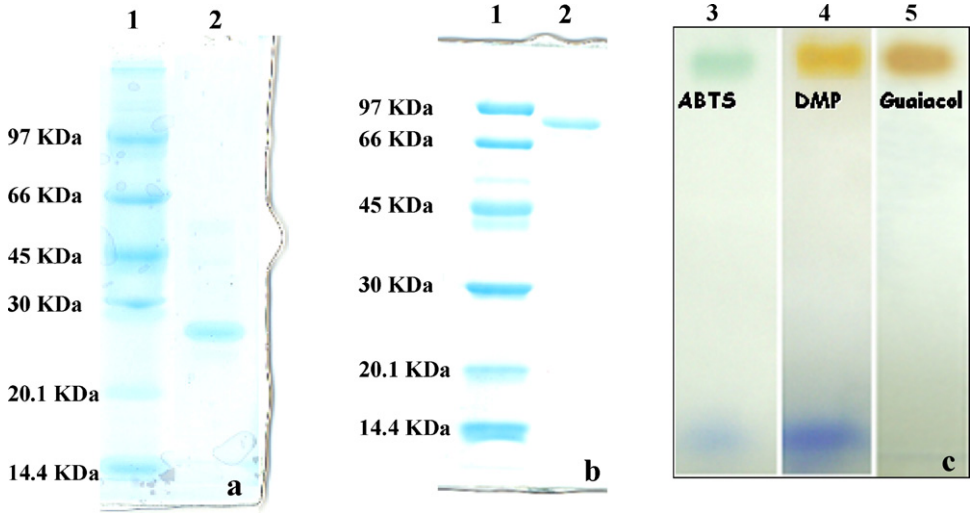

Fig. 3. Electrophoretic analysis of the purified *Scytalidium thermophilum* laccase. (a) Determination of molecular weight on SDS-PAGE (12% polyacrylamide gel). **Lane 1**: lower-molecular-mass standards: phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsine inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). **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. (c) The laccase activity band was detected by zymogram with ABTS (lane 3), DMP (lane 4) and guaiacol (lane 5) as substrates.

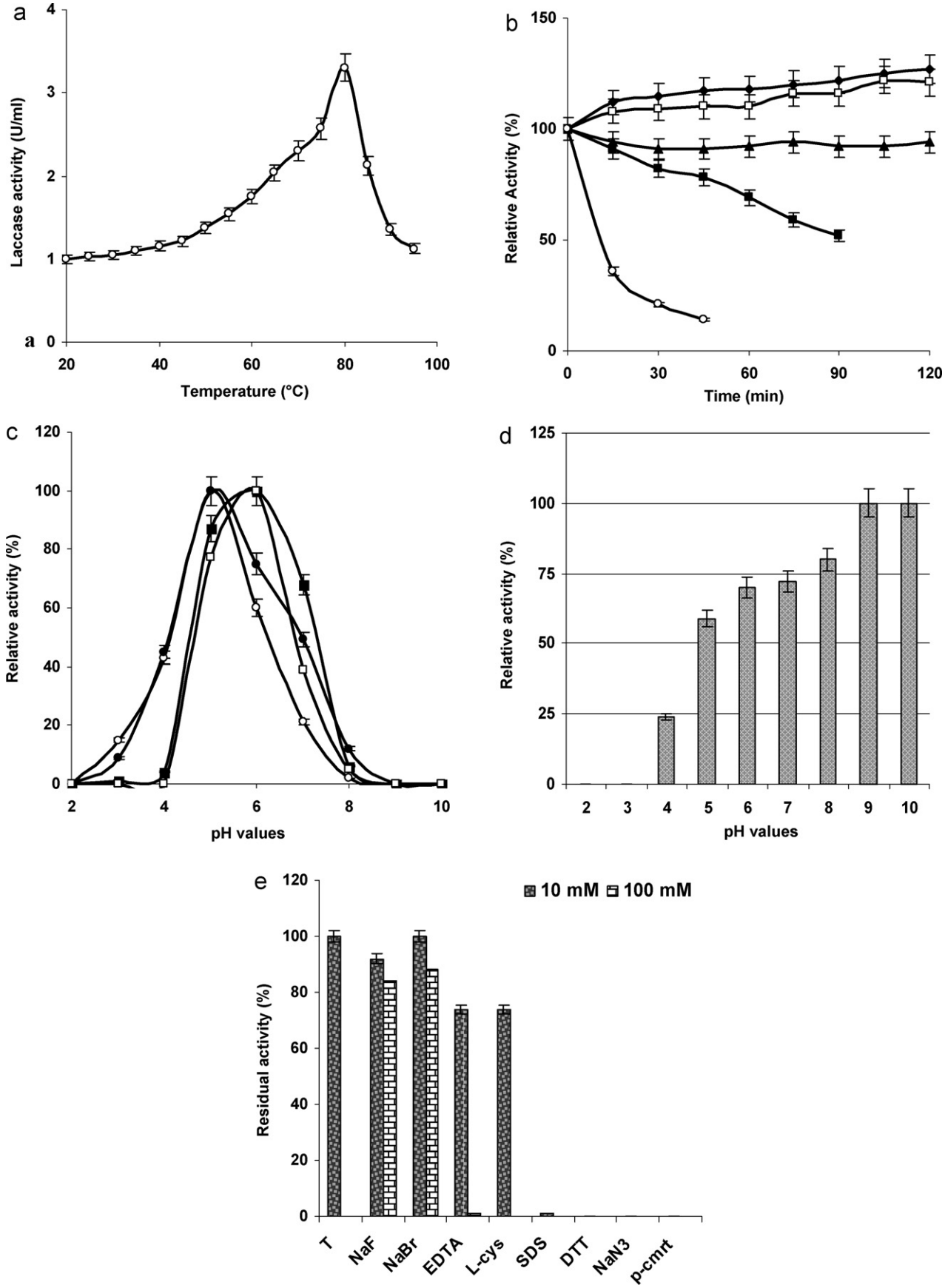

Fig. 4. (a) Optimum temperature of laccase from *Scytalidium thermophilum* using 2,6-DMP as substrate; (b) thermotolerance of purified laccase at 40 °C (-♦-), 50 °C (-□-), 60 °C (-▲-), 70 °C (-■-) and 80 °C (-○-); (c) pH stability of *Scytalidium thermophilum* laccase after 5 days of incubation; (d) optimum pH using 2,6-DMP (-●-), ABTS (-○-), hydroquinone (-■-) and guaiacol (-□-) as substrates; and (e) effect of various inhibitors on oxidation of 2,6-DMP by purified *Scytalidium thermophilum* laccase (*p*-cmrt = *p*-coumarate).

Table 3
Kinetic constants of laccase from *Scytalidium thermophilum*.

Substrates	V_{max} (mM min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
2,6-DMP	77.88	0.36	20.97×10^4	82.5×10^3
ABTS	31.91	0.26	8.59×10^4	330.3×10^3

remained after 48 h incubation at pH 7 and 8, respectively (Fig. 4d). This stability is rare among fungal laccases, for example laccases from *M. albomyces*, *Pleurotus ostreatus*, *Pleurotus erengii*, *Trichoderma atroviride* [16] and *Perenniporia tephropora* [14] have good stability at neutral and slightly alkaline pH values. In fact, these properties could potentially be exploited in the textile industry where alkaline conditions predominate in jeans dyeing [1,14,16].

3.4. Substrate specificity and inhibition pattern

The substrate specificity of the purified laccase was examined with various phenolic and non-phenolic compounds. *S. thermophilum* laccase oxidizes ABTS and various phenolic compounds including DMP, pyrogallol, guaiacol, hydroquinone, catechol and ferulate. But, no activity was observed using ferulate as substrate (Table 2). In Ogel et al.' studies, *S. thermophilum* laccase did not oxidize neither guaiacol nor ABTS [23]. On the other hand, laccase oxidized much faster a monophenolic substrate with a double methoxyl group such as 2,6-DMP than a monophenolic substrate having only one methoxyl group such as guaiacol [14]. It was also noticed that the enzyme showed a less reactivity with ABTS, which is a nonphenolic substrate, than that with 2,6-DMP (Table 2). As a typical laccase, the enzyme secreted by *S. thermophilum* had a wide substrate specificity oxidizing hydroxy- and methoxy-substituted phenols. Similar results were also shown with laccases from *Coriolopsis rigida* [14].

The effects of several laccase inhibitors were determined with 2,6-DMP (5 mM) as a substrate in 100 mM citrate buffer (pH 5.0). Lower concentration of 10 mM of NaN_3, DTT, SDS and *p*-coumarate completely inhibited the laccase of *S. thermophilum*, while other inhibitors required a higher concentration for complete inhibition (Fig. 4e). For example L-Cysteine and EDTA completely inhibited laccase activity only at a 100 mM concentration. On the other hand, the NaF and NaBr did not inhibit the laccase activity because even at a concentration higher than 100 mM no inhibition effects were observed. Pure laccase was strongly inhibited by sodium azide, DTT, SDS and *p*-coumarate but it was not sensitive to EDTA, L-Cys, sodium bromide and fluoride. In fact, the binding of NaN_3 to the type 2 and 3 copper sites affects internal electron transfer, thus inhibiting the activity of the laccase. These findings are in accordance with the general properties of laccase from a diverse range of fungal sources [14,16].

3.5. Effect of heavy metals on laccase activity and stability

Laccase activity was tested in the presence of several metal ions including Li^+, F^-, Cu^{+2}, Mg^{2+}, Zn^{2+}, Ni^{+2}, Co^{+2}, Ca^{+2}, Mn^{+2}, Hg^+, Cr^{3+}, Fe^{2+}, Cd^{+2} and Pb^{2+}. Two concentrations were tested: 10 and 100 mM. It was found that for a concentration of 10 mM, laccase activity remained unchanged except for Hg^+, Cr^{3+} and Fe^{2+} which showed a decrease of the activity of 48%, 99% and 98%, respectively. When the concentration was increased to 100 mM, the laccase activity decreased in the presence of Hg^{2+} which showed 97% of inhibition. The other ions have a variable inhibiting effect: Cu^{2+} (67%), Cd^{+2} (48%), Pb^{+2} (44%), Ca^{+2} (43%), Co^{+2} (29%) and the rest of the metal ions caused an inhibition of more than 80%. Laccase was not inhibited by fluor and zinc.

Effluents from textile dyeing facilities contain metals used in dye production technologies or in dyes molecules themselves. The interaction of metals with extracellular ligninolytic enzymes from white-rot fungi is particularly important for the understanding of the regulation of biotechnological processes of fungal xenobiotics degradation. Therefore, the stability of laccase activity against several metal ions, some of them normally present in waste water effluents, was assessed.

For stability assays, laccase was incubated with these metals (Cu^{2+}, Co^{2+}, Pb^{2+}, Ca^{2+}, Cd^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, F^-, Zn^{2+} and Li^+) at a concentration of 10 and 100 mM. It was found that for those concentrations, laccase activity remained stable for 24 h against all the metal ions tested. Fe^{2+} inhibited completely laccase activity at a concentration of 10 mM. The stability of this laccase makes it an efficient agent in the treatment of waste waters containing heavy metals.

3.6. Catalytic properties

Different kinetic parameters of *S. thermophilum* laccase were studied with two different substrates ABTS and 2,6-DMP. The K_m and V_{max} values for laccase were found to be 0.36 mM and 77.88 mM min^{-1} for 2,6-DMP and 0.26 mM and 31.91 mM min^{-1} for ABTS, respectively. Purified laccase showed notable differences in its catalytic efficiencies (k_{cat}/K_m) when substrates were varied (Table 3). These parameter values were determined to be 330.61 min^{-1} M^{-1} and 82.5 min^{-1} M^{-1} in the presence of ABTS and 2,6-DMP, respectively. All the kinetic parameters of *S. thermophilum* laccase suggest that the enzyme had a higher affinity towards ABTS than 2,6-DMP. Fungal laccases are known to possess a very wide range of substrate affinities and even the laccase isoenzymes of a same strain may also show different substrate affinities [14]. These differences could be related to the redox potential of this enzyme and its role in the degradation of recalcitrant compounds present in contaminated soils or residual waste water [14].

4. Conclusions

In this study, we report the purification and characterization of a novel laccase from the thermophilic ascomycete *S. thermophilum* strain. Characterization studies showed that the enzyme has a homotrimeric structure, possesses a total molecular-weight of 82 kDa, an acidic optimum pH, and an optimum activity temperature at 80 °C. This laccase showed a great thermostability at 70 °C. The enzyme manifests activity towards a variety of phenolic compounds. It has shown a high sensitivity to DMP, with a K_m of 0.36 mM at pH 5.0 and 25 °C. The laccase was inhibited by NaN_3, DTT, SDS and *p*-coumarate but not EDTA, L-Cys, NaF and NaBr. Finally this laccase exhibited a noticeable stability in the presence of some metal ions.

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