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Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1

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ABSTRACT

A new strain of *Trichoderma harzianum* WL1 was isolated from the Western Ghats region of Tamilnadu, India and assayed for laccase activity by ABTS oxidation. Supplementation of $CuSO_4$ (1 mM) in liquid medium yielded high amounts of laccase (4.36 U ml^{-1}) at an incubation period of 4 days. Laccase enzyme produced by *T. harzianum* in cultures supplemented with copper sulphate has been purified by ultrafiltration, Sephadex G-100 column chromatography and Concanavalin-A affinity chromatography with a final purification fold of 151.7 and a yield of 0.39%. The purified enzyme was identified as a glycoprotein with a molecular mass of 79 kDa by SDS gel electrophoresis. The UV–vis spectrum of the purified laccase had a peak at 608 and 325 nm suggesting the presence of types I and III Cu centers. The redox potential of the enzyme was found to be 692 mV for the type 1 Cu (T1) site. The optimum pH and temperature for enzyme activity were 4.5 and 35 °C, respectively. Laccase was stable for 24 h at 35 °C and had half-life of 60 min at 65 °C. Purified laccase showed K_m values of 180 and 60 μM, respectively, and V_{max} values of 3.95 and 1.42 U mg^{-1} protein, respectively, for the substrates ABTS and guaiacol. The susceptibility of laccase towards several putative inhibitors and metal cations was also assessed. The enzyme activity was completely inhibited by sodium azide (NaN_3) at the concentration of 20 μM. Laccase activity was also inhibited by several metal cations, especially Hg^{2+}.

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper blue oxidases widely distributed in higher plants, in some insects and in a few bacteria. However the best known laccases are of fungal origin. Laccase production occurs in various fungi over a wide range of taxa. Fungi from the deuteromycetes, ascomycetes as well as basidiomycetes are known producers of laccase [1]. Among them, basidiomycetes are considered to be efficient laccase producers, especially white rot fungi [2]. Well known laccase producers include *Trametes versicolor*, *Chaetomium thermophilum* and *Pleurotus eryngii*. It has been reported that some *Trichoderma* species, including *T. harzianum* has the ability to produce polyphenol oxidases [3]. In fungal physiology, laccases are involved in plant pathogenesis, pigmentation, detoxification and lignin degradation. All of these functions are related to the oxidation of various organic compounds by means of dioxygen (O_2), including monophenols, polyphenols, aromatic amines and their derivatives, to free radicals, which in turn can undergo both spontaneous chemical and enzymatic reactions [4].

In structural terms, these enzymes can be either monomeric or multimeric glycoproteins, which may exhibit additional heterogeneity because of variable carbohydrate content or differences in copper content [5]. There are many reports on the purification and biochemical characterization of fungal laccases [6,7]. The key characteristics of laccase are the standard redox potentials of its redox centers, the T1, T2 and T3 sites. The types of copper centers (T1, T2 and T3) can be distinguished using UV/vis and electron paramagnetic resonance (EPR) spectroscopy [8]. The redox potential of laccases vary from 0.4 to 0.8 V and the most critical factor determining the E° (laccase) is the coordination sphere of the T1 copper [9].

Laccases catalyze the oxidation of a broad range of substrates such as *ortho* and *para*-diphenols, methoxy-substituted phenols, aromatic amines, phenolic acids and several other compounds coupled to the reduction of molecular oxygen to water with the one electron oxidation mechanism. The substrate specificity of laccases varies from one organism to another. The spectrum of laccase-oxidizable substrates can be expanded considerably in the presence of appropriate redox mediators [10]. Due to their interesting catalytic properties laccases have gained considerable interest in

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various industrial areas. The most intensively studied applications have included development of oxygen cathodes in biofuel cells, biosensors, labeling in immunoassays and organic synthesis through biocatalysis. Due to their broad substrate specificity, laccases might have great potential in varied environmental applications including pulp delignification, textile dye bleaching and xenobiotics degradation [11,12]. These applications stimulate new waves of fundamental research concerning this enzyme.

The current research activity of laccases includes screening of laccase sources, studying new laccases and investigating the electrochemical properties of laccases. Laccases of basidiomycetes and ascomycetes are extensively studied and only few reports are available with respect to deuteromycetes laccase. Although there are also some reports on laccase activity in *Trichoderma* species, the purification and characterization of the purified laccase have not yet reported [13,14]. In this study, the biochemical and physicochemical characterization of laccase from the fungus *Trichoderma harzianum* WL1 with respect to production, purification, redox potential, kinetic and stability properties were reported.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated all chemicals were purchased from Sigma–Aldrich, USA and were certified reagent grade.

2.2. Microorganism and growth conditions

The test fungus *Trichoderma harzianum* WL1 was isolated from the Western Ghats region of Tamilnadu, India [15] and is placed in microbial culture collection, Microbial Biotechnology Laboratory, Department of Biotechnology and Bharathiar University, India. The fungus was identified as *Trichoderma harzianum* according to its morphological characteristics [16]. The fungus was maintained on Czapek Dox agar plates at 30 °C. After 6 days of incubation, the spores were harvested without disturbing the mycelial growth using a camel hairbrush and filter-sterilized. The inoculum volume (2.0 ml of 10^5 spores ml^{-1}) was used as standard inoculum for further studies.

2.3. Production of laccase

For laccase production, *T. harzianum* grown on Czapek-Dox agar plates were used to inoculate 250 Erlenmeyer flasks containing 100 ml of liquid culture medium [17]. The medium used had the following composition ($g\,l^{-1}$): 3.0 glucose, 1.0 KH_2PO_4, 0.26 NaH_2PO_4, 0.317 $(NH_4)_2SO_4$, 0.5 $MgSO_4$, 0.5 $CuSO_4$, 2.2 dimethyl succinic acid, 0.74 $CaCl_2{\cdot}2H_2O$, 0.006 $ZnSO_4{\cdot}7H_2O$, 0.005 $FeSO_4{\cdot}7H_2O$, 0.005 $MnSO_4{\cdot}4H_2O$, 0.001 $CoCl_2{\cdot}6H_2O$, 500 μl of trace elements and vitamin solution [18]. The pH of the medium was adjusted to 4.5 with 0.1N NaOH. Cultures were incubated at 27 °C on a rotary shaker (125 rpm) for 14 days. After 24 h from the inoculation, the putative inducer (i.e. $CuSO_4$) was added to liquid cultures at a 1 mM concentration and extracellular fluids were sampled on a daily basis in order to assay laccase activity.

2.4. Enzyme assay

Laccase activity was determined spectrophotometrically with 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate and the oxidation was monitored at 436 nm (ε 436 = 36 $mmol^{-1}\,cm^{-1}$) [19]. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS min^{-1}.

2.5. Protein determination

The protein content of the culture filtrate was estimated by Lowry's method [20] with bovine serum albumin as the standard.

2.6. Enzyme purification

For enzyme purification, pre-chilled acetone (4 °C) was added to the culture filtrate (66% v/v) and incubated for 6 h at −20 °C. After 6 h, the culture filtrate was centrifuged at 5600 × *g* for 10 min. The supernatant was decanted and the pellet was dissolved in 0.1 M sodium acetate buffer (pH 4.5) and dialyzed against the same buffer. The dialyzed enzyme preparation was loaded onto a Sephadex G-100 (Fine) column (45 cm × 2.5 cm) previously equilibrated with 0.1 M sodium acetate buffer (pH 4.5) containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 10.0 ml h^{-1}. In each step, the protein content and laccase activity were determined. The active fractions were pooled, dialyzed against distilled water using a 12,000 Da cut-off dialysis tubing (Sigma, USA) and concentrated by freeze-drying. The concentrated laccase fractions were dissolved in 3.0 ml of 0.1 M sodium acetate buffer (pH 4.5) and loaded onto a Con-A CL Agarose column (5 cm × 2.5 cm) (Genei, Bangalore, India) pre-equilibrated with 0.1 M sodium acetate buffer containing 0.5 M NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. After being washed with the same buffer, bound proteins were eluted by a linear gradient of α-D-methylmannopyranoside from 0 to 0.5 M in equilibrating buffer with a total volume of 30 ml. Fractions were collected at a flow rate of 20 ml h^{-1}. The fractions with high laccase activity were pooled, concentrated and stored at −4 °C. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.7. Molecular mass

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 10% resolving gel and a 4% stacking gel according to the method of Laemmli [21]. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

2.8. Absorption spectrum

The UV–vis absorption spectrum of purified laccase (1 mg ml^{-1} in 0.1 M sodium acetate buffer, pH 4.5) was recorded at 25 °C on a spectrophotometer (Hitachi, model U-3210, Tokyo) in 2 cm path length quartz cells.

2.9. Redox potential

Cyclic voltammetry (CV) was performed on a CH instruments electrochemical analyzer, with a platinum disc-working electrode, an AgCl/Ag reference electrode and a platinum wire counter electrode. Prior to each voltammetric run, surface cleansing of the working electrode was carried out with alumina according to the manufacturer's instructions. To determine the redox potentials of laccase T1 centers, protein redox titration method [22] was employed with potassium octocyanomolybdate (IV and V) mediators. Laccases were placed anaerobically into a cell containing a high concentration of $K_3Mo(CN)_8$ in 0.1 M sodium acetate buffer, pH 4.5. Redox potentials were registered with platinum electrodes before and after the addition of enzymes. Further titration was performed with the reduced form of the mediator ($K_4Mo(CN)_8$) in 0.1 M sodium acetate buffer, pH 4.5. The voltammogram was obtained at the scanning rate of 0.1 V s^{-1}.

2.10. Effect of pH and temperature

The optimum pH of the purified enzyme was studied over a pH range of 2.0–10.0. To determine the pH stability, enzyme was kept at 4 °C for 1 h in different buffers (100 mM) and the residual laccase activity was determined under standard assay conditions. The buffer systems used were citrate buffer for pH 2.0–3.5; acetate buffer for pH 4.0–5.5; phosphate buffer for pH 6.0–7.5; Tris–HCl buffer for pH 7.5–9.0; carbonate–bicarbonate buffer for pH 9.5–10.0. To analyze the effect of temperature, the activity was tested at different temperatures (25–80 °C) by standard enzyme assay (100 mM sodium acetate buffer, pH 4.5).

2.11. Thermal stability

Thermal stability was determined after different pre-incubation times (1–24 h, at 35, 55 and 65 °C) as the residual activity detectable with ABTS in sodium acetate buffer (100 mM, pH 4.5).

2.12. Kinetic constants of T. harzianum laccase

Kinetic constants of laccase for the most commonly used substrates 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and guaiacol was investigated. The rate of aromatic substrate oxidation was determined by spectrophotometry, using reported molar extinction coefficients (ε). The substrate concentration ranges used were of 100–600 μM and 50–300 μM for ABTS and guaiacol, respectively. The reactions were conducted at 35 °C. Kinetic studies were conducted for both the substrates and the V_{max} and K_m values were calculated using the Lineweaver–Burk transformation of Michaelis–Menten equation.

2.13. Effect of inhibitors on enzyme activity

The effect of several potential inhibitors such as diethyldithiocarbamic acid (DDC), ethylenediaminetetraacetic acid (EDTA), ethanol, sodium azide (NaN_3) and thioglycolic acid on laccase activity was monitored. To 1.0 ml of reaction mixture, 800 μl of 0.1 M sodium acetate buffer containing ABTS (0.18 mM, pH 4.5), 100 μl of enzyme (4.1 U ml^{-1}) and 100 μl of inhibitor at various concentrations were added. The reaction mixture was incubated at 35 °C and the change in absorbance was measured spectrophotometrically at 436 nm. A control test was conducted in parallel in the absence of the inhibitor.

Table 1
Purification table for the purification of laccase from *T. harzianum*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification factor (fold)
Culture filtrate	752.0	653.0	0.86	100	1.00
Acetone precipitation	128.8	168.6	1.30	25.8	1.51
Membrane filtration	20.4	52.6	2.57	8.05	2.98
Sephadex G-100 column chromatography	0.45	11.9	26.4	1.82	30.6
Concanavalin-A affinity chromatography	0.02	2.61	130.5	0.39	151.7

2.14. Effect of metal ions on enzyme activity

To determine the effect of metal ions on enzyme activity, the reactions were performed by incubating the reaction mixture containing 100 μl of enzyme, 800 μl of 0.1 M sodium acetate buffer (pH 4.5) containing ABTS (0.18 mM), 100 μl of metal ion solution at 35 °C. Metals, Co, Sn, Hg, Fe, K, Zn, Mg, Mn, Na, Ba, Cr and Ca were used at the concentrations of 1 and 5 mM. After incubation, the remaining enzyme activity was assayed. The reaction mixture with heat denatured enzyme served as control.

3. Results and discussion

3.1. Production of laccase

Laccase production by fungi has been found to be largely affected by culture conditions, such as carbon and nitrogen source and related concentrations and presence or absence of microelements. Laccases are generally produced in low concentrations by fungi, but higher concentrations could be obtained by adding various supplements to liquid growth media [23,24]. The onset of laccase activity in *T. harzianum* occurred on day 2 and reached its maximum on day 4 and then the rate of enzyme production declined gradually. No enzyme production was observed after 8th day (Fig. 1). Supplementation of $CuSO_4$ at 1 mM concentration yielded high amounts of laccase (4.36 U ml^{-1}) at an incubation period of 4 days. These findings are in agreement with previous reports showing that the addition of 2 mM $CuSO_4$ during the exponential growth phase of the fungus led to a remarkably increased laccase production [25,26]. A similar effect was observed in the cultures of *Trametes multicolor* MB 49 and *T. trogii* BAFC 463 with copper concentrations ranging from 0.5 to 2.0 mM. Addition of inducers enhanced the production of laccase at the level of gene transcription. The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals. It has been demonstrated that the *Pleurotus ostreatus* laccase genes *poxc* and *poxa1b* are transcriptionally induced by copper, and several putative metal responsive elements (MREs) were found in the promoter regions of these genes [27]. The white rot fungus *Pycnoporus cinnabarinus* produced maximum level of laccase on day 5 and accounted for about 70% of the total extracellular protein [28]. Under static condition laccase production from *Cyathus bulleri* was detectable after 2 days and reached maximum on day 7 [29]. The difference in time course of laccase production by the various fungal systems mainly depends on the source, media composition and type of inducers.

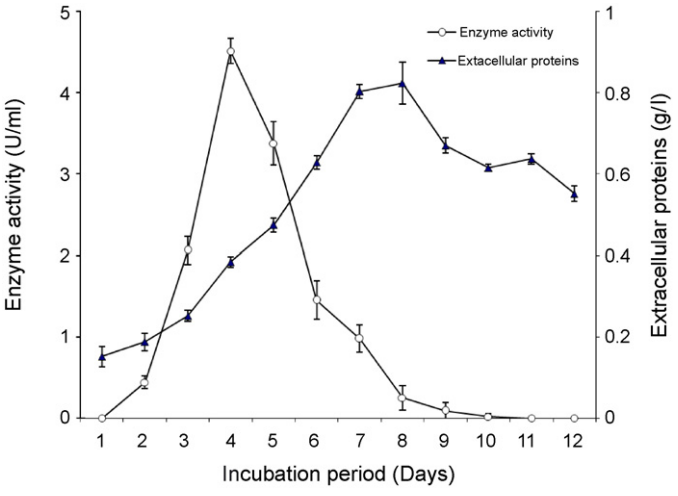

Fig. 1. Time course of extracellular laccase activity. Data points represent the means of three replicates with S.D. of ±5%.

3.2. Purification of laccase

After removal of the biomass, the supernatant was frozen and thawed. This was necessary in order to remove long chain polysaccharides from the supernatant, which was produced during the growth of the fungus. *T. harzianum* laccase was purified to homogeneity using a three-step purification procedure as summarized in Table 1. Acetone precipitation followed by Sephadex G-100 column and affinity chromatography on Con-A Agarose enriched the purification of laccase by 151.7-fold. In Sephadex G-100 column chromatography, the specific activity was increased to 26.4 U mg^{-1} protein and the yield was 1.82% with a purification factor of 30.6-fold. The major laccase containing fraction was further purified by Con-A affinity chromatography using 0.25 M α-D-methylmannopyranoside as eluent. Among fraction exhibiting laccase activity, only the most active one was collected and displayed a specific activity of 130 U mg^{-1} protein with a final yield 0.39%. Further, binding of the enzyme to the Concanavalin-A column indicated that the purified laccase might be a mannose-containing glycoprotein. Laccase from *Pycnoporus cinnabarinus* was purified by Sephacryl S-400 gel filtration chromatography with a final purification of 36-fold [28]. Laccase purified from *Chaetomium thermophilium* by Concanavalin-A affinity chromatography had a specific activity of 37 U mg^{-1}, yield of 40% with the purification of 10-fold [30]. Laccase purified from *Chalara paradoxa* CH32 by Superose 12 has showed a specific activity of 142.4 U mg^{-1}, yield of 7.20% and a purification factor of 7122.5-fold [31]. Laccase of the ascomycete *Mauginiella* sp. was purified by ammonium sulfate precipitation followed by anion exchange and hydrophobic interaction chromatography [6].

3.3. Molecular mass

The purified *T. harzianum* laccase showed a single band on SDS-PAGE with a mobility corresponding to the molecular mass of 79 kDa as visualized by Coomassie brilliant blue staining (Fig. 2). Its molecular mass appears similar to that of other fungal laccases, which were in the range of 70–80 kDa [28,30]. It was reported that the molecular weights of laccases usually ranges from 55 to 90 kDa including carbohydrates [32].

3.4. Absorption spectrum of *T. harzianum* laccase

The UV–vis spectrum of the purified *T. harzianum* laccase (Fig. 3) showed a peak of absorption at around 608 nm, typical for the type I Cu(II), which is responsible for the deep blue colour of the enzyme [33]. A shoulder at around 325 nm suggests the presence of the

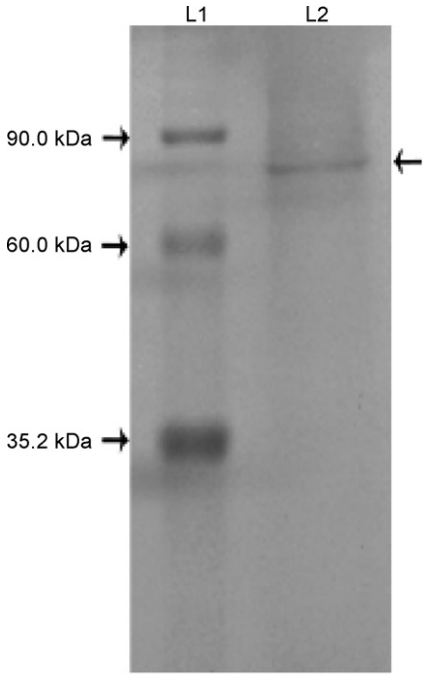

Fig. 2. SDS-PAGE of purified laccase. L1, molecular mass protein markers; L2, purified ***T. harzianum*** laccase (10 μg).

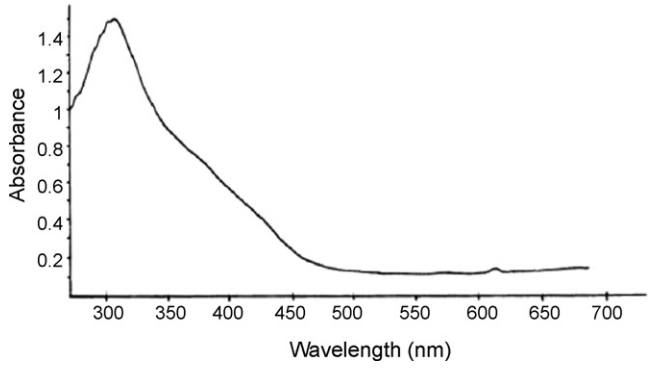

Fig. 3. UV–vis absorption spectrum of the purified laccase from ***T. harzianum***.

type III binuclear Cu(II) pair [34]. The spectral characteristics of laccase from *T. harzianum* were similar to that observed for other fungal laccases [6,8].

3.5. Redox potential

From an electrochemical point of view as well as from the analysis of the primary structure of the enzymes, laccases can be divided into three groups as a function of the potential of the T1 site: low, middle and high potential laccases. The low-redox potential laccases was mainly found in trees and fungi. For an example *Rhus vernicifera* laccase had a T1 site potential of about 430 mV vs. normal hydrogen electrode (NHE) [35]. The middle group includes laccases from ascomycetes and basidiomycetes fungi, *Myceliophthora thermophila* and *Coprinus cinereus*, respectively [36,37]. These enzymes have T1 site potentials ranging from 470 to 710 mV vs. NHE. All the high potential laccases (those from *T. hirsuta, T. versicolor* and *Trametes villosa*) have T1 site potentials

Table 2
Comparison of the redox potentials (E°) of the T1 site of laccases from different sources

Source	E°, T1 (mV)	Reference
Trametes ochracea	790 ± 10	[8]
Cerrena maxima	750 ± 5	[8]
Trametes versicolor	780	[35]
Rhus vernicifera	430	[35]
Trametes villosa	780	[36]
Rhizoctonia solani	710	[36]
Coprinus cinereus	550	[37]
Trametes hirsute	780	[38]
Marasmius quercophilus C30 (I)	730	[39]
Trametes pubescens Lac I	746	[40]
Trichoderma harzianum	692	Present study

of about 780 mV vs. NHE [35,36]. The structural reasons underlying the variability in redox potentials among laccases from different sources has not been fully elucidated. Laccase of *T. harzianum* was observed to have redox potential (E°) of 692 mV for the type 1 Cu (T1) site. Hence, *T. harzianum* laccase seems to belong to a group of middle redox potential laccases. A comparison of the redox potential of *T. harzianum* laccase isolated in the present study with laccases from different sources was presented in Table 2.

It was suggested that the value of the redox potentials of the copper-containing oxidases depends on the ligands of the T1 copper and on the amino acids, which form the T1 pocket [41]. The middle redox potentials of *Trametes ochracea* and *Coriolopsis fulvocinerea* laccases were mainly due to the significant distances between the amino acids, glutamic acid and alanine [8].

3.6. Effect of pH and temperature on enzyme activity

The optimum pH for the maximum laccase activity was observed at 4.5 when ABTS was used as substrate. At pH values larger than 4.5, the enzyme activity decreased gradually and completely inactivated at higher alkaline pH (Fig. 4). 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 [42]. Studies with laccases from *Coriolus hirsutus*, *Trichoderma atroviride*, *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH 32 and *Cerrena unicolor* 059 showed that the optimal pH range for fungal laccase was from 4.0 to 6.0 [13,31,43].

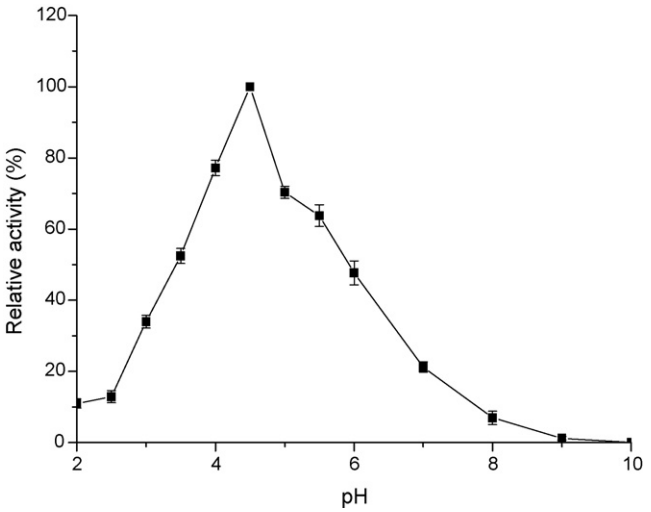

Fig. 4. The effect of pH on the activity of ***T. harzianum*** laccase. Data points represent the means of three replicates with S.D. of ±2.5%.

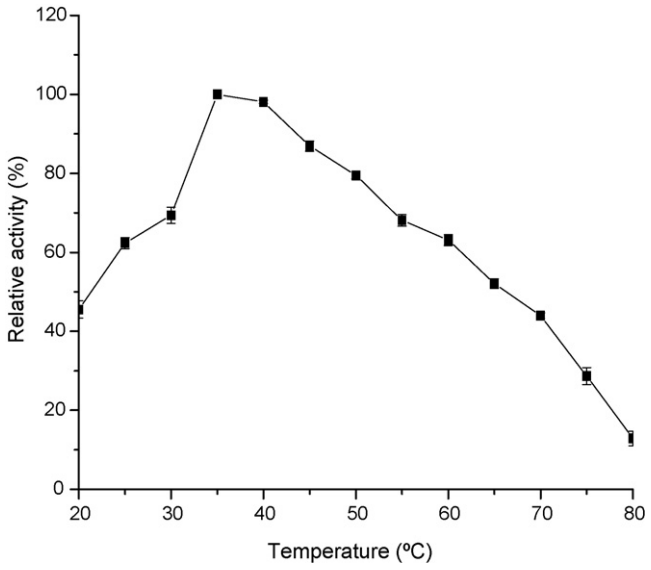

Fig. 5. The effect of temperature on the activity of ***T. harzianum*** laccase. Data points represent the means of three replicates with S.D. of ±2.5%.

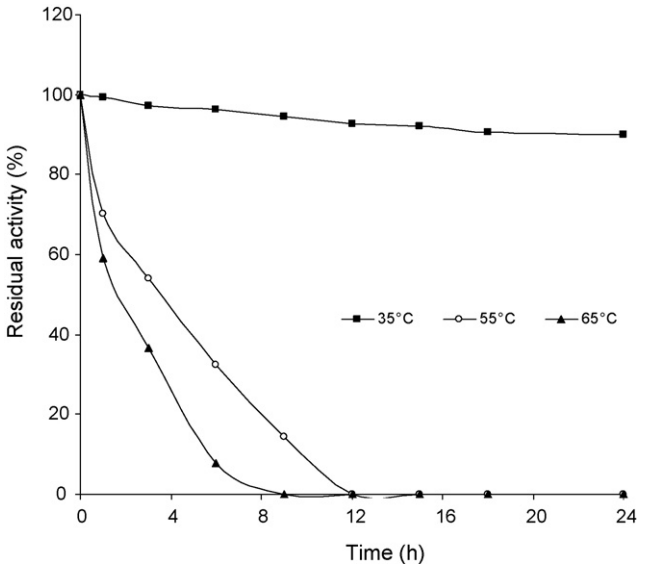

Fig. 6. Thermal stability of ***T. harzianum*** laccase. Data points represent the means of three replicates with S.D. of ±2.5%.

When laccase activity was studied as a function of temperature, the enzyme was found to be active in a temperature range of 30–50 °C, with the maximum activity at 35 °C (Fig. 5). In general, laccases are stable at 30–50 °C and rapidly lose activity at temperatures above 60 °C [6,36]. The laccase from *Rigidoporus lignosus* was almost fully active in a temperature range of 30–50 °C, with the maximum activity at 40 °C [44]. The optimum temperature for laccase from the edible mushroom *Lentinula edodes* at 40 °C [45].

The stability of the enzyme with respect to temperature was also studied. The *T. harzianum* laccase retained 70% of its initial activity after 1 h incubation at 55 °C; whereas the half-life ($t_{1/2}$) of enzyme was about 1 h at 65 °C and it was completely inactivated after 6 h incubation. No significant decrease over enzyme activity was observed even after 24 h incubation at 35 °C (Fig. 6). It has been reported that temperature stability of laccases varies considerably depending on the source organism. The typical half-life of fungal laccases is below 1 h at 70 °C and below 10 min at 80 °C [46]. *Pycnoporus cinnabarinus* laccase was very stable below 50 °C and at 70 °C the half-life of the enzyme was about 60 min; whereas at 80 °C, the laccase was completely inactivated [28]. The results obtained in the present study, concurred with these observations.

3.7. Kinetic constants of *T. harzianum* laccase

Laccases are considered to be non-specific to their substrates, being able to oxidize a wide range of aromatic compounds is of interest in delignification, textile dye bleaching and the detoxification of contaminated soil and water. For this reason, in the present work to study the kinetics of laccase activity two different substrates ABTS and monomethoxy-substituted phenolic substrate guaiacol were used. The main kinetic parameters, V_{max} (maximum enzyme velocity) and K_m (affinity constant) were determined (Table 3). The V_{max} and K_m values were 3.95, 1.42 μmol min^{-1} mg^{-1} and 180 and 60 μM, respectively, for ABTS and guaiacol. ABTS was more efficiently oxidized than guaiacol. This suggests a different behavior of the enzyme for these substrates, which may be correlated to their structures. Laccase from *Chaetomium thermophilum* had V_{max} value of 2.6 μmol min^{-1} mg^{-1} for the substrate ABTS [30]. The fungus *Coriolus hirsutus* laccase had K_m value of 62.9 μM for guaiacol [43]. The *Ganoderma lucidum* laccase had K_m value of 3.7 μM for ABTS [47]. The K_m value for laccases from *Pleurotus sajor-caju* was 0.092 mM against ABTS [48]. *Pleurotus pulmonarius* laccase showed the apparent K_m value for ABTS as 210 μM [49]. The values of the catalytic constants obtained for *T. harzianum* laccase were markedly higher than the above reported fungal laccases, which indicates the higher substrate specificity of laccase from *Trichoderma harzianum*.

3.8. Effect of inhibitors on laccase activity

Effect of a range of potent laccase inhibitors on the enzyme activity was tested with ABTS as substrate and the results were presented in Table 4. Addition of water miscible solvents such as ethanol caused a net decrease in enzyme activity, when its concentration exceeds above 20%. Thioglycolic acid inhibited 92.4% of enzyme activity at the concentration of 750 μM. In general, organic solvents alter the pH of aqueous solution and there by affect the enzyme activity [50]. Laccase activity was completely inhibited by the common metalloenzyme inhibitor, sodium azide (NaN_3) at the concentration of 20 μM. It was reported that the binding of sodium azide to the types 2 and 3 copper sites affects internal electron transfer, thus inhibiting the activity of laccase [51]. *T. harzianum* laccase was mildly inhibited by the metal chelator EDTA (5 mM) and more strongly inhibited by the copper chelator diethyldithiocarbamic acid (DDC) at 1 mM concentration.

Table 3
Kinetic parameters for laccase from *T. harzianum*

Substrate	Wavelength (λ_{max}, nm)	Assay pH	Molar extinction coefficient ε (M^{-1} cm^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	V_{max}/K_m
ABTS	436	4.5	29,300	180	3.95	21.94
Guaiacol	470	4.5	12,000	60	1.42	23.66

Table 4
Effect of inhibitors on laccase activity

Inhibitor	Concentration	% Inhibition
Diethyldithiocarbamic acid (DDC) (mM)	0.1	25.9
	0.2	34.5
	0.5	59.2
	1.0	99.8
Ethylenediaminetetraacetic acid (EDTA) (mM)	1	16.8
	5	40.3
	10	62.8
	25	94.5
Ethanol (%)	10	3.4
	20	8.9
	50	48.9
	70	100
Sodium azide (NaN_3) (μM)	2	14.0
	5	38.7
	10	69.2
	20	100
Thioglycolic acid (TGA) (μM)	100	13.3
	250	32.6
	500	54.1
	750	92.4

Laccase activity was strongly inhibited by metal specific chelators rather than by general metal chelators.

3.9. Effect of metal ions on laccase activity

The interaction of metals with extracellular laccase was particularly important for the better understanding of the biotechnological processes of xenobiotic degradation. Therefore, the stability of laccase activity against several metal compounds was tested (Table 5). Metals such as Co, Sn, Hg, Fe, K, Zn, Mg, Mn, Na, Ba, Cr and Ca were assessed. Metal ion concentration of 1 mM had no significant effect over laccase activity except Hg, which caused 17.2% inhibition. When the metal ion concentration was increased to 5 mM, Cr, Zn and Sn inhibited laccase activity by 13.8, 11.4 and 9.7%, respectively; whereas the laccase activity was highly sensitive to 5 mM Hg showing 25.4% inhibition, indicating the presence of thiol groups, essential for its activity. The purified laccase from the edible mushroom *Lentinula edodes* was inhibited in the presence of 1 mM Sn^{2+} (99%), Ca^{2+} (70%), Zn^{2+} (64%), Hg^{2+} (55%), K^{+} (54%) and Cd^{2+} (45%) and it was activated by 40% in the presence of 10 mM Cu^{2+}. The activation of laccase by Cu^{2+} may be due to the filling of type-2 copper binding sites with copper ions [45]. The observations indicated that the effect of metal ions on laccase activity was highly dependent on its source and the type of metals used, which had a great influence on the catalytic activity of the enzyme. The activation or inhibition of proteolytic enzymes by metals could change the turnover rate of extracellular enzymes.

Table 5
Effect of metal ions on laccase activity

Metal ion	Concentration (mM)	% Inhibition
Cobalt	1	1.8
	5	3.6
Tin	1	1.1
	5	9.7
Mercury	1	17.2
	5	25.4
Ferric	1	1.5
	5	2.0
Potassium	1	3.3
	5	3.9
Zinc	1	7.6
	5	11.4
Magnesium	1	1.4
	5	2.8
Manganese	1	0.8
	5	1.2
Sodium	1	Nil
	5	Nil
Barium	1	Nil
	5	0.6
Chromium	1	1.7
	5	13.8
Calcium	1	0.6
	5	3.5

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