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Biochemical characterization of three phase partitioned laccase and its application in decolorization and degradation of synthetic dyes

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ABSTRACT

Growing laccase utilization in numerous biotechnological applications encourages the search for high benefit/cost ratio purification methods such as Three Phase Partitioning (TPP). TPP was employed to the crude extract of *Pleurotus ostreatus*, gave yielded nearly 161% with 27.8 fold purity. The specific activity of the TPP purified laccase was 91 U/mg and the molecular mass of isozymes was estimated to be 72 kDa and 40 kDa. An extracellular laccase acted optimally at pH 6.0 and exhibited an optimum temperature of 45 °C. The enzyme was able to sustain its activity even at elevated temperatures (50–60 °C) for extended periods (2–3 h). Laccase displayed a high affinity towards syringaldazine and oxidizes other phenolic compounds. This laccase was activated by Mn^{2+} and glycerol, but completely inhibited by the classic laccase inhibitors, oxalic acid, Hg^{2+}, Pb^{2+} and Fe^{2+}. The purified laccase could efficiently decolorize triphenyl methane (TPM), azo and indigo dyes without addition of redox mediators. The biodegradation of malachite green (MG) was monitored by UV–vis, IR-spectroscopy and HPLC. The thermostable and rapid dye decolorizing property makes it an attractive and promising biocatalysts for the environment biotechnological applications.

1. Introduction

Oxidative enzymes in the lignolytic systems of white-rot fungi are the most important class of enzymes suited for enzymatic industrial pollutants degradation [1–3]. They can be produced on a large scale and may be applied in crude form [4]. Besides peroxidases [5], laccases seem to be the most promising enzymes. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are copper-containing oxidases that accept a wide substrate range, which can serve industrial purposes and/or bioremediation processes [4]. The simple requirements of laccase catalysis (no cofactor requirement, presence of substrate and O_2), as well as its apparent stability and lack of inhibition (as has been observed with H_2O_2 for peroxidase), make this enzyme both suitable and attractive for industrial applications [6]. Laccases have numerous industrial applications, e.g. for dye decolorization, for biological bleaching in pulp and paper industries, synthesis of new hybrid molecules and biomaterials, detoxification of recalcitrant biochemicals, ethanol production, coal solubilization, and food and beverage industry [4,6–8].

The practical applications of laccases in industrial biotechnology are broadening, it is important to discover new strains producing laccase and isolating novel laccase with different physicochemical and catalytic properties [9]. In our screening studies on laccase activities of various fungi, *Pleurotus ostreatus* showed remarkably high activities and found to be capable of decolorizing different groups of synthetic dyes. Although the laccases from *P. ostreatus* have been already well characterized [10], there is still a lack of information on the enzyme's kinetic properties and factors that influence stability. Enhancing the stability and maintaining the desired level of activity over long period of time are two significant points, which are taken into account for the selection of laccases in industrial applications. The purpose of this work is to present a concise study of the laccase of *P. ostreatus* showing biochemical characteristics and parameters that improve the activity and stability of this metallo-enzyme.

Synthetic dyes are being increasingly used in the textile, paper, cosmetics and pharmaceutical industries. These compounds chemically classified as anthraquinone, azo, heterocyclic, triphenylmethane (TPM) dyes, cause serious environmental pollution. Most of them are toxic, mutagenic and carcinogenic. Moreover, they are resistant to degradation. At present, a number of studies have focused on white-rot fungi that can decolorize and biodegrade these dyes [1–3,11]. The most recent research in this area has focused on the enzymatic process for the treatment of wastewater and soil [12,13]. The potential advantages of the enzymatic treatment, as compared with conventional treatments, include from application to recalcitrant materials, operation at high and low contaminant concentration over a wide pH, temperature and salinity range, the easy control process among others and less sludge

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formation. The process of dye decolorization based on laccase is an efficient method and is attracting increasing interest [4]. By means of enzymatic catalyzed oxidative reactions, laccase can detoxify phenolic contaminants, such as aromatic amines, to harmless/less harmful products [12].

Despite many attractive features, the enzymatic decolorization of dyes or effluents is limited at present because of the following factors: (1) procurement of enzymes is expensive for processing large volumes of effluent; (2) the inability of the available enzyme preparations to complete decolorization; and (3) lack of commercially viable processes for large-scale purification of enzyme and decolorization of textile effluents [14]. Current trends in down stream processing of laccase have conventional purification procedures such as ammonium sulfate precipitation or ultrafiltration, followed by anion exchange chromatography and gel filtration [15]. Although these methods yield a quality product they are unscalable, expensive, time consuming and have multi-step protocol. Use of robust and inexpensive commercial laccase product with high prospecting means that the major barriers that restrict use of enzymes in waste water treatment can be overcome. TPP is a relatively novel bioseparation technique, which employs the collective operation of principles involved in numerous techniques for proteins precipitation [16,17]. It has been used for the extraction and purification of enzymes and inhibitors [16–22]. The suitability of laccases for TPP purification has been known very recently [20] and successful results achieved have been well documented by our group [22].

TPP is being employed today at process scale by a few industries for protein recovery. However, in spite of the definite advantages of the technique, the large-scale application of TPP of laccase has not been exploited and needs to be given importance. Further investigations on laccase purification by TPP at a pilot scale could be highly beneficial. In this present study, we have shown the pilot scale TPP for purification of laccase and its biochemical characterization. We have also investigated the feasibility of utilizing TPP purified laccase for the decolorization of structurally different dyes. The reported biochemical properties of the purified laccase and their rapid decolorizations of dyes suggest that it might be suitable for industrial wastewater bioremediation.

2. Materials and methods

2.1. Chemicals and microorganism

2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and syringaldazine were obtained from Sigma chemical Co., St Louis, USA. The TPM and azo dyes were obtained from the Himedia India Private Limited, India. All chemicals used were of the highest purity available and of the analytical grade. *P. ostreatus* was obtained from Tamil Nadu Agriculture University (TNAU), Coimbatore, India and maintained on Malt extract agar slants and stored at 4 °C [21].

2.2. Production and purification of laccase

Previously, we showed that *P. ostreatus* has ability to produce laccase and it was further purified using TPP [21–23]. Pilot scale solid state fermentation was carried out in enamel coated metallic trays (45 × 30 × 4 cm) containing KOH treated 300 g of Sago hampas moistened with 300 mL mineral salt solution (1.0:1.0 w/v) composed of malt extract 1%; yeast extract 0.4%; glucose 0.4%; 0.1% veratryl alcohol; 10 mM $CuSO_4$; 5 mM L-tyrosine and the initial pH of the cultures were 6.3, autoclaved and inoculated with *P. ostreatus* 10% (w/v). The contents of the trays were mixed before and after inoculation. The trays were covered with aluminium foil and incubated in a temperature control chamber at 30 °C. At selected times duplicate trays were taken out of the chamber, buffer was added to the fermented mass, the suspensions were stirred and centrifuged at 10,000 × *g* for 30 min and the supernatant was filtered through Whatman No. 1 filter paper and the enzyme activities were determined in the filtrate. Three phase partitioning (TPP) was performed in batch type (small scale – 50 ml centrifuge tubes; pilot scale – 5 l separation flasks). The filtrate (crude enzyme) was saturated with 60% (w/v) of ammonium sulfate and *t*-butanol was added in the ratio of 1.0:1.8 (v/v) and the tubes were kept at 42 ± 3 °C for 1 h for complete phase formation [22]. The tubes were centrifuged at 2000 × *g* for 10 min and the three phases formed were collected separately. The enzyme is usually precipitated in the middle layer. To enrich the enzyme concentration, the interfacial precipitate was collected and adjusted to pH 4.0 (0.1 M acetate buffer).

2.3. Effect of pH on activity and stability of the purified laccase

The effect of pH on the activity of the purified laccase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 9.0 using 0.1 M of the following buffer systems: acetate (3.0, 4.0, and 5.0), phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0 and 9.0) buffer systems. The pH stability of *P. ostreatus* laccase was investigated in the pH range of 3.5–7.5. Therefore, 1 ml of the enzyme was mixed with 1 ml of the buffer solutions mentioned above and incubated at 40 °C for 60, 120, 180, 240 and 300 min. Afterwards aliquots of the mixtures were taken to measure the laccase activity (%) with respect to control, under standard assay conditions.

2.4. Effect of temperature on activity and stability of the purified laccase

The effect of temperature on the activity of the purified laccase was determined by performing the standard assay procedure at different temperatures ranging from 28 to 75 °C (28, 37, 40, 45, 55, 65 and 75 °C). Before the addition of enzymes, the substrate (0.2 mM) syringaldazine was preincubated at the respective temperature for 10 min. Thermal stability studies were conducted without any additives. The enzyme solution was incubated at various temperatures ranging from 25 to 60 °C (25, 37, 45, 50 and 60 °C) in a temperature-controlled water bath for 15–16 h and the relative enzyme activity was measured at regular intervals of time. The relative activity was calculated as the percentage ratio of activity at a given temperature to the activity at optimum temperature.

2.5. Effect of metal ions, organic acids and various compounds on the laccase activity

Metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Fe^{2+}), organic acids (citric acid and oxalic acid) and various compounds (EDTA, glycerol, SDS and β-mercaptoethanol) were tested for their effect on purified laccase activity in phosphate buffer, pH 6.0. The enzyme was preincubated for 30 min at 40 °C with 1 mM and 10 mM of listed metal ions as a final concentration prior to the substrate addition.

2.6. Protein determination and enzyme activity

Protein concentration was determined by the Lowry's method using bovine serum albumin as the standard [24]. The laccase activity was determined using ABTS as the substrate [22]. Enzymatic activity was expressed as 1 U = 1 μmol of ABTS oxidized per min at 45 °C (±1). The kinetic constants (V_{max} and K_m) were determined using Lineweaver–Burk double reciprocal ($1/v$ versus $1/S$)

plot, where different concentrations of syringaldazine, ABTS and guaiacol were used as substrate (0.1–1 mM) at pH 6.0 and 40 °C.

2.7. Laccase purity analysis using RP-HPLC and SDS-PAGE

Purity of the enzyme was confirmed by RP-HPLC (Reverse Phase–High Performance Liquid Chromatography) analysis using Agilent 1100 HPLC system. RP-HPLC was carried out according to the method of Divakar et al. [25]. The purified laccase was applied on to C-18 column (Zorbax C-18, 4.6 mm × 250 mm i.d., 5 μm particle size, Agilent technologies). For calculation of the protein molecular mass, SDS-PAGE was carried out with a 10% gel, as described by Laemmli [26]. After running the gel, the proteins were stained by Coomassie brilliant blue R-250.

2.8. Laccase spectroscopic studies and determination of copper content

Purified enzyme was concentrated by lyophilization and dialyzed against deionized water. The concentration of purified laccase preparation was adjusted to 1 mg protein per ml in 0.1 M phosphate buffer (pH 6.0). The absorption spectrum of purified laccase preparation was determined at room temperature (28 °C) on Shimadzu UV-1800 spectrophotometer (Tokyo, Japan) in the range of 300–700 nm. The copper content of the protein was determined by atomic absorption spectrometry, using Shimadzu AA-6300 Model atomic absorption spectrophotometer.

2.9. Decolorization of azo and TPM dyes by purified laccase

The concentration of MG, fuchsine, crystal violet, methyl green, indigo carmine, methylene blue, reactive green, reactive yellow, reactive orange, congo red, reactive violet, diamond black and methyl red in the experimental solution was determined from the calibration curve prepared by measuring absorbance of different determined concentrations of dye solutions at λ_{max} 618 nm, 570 nm, 592 nm, 632 nm, 610 nm, 490 nm, 580 nm, 410 nm, 525 nm, 497 nm, 607 nm, 540 nm and 520 nm, respectively using a UV-1800 UV-visible Spectrophotometer (Shimadzu, Japan). The pH of solution was measured with a Hanna pH meter using a combined glass electrode (Model HI 9025C, Singapore).

Decolorization of dyes was examined using the purified *P. ostreatus* laccase. Unless otherwise indicated, all experiments were performed in 3 mL disposable cuvettes in a 2 mL final reaction volume. The reaction mixture contained 0.1 M acetate buffer pH 4.5, initial dye concentration (100 ppm for azo dyes; 1000 ppm for TPM dyes), and 10 U ml^{-1} purified laccase. The reaction was initiated by the addition of laccase and incubated in the dark at 30 °C. Decolorization of dyes was followed by measuring the λ_{max} using UV–vis spectrophotometer at 10 min intervals. Controls used heat killed enzyme solution whereas blanks contained all components of the reaction mixture except the dyes. All experiments were performed in duplicate.

2.10. Decolorization and degradation of MG by purified laccase

One TPM based dye, MG (λ_{max} 618 nm) was used in this study. The decolorization reaction was carried out at 37 °C for 2 h in 2 ml reactions mixture containing 1000 mg l^{-1} dye prepared in 0.1 M acetate buffer (pH 5.0) and 10 U ml^{-1} purified laccase. Control containing heat-denatured enzyme was used to measure decolorization of dye at different time interval. The decolorization was monitored by scanning the UV–vis spectrum between 400 and 800 nm using Shimadzu UV-1800 spectrophotometer (Tokyo, Japan). The laccase decolorized MG solution was centrifuged at 1000 × *g* for 1 min and the metabolites of MG were extracted by adding ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and solvent was evaporated in rotary evaporator. The dried residues obtained were dissolved in small volume of HPLC grade methanol and the sample was used for further analysis. The biodegraded metabolites were characterized by Fourier Transform Infrared Spectroscopy (FTIR) (PerkinElmer, Spectrum one) and compared with control dye. The FTIR analysis was done in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. HPLC (High performance liquid chromatography) analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector, using C_{18} column (4.6 × 250 mm) and HPLC grade methanol as a mobile phase.

2.11. Phytotoxicity studies

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye. The ethyl acetate extracted products of MG degradation were dried and dissolved in 10 ml sterile distilled water to make a final concentration of 1000 ppm for phytotoxicity studies. The phytotoxicity study was carried out (at room temperature) on two kinds of seeds commonly used in the Indian agriculture *Triticum aestivum* and *Phaseoulus mungo* (10 seeds) by watering separately 10 ml sample of control MG and its degradation products (1000 ppm) per day. Control set was carried out using distilled water at the same time. Germination (%) as well as the length of root and shoot was recorded after 7 days.

3. Results and discussion

3.1. Purification of laccase

TPP is an efficient protein purification technique, where remarkable increase in the yield of various enzymes has been reported [16,18,19,27]. The overall purification of laccase by TPP is given in Table 1. TPP was applied to the crude extract (20 ml) of *P. ostreatus*, wherein 161.4% laccase yield, 27.8 fold purity and specific activity of 91 U/mg of protein were observed in the interfacial precipitate. The molecular mass of laccase isozymes was estimated by SDS-PAGE and found to be 72 kDa and 40 kDa (Fig. 1) which is similar to that of the previously reported results [33,34]. Purity of the laccase enzyme was also confirmed by RP-HPLC analysis, which got eluted

Table 1
TPP of laccase from *Pleurotus ostreatus*.

Steps	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude	126.2	38.6	3.27	1	100
TPP-Precipitate[a] 20 ml scale	203.7	2.24	90.93	27.8	161.4
TPP-Precipitate[a] 2 l scale	17360	203	85.5	26.1	137.5

[a] The crude extract of *Pleurotus ostreatus* laccase (20 ml containing 126.2 U) was brought to 60% (w/v) ammonium sulfate saturation. This was followed by addition of ratio of crude extract to *t*-butanol in a ratio of 1:1.8 (v/v) (crude extract to *t*-butanol) and the reaction mixture was incubated at 42 ± 3 °C. Three phases formed were collected separately. The upper phase was removed and then the lower aqueous phase and interfacial precipitate were tested for enzyme activity and protein amount. The interfacial precipitate (7 ml containing 203.7 U) solution was adjusted to pH 4.0. Each experiment was carried out in duplicate and the difference in the readings was less than ±5%.

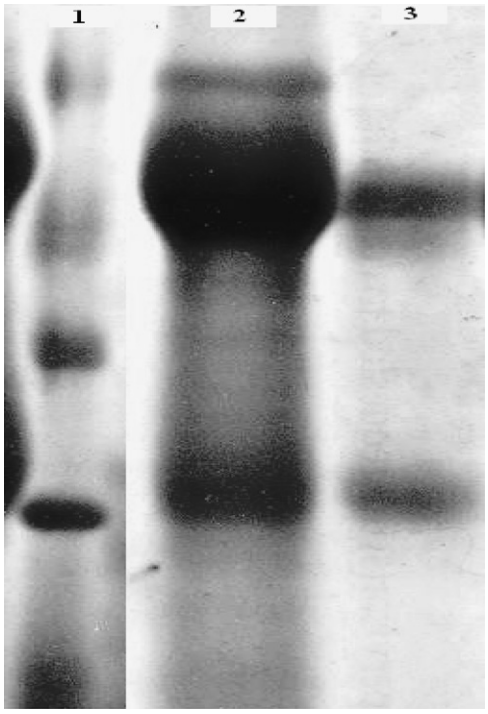

Fig. 1. SDS-PAGE of TPP purified laccase.

as a double peak with retention time of 12.42 and 14.09 min in a reverse phase C-18 column as shown in Fig. 2. To test the validity of the outcome found in the small scale TPP purification, we subjected the procedure to a 100-fold scale up. In comparison with the small scale, the scaled-up method resulted in a similar purity but with a small decrease in yield which may be attributed to the stirring and settling procedures. The reduction of unit operations with a higher enzyme recovery from the TPP process is the major advantage when compared to that of the multi-step chromatography process. Although TPP produces a less favorable increase in the purity of laccase compared to chromatography process, this difference is negligible when the cost aspect is taken into account. A complete comparison of the TPP with other techniques has also been made (Table 2). It can be seen from the table that the present work is an excellent technique for purification of laccase. Cheap, simple and efficient laccase purification procedures are proving essential to make wastewater bioremediation economically feasible, and it can be concluded that TPP is a one such highly promising method.

3.2. Effect of pH on laccase activity and stability

The effect of pH on the laccase activity is presented in Fig. 3. As it can be observed, the enzyme was active over a broad pH range, displaying over 62% of its activity in the pH range of 4.0–8.0 with an optimum pH of 6 using syringaldazine as substrate. A further variation from these pH values decreased the relative activity down to 28%. For ABTS, an optimum pH was 4.0 and the lower relative activity was obtained at pH 7.0. These findings were in correlation with several earlier reports for *P. ostreatus* laccase, showing pH optima of 6.0–6.2 for syringaldazine and pH optima of 3.0–3.6 for ABTS [10,35]. The majority of fungal laccases have been found to function as laccase under mild acidic conditions (pH 4–6) [36,37]. The high relative activity in the neutral pH region in addition to the acidic pH optima will open up new application windows for the current purified laccase. Investigations on pH stability by incubating the enzyme in suitable buffer systems for various incubation times at 40 °C (Fig. 3) revealed, that the laccase enzyme was very stable at the broad pH range of pH 4–8. After 5 h of incubation, laccase maintained 97% of relative activity at its optimum pH of 6. However, the stability was significantly reduced to 22% of pH 8. It is reported that the inactivation process is found to be faster at high alkaline

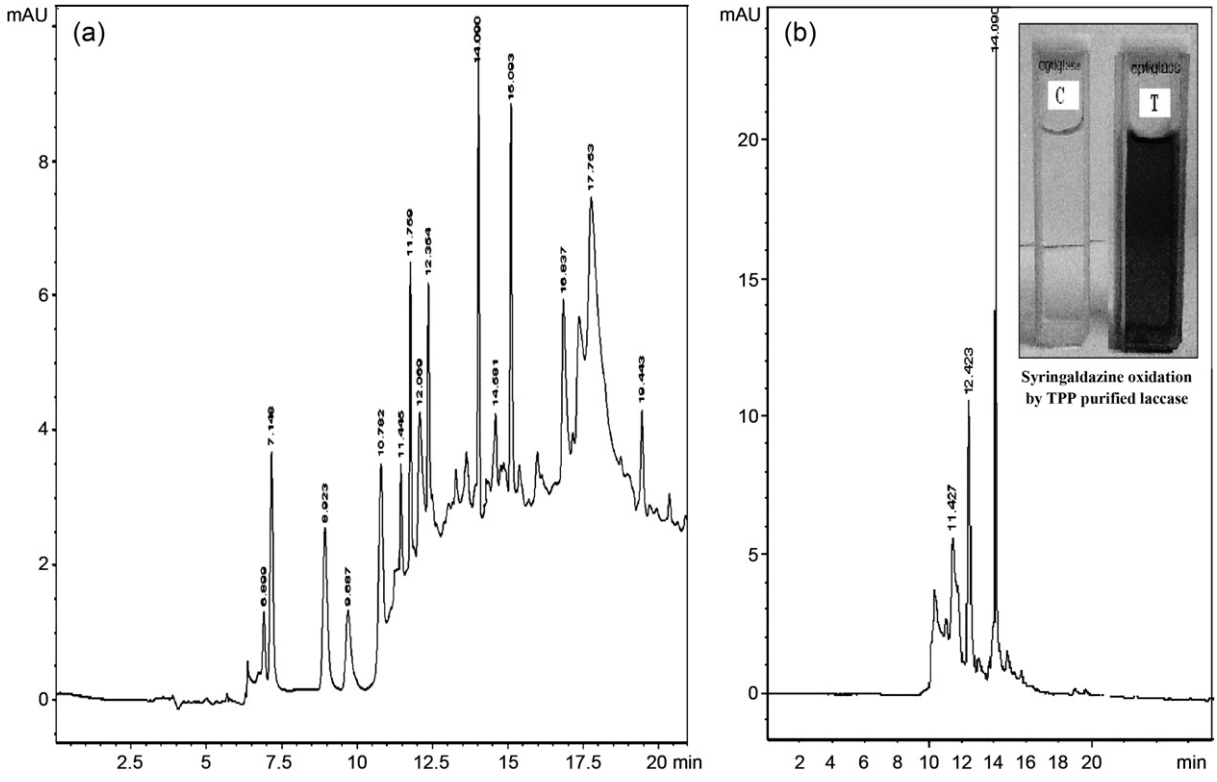

Fig. 2. RP-HPLC of crude and purified *Pleurotus ostreatus* laccase.

Table 2
Comparison of the previously reported processes for the laccase purification from various sources.

Micro organism	Unit operations	Yield (%)	Purity (fold)	References
Pycnoporus sanguineus	UF	87	2.14	[28]
Ganoderma sp.	DF	90.4	3.7	[29]
Tramates sp.	FF	72	11.7	[30]
Agaricus bisporus	ATPS	95	2.48	[31]
Ganoderma sp.	TPP	60	13.1	[20]
Pleurotus ostreatus	TPP	161	27.8	Present study
Pleurotus ostreatus	ASP–IMAC	57.4	46	[32]

UF – Ultrafiltration; ATPS – Aqueous Two Phase System; DF – Diafiltration; ASP – Ammonium sulpahte precipitation; FF – Foam fractioning; IMAC – Immobilized metal affinity chromatography.

pHs due to disulfide exchange, which usually occurs at near neutral and alkaline conditions [36,37]. Therefore acid stable laccase characterized in this study can be used for the decolorization and degradation of acidic (pH 3.8–4.4) wool dyeing effluent. In addition, with the broad pH stability range, this enzyme can be considered suitable for applications requiring long exposure times.

3.3. Effect of temperature on laccase activity and stability

The *P. ostreatus* laccase was active over a broad temperature range of 28–75 °C with an optimum temperature of 45 °C (Fig. 3). It retained more than 60% of its activity at 60 °C. This optimum temperature was in agreement with the studies conducted by several authors using different strains. For example Xiao et al. found the optimum temperature of laccase from *Trametes* sp. as 45 °C [33], similarly, maximum activity for *P. ostreatus* laccase was determined at 45 °C [38]. Furthermore, the laccase from *Cyathus bulleri* and *Volvariella volvacea* was optimally active at 45 °C as well [39,40]. The catalytic activity of fungal laccases is optimum in the temperature range between 30 and 55 °C [36,37]. The thermostability of the TPP purified laccase was measured during incubation at different temperatures (25–60 °C). As seen from Fig. 3, the increase in temperature and time causes an overall increase in the stability. The enzyme was stable at 25 °C (ambient temperature) for a longer duration with no inactivation at 37 °C, as activity was stable up to 4 h. At 37 °C the enzyme showed 50% inactivation after 14 h incubation. Thermostability results from co-action of four Cu ions in laccases, and internal protein features, such as existence of ionic bonds (salt bridges) and dense hydrogen bonding network [41]. At its optimum temperature 45 °C, the rate of inactivation was

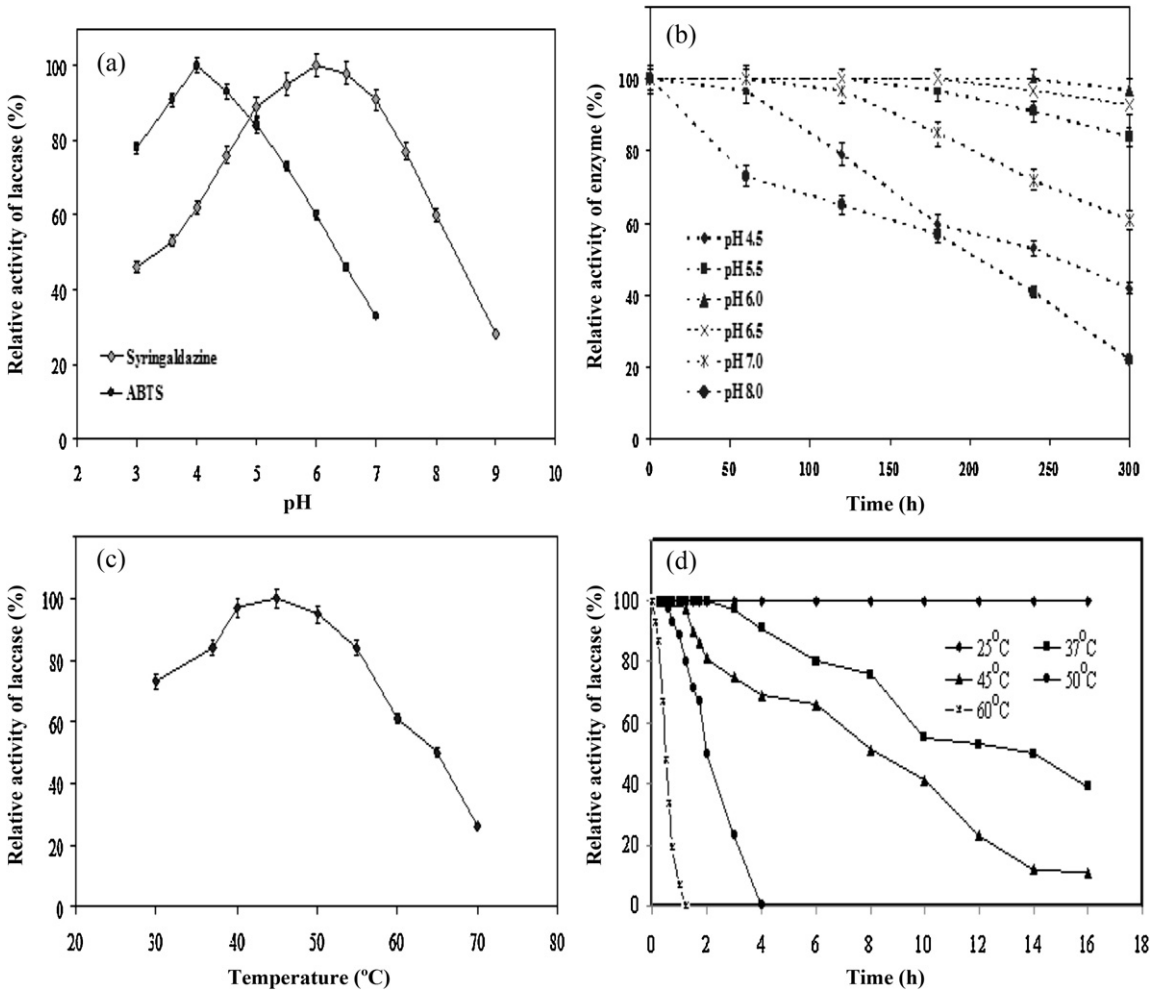

Fig. 3. Effect of pH and temperature on activity and stability of laccase from *P. ostreatus*.

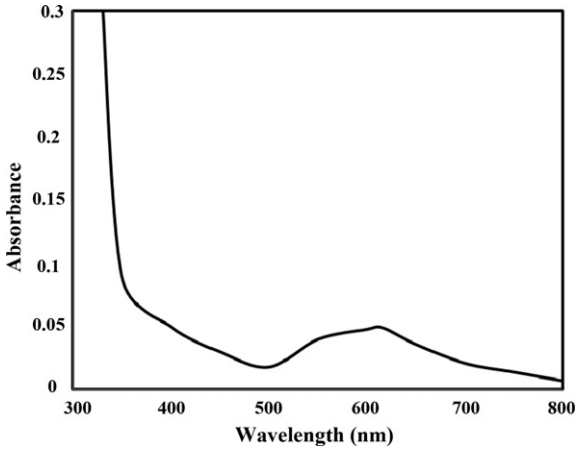

Fig. 4. UV–vis absorption spectrum of *P. ostreatus* laccase.

higher, with a calculated half-life of 8 h. At 60 °C the half-life was 30 min (Fig. 2). Fungal laccases usually rapidly drop their activity at temperatures above 60 °C [36].

3.4. Kinetics of laccase

The kinetics parameters K_m were determined by measuring the activity of the laccase at different substrate (guaiacol, ABTS, syringaldazine) concentrations ranging from 0.1 to 1 mM. The parameters were determined by fitting the experimental data to the Michaelis–Menten model. The K_m value of guaiacol, ABTS, syringaldazine were 0.69 ± 0.11, 0.13 ± 0.02 and 0.051 ± 0.01 mM, respectively. The estimated K_m values which indicate the affinity of the *Pleurotus pulmonaris* laccase towards the same substrates are 0.55, 0.21 and 0.012 mM, respectively which seem to be slightly differ from earlier reports [42]. This indicates that the current laccase has a higher affinity. While several of the compounds tested were oxidised by the laccase, syringaldazine appeared to be the best substrate.

3.5. Spectral characteristics of laccase and its copper content

The nature of the catalytic center of *P. ostreatus* laccase was determined spectrophotometrically in native form (Fig. 4). The laccase monomer generally requires four copper atoms for functional catalytic activity. The UV–vis spectrum of the purified native laccase showed an absorption peak at 610 nm, typical for the type I Cu^{2+} center that is responsible for the blue color of the enzyme. The shoulder at around 326 nm suggests the presence of the type III binuclear Cu^{2+} center. The spectral characteristic obtained in the present study was similar to previous studies [43,44]. The purified laccase contained 6.1 μg copper per mg enzyme, determined by atomic absorption spectrometry, which corresponds to 3.66 copper atoms per molecule. The copper content of the *Pleurotus florida* laccase was 3.7 molecules of copper per molecule of protein [34]. Similarly, *Streptomyces griseus* laccase showed the presence of 0.3 μg of copper per 45 μg of protein, which corresponds to a content of four copper ions per subunit [45].

3.6. Effect of metal ions on laccase activity

The effect of metal ions which are concentration dependent could be important, where textile effluents are high salt content. As it can be observed from Table 3, the enzyme was slightly inhibited in the presence of 2 mM Mg^{2+}, Zn^{2+} and Cu^{2+} whereas K^+, Na^+, Co^{2+} and Cd^{2+} did not have any profound effect. The effect of these metal ions was concentration dependent. For example increasing the concentration of metal ions from 2 to 10 mM, reduced the enzyme activity by many folds. Furthermore the enzyme activity was activated by 2 mM of Mn^{2+} ions, and completely inhibited in the presence of 10 mM Hg^{2+}, Pb^{2+} and Fe^{2+}. Similar results were obtained by various authors [15,46,47], which indicated the key role of the thiol groups in laccase activity. Ferric and lead ions (2 mM) inhibit the activity of laccase to 90% and 89%, respectively (Table 4). A similar Fe^{2+} inhibitory effect was reported by *Paraconiothyrium variabile* [15]. The activity of laccase in the presence of copper decreased up to 32% at 2 mM concentration, whereas at 10 mM 61% inhibition was obtained. Although laccase is a copper containing protein, excess supply of Cu^{2+} ions might cause a change in laccase structure. This phenomenon was also reported in the laccase from *P. ostreatus* K16-2 [44].

Table 3
Effect of metal ions and various compounds on the activity of the purified laccase.

	Relative activity (%)	
Control	100	
Metals ions (mM)	2	10
Na^+	100 ± 1.60	100 ± 1.19
K^+	100 ± 1.30	100 ± 0.91
Mn^{2+}	132 ± 3.97	120 ± 1.14
Cu^{2+}	68 ± 7.12	39 ± 5.40
Mg^{2+}	87 ± 3.10	59 ± 1.82
Fe^{2+}	9 ± 0.95	2 ± 0.60
Pb^{2+}	11 ± 0.82	3 ± 0.90
Zn^{2+}	87 ± 2.14	63 ± 1.77
Hg^{2+}	ND	ND
Co^{2+}	100 ± 1.85	81 ± 7.40
Cd^{2+}	100 ± 2.11	90 ± 3.86
Glycerol	98 ± 3.19	193 ± 1.80

After pre-incubation of enzyme with metal ions and chemical reagents at different concentrations at 30 °C for 30 min, the remaining enzyme activity was measured. Activity without added metal ions taken as 100% activity. Data represent the means of three determinations ± SE. ND – Not detected.

3.7. Effect of various compounds on laccase activity

Substances that inhibit laccase are of great consequence to the industrial application of the enzyme. If they are present in sufficient concentrations enzymatic degradation of the substrate will not occur. Laccase inhibition may occur through amino acid residue modification, copper chelation or conformational change of the enzyme [48]. The effects of a number of possible laccase inhibitors are shown in Table 3. Among the different inhibitors assessed the

Table 4
Effect of various compounds on the activity of the purified laccase.

Compounds	Concentration (mM)	Inhibition (%)
EDTA	10	29 ± 1.24
	100	77 ± 2.93
Sodium azide	0.001	68 ± 3.77
	0.005	N.D
Oxalic acid	10	90 ± 7.3
Citric acid	10	67 ± 2.91
L-cysteine	0.1	80 ± 0.82
	0.25	N.D
1,4 Dithiothretiol	0.01	65 ± 2.07
	0.1	N.D
SDS	(0.01%)	N.D
β-Mercaptoethanol	(0.1%)	91 ± 1.01

After pre-incubation of enzyme with compounds at different concentrations at 30 °C for 30 min, the remaining enzyme activity was measured. Data represent the means of three determinations ± SE. ND – Not detected.

most potent was sodium azide (NaN_3), and complete inhibition was obtained at 5 μM concentration. The binding of sodium azide to the types 2 and 3 copper sites effects internal electron transfer, thus inhibit the activity of the laccase [48]. Laccase was inhibited by copper chelators such as EDTA, oxalic acid and citric acid the % of inhibition being 29%, 90% and 67% respectively at a concentration of 10 mM. SDS a protein denaturant inhibits enzyme activity of the laccase completely, whereas β-mercaptoethanol decreases the enzyme activity suggesting that disulphide linkages have a critical role in maintaining an appropriate conformation of the enzyme for its catalytic activity. In the study conducted by Kiiskinen et al., it has been reported that the activity of laccase was inhibited strongly by SDS, sodium azide and L-cysteine [9]. These results are in good agreement with our results. The current results are also comparable to a study conducted by *P. variabile* laccase [15]. The shelf life of purified enzymes remains an important exploratory field, requiring the development of not only stable, but also economical and eco-friendly formulations. Glycerol which is mostly used as protective agent in preservation promoted laccase activity significantly with an increase in the concentration from 1 to 10 mM. Increasing the concentration of glycerol to 10 mM resulted in 1.99 fold increase in activity. Therefore, glycerol can be recommended for preservation of laccase enzyme during storage.

3.8. Decolorization of azo and TPM dyes using purified laccase

The decolorization of various dyes with different structural patterns was investigated using pure laccase from *P. ostreatus*. Our system was able to efficiently degrade a number of commercial textile dyes at pH 4.5. The addition of redox mediators was not necessary for the decolorization process in our study. TPM dyes represent an especially recalcitrant class of compounds [49]. The present study confirms the ability of TPP purified laccase to decolorize TPM dyes with decolorization efficiency of more than 90% in short time (Fig. 5). This could be due to the presence of electron donating methyl and methoxy groups on the TPM dyes. Fuchsine and MG was very efficiently decolorized with enzymatic treatment, showing complete absence of color in the treated sample. The results obtained in this study were in agreement with results reported previously for *P. variabile* laccase [15], *C. bulleri* laccase [50], *Pycnoporus sanguineus* laccase [51] and *Trametes* sp. SQO1 laccase [52]. They also demonstrated that different decolorization rates were attributed to the specific catalytic properties of the individual enzymes and to the structure of dyes. With respect to the molecular structure, all the TPM based dyes were oxidized regardless of whether dissolved in water or buffer.

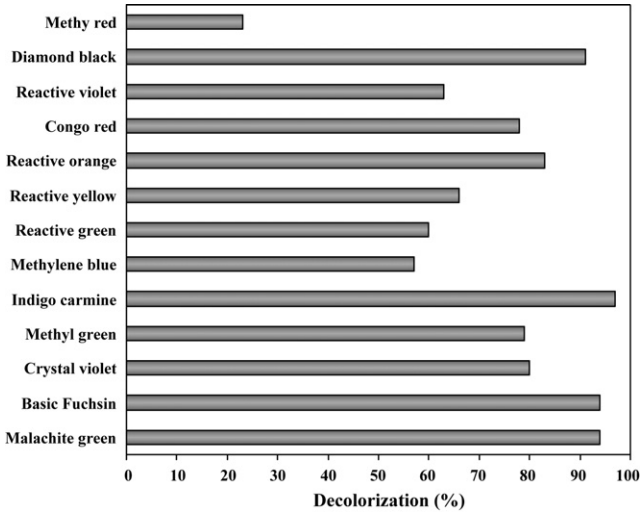

Fig. 5. Decolorization efficiency of laccase on various types of dyes.

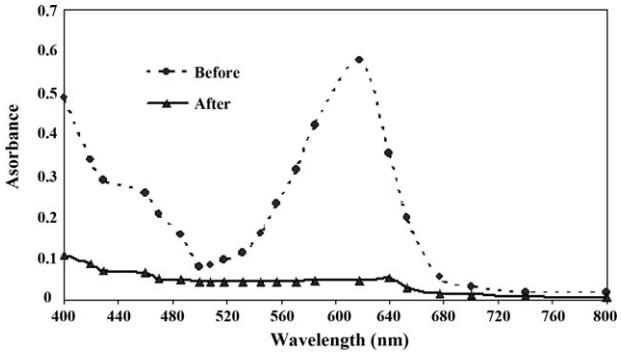

Fig. 6. UV–vis absorbance spectrum of undecolorized MG and decolorized in the presence of purified laccase.

The most employed dyes belong to the azo class which accounts for the 70% of all textile dyes produced. The best decolorization overall (80–97%) was obtained with reactive orange, congo red, indigo carmine and diamond black (Fig. 5). Within a 100 min period these dyes appeared to be completely degraded due to the laccase activity. Somewhat lower decolorization (20–70%) was obtained with methyl red, reactive violet, reactive yellow 2, reactive green and methyl green. Laccases modify azo dye structures by destroying their chromophoric assemblies, phenoxyl radicals are generated in the reaction course [53]. The decolorization of numerous dyes of different structural patterns was studied using a laccase from *Trametes modesta* [54]. The results obtained in this study were in agreement with results reported previously for *Pyricularia oryzae* laccase [53], *P. sanguineus* laccase [51] and *Trametes* sp. SQO1 laccase [52]. From an industrial point of view it would be simpler to add a laccase solution directly into the wastewater. An attractive feature of this enzyme is the fact that it does not require redox mediators to function efficiently in dye biotransformation. Consequently, the laccase from *P. ostreatus* which is very stable in the acidic pH range may prove to be successful on an industrial scale. Thus TPP purified laccase could be successful employed for the treatment of TPM, azo and indigo dyes bearing industrial wastewater as it has prominent capacity to degrade other different dyes.

3.9. Analysis of metabolites resulting from decolorization and biodegradation of MG by purified laccase

In order to disclose the possible mechanism of dye decolorization, we have also analysed the products of biotransformation of MG by UV–vis spectroscopy, FTIR and HPLC [55,56]. UV–vis spectroscopy is the primary technique to determine dye decolorization. For purified laccase, UV–vis scan (400–800 nm) of enzyme treated dyes withdrawn at different time interval indicated decolorization and decrease in dye concentration (Fig. 6). Peak observed at 618 nm (0 h) decreased without any shift in λ_{max} upto complete decolorization of the dye (2 h) incubation. Evidence of the removal of dye can be observed with absorbance at λ_{max} being virtually zero after 2 h and an increase in absorbance towards UV region. The absorbance peaks, corresponding to dyes, diminished indicating that the dye had been removed. The decrease of absorbance peak of dyes indicated a rapid degradation of the dye. Similar to our work, many researchers have shown the decolorization of industrially important dyes by laccase [55].

The nature of degradation products was confirmed from FTIR analysis. Comparison of FTIR spectrum of control dye with metabolites extracted after complete decolorization clearly indicated the biodegradation of parent dye compound by purified laccase (Fig. 7).

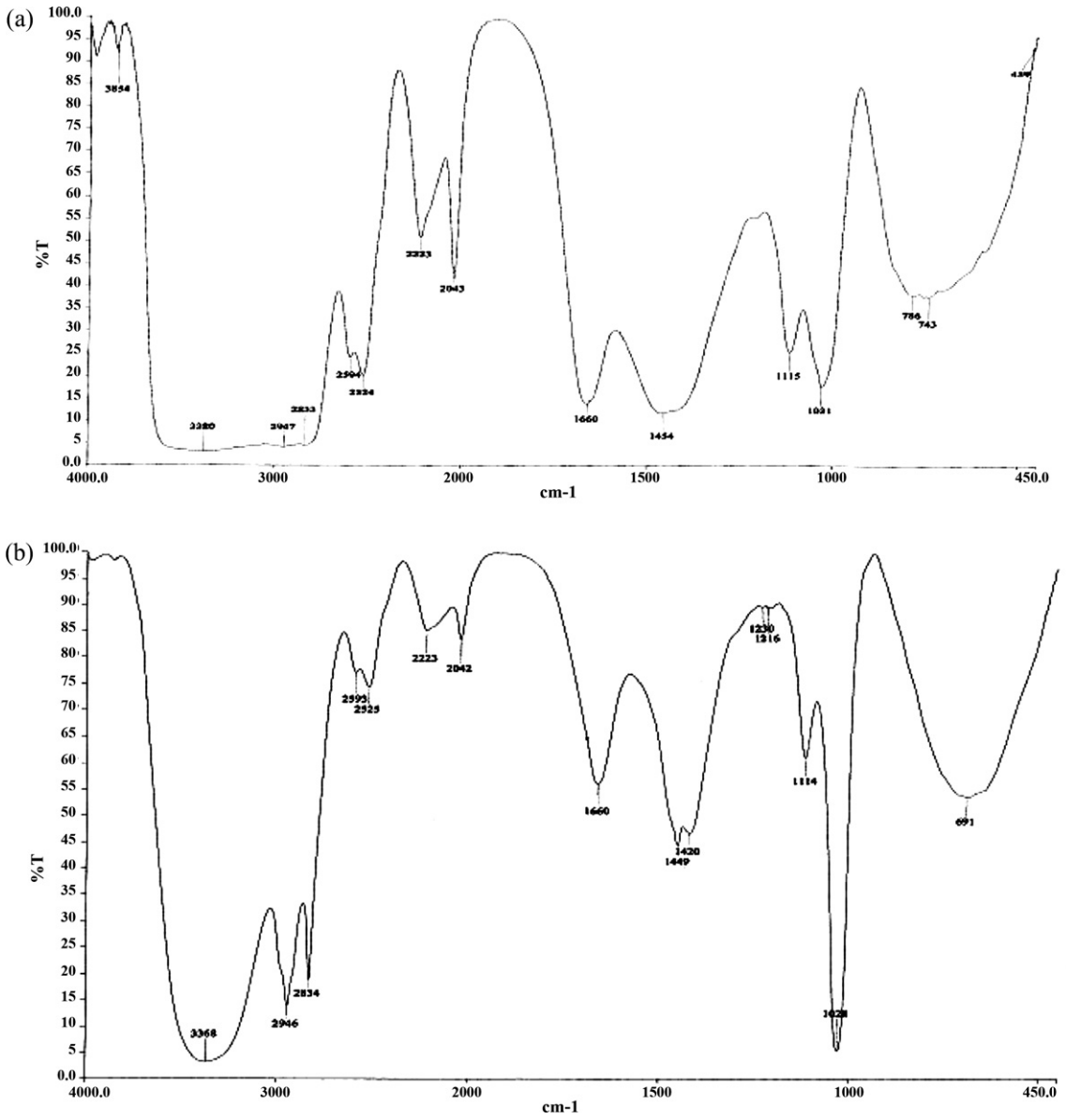

Fig. 7. FTIR spectra of undecolorized MG and decolorized in the presence of purified laccase.

An obvious difference was noticed between the FTIR spectra of MG and its metabolites. FTIR spectra of MG is presented in Fig. 6, it shows specific peaks in fingerprint region (1500–500 cm^{-1}) for the mono-substituted and para disubstituted benzene rings which are support the peak at 1660 cm^{-1} for the C=C stretching of the benzene ring [56]. Also, the peak at 1115 cm^{-1} indicates aromatic C–N stretching vibrations. Furthermore, at 2947 cm^{-1} showed C–H asymmetric stretching and free $-NH_2$ group showed amide anti-symmetric stretching vibration at 3380 cm^{-1}. In addition, the peak at 743 cm^{-1} indicates symmetric out of plane bending of the ring hydrogens. The peaks at 1454 cm^{-1}, 1376 cm^{-1}, 1741 and 786 cm^{-1} were observed for CH_2 scissoring and $-NH_2$ wag. The absorption bands below 900 cm^{-1} indicate the aromatic nature of the compound. Virtually all the peaks in the FTIR spectrum of MG (control) were found to be disturbed in that of the 2 h metabolite (Fig. 6). FTIR spectra of degradation product display a peak at 3368 cm^{-1} for C=O overtone, a peak at 1660 cm^{-1} for C=C, peak at 1420 cm^{-1} for CH deformation in asymmetric CH_3, peak at 1028 cm^{-1} for $=CH_2$ out of plane twist, peak at 691 cm^{-1} for CH bend and peak at 1114 cm^{-1} for the C–O stretch antisymmetrically coupled to the C–C stretch. Absence of peaks at 459 cm^{-1}, 743 cm^{-1} and 786 cm^{-1} indicates loss of aromaticity or benzene ring.

Table 5
Phytotoxicity studies of MG and its degradation products with *Triticum aestivum* and *Phaseoulus mungo*.

Parameter	*Phaseoulus mungo*			*Triticum aestivum*		
	Water	MG	Degraded Products	Water	MG	Degraded Products
Germination (%)	100	18	69	100	47	81
Shoot (cm)	8.14 ± 0.31	2.7	5.1	9.76 ± 0.73	3.98 ± 0.33	7.20 ± 1.14
Root (cm)	6.80 ± 0.21	3.6	5.67	9.60 ± 0.65	5.21 ± 0.60	8.11 ± 1.30

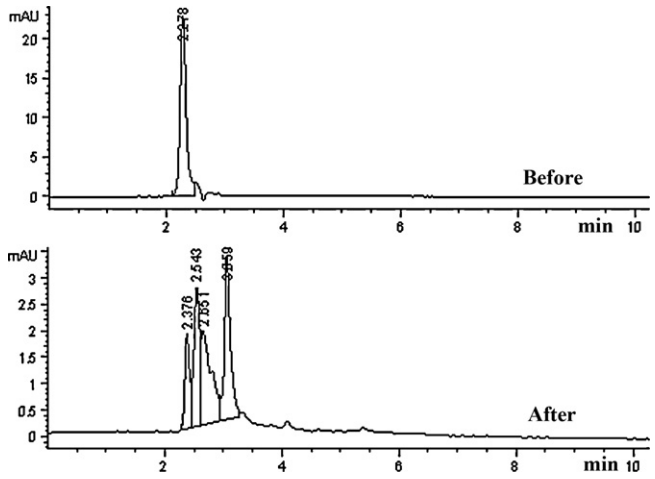

Fig. 8. HPLC of undecolorized MG and decolorized in the presence of purified laccase.

To ensure that the dye decolorization catalyzed by laccase is a real chemical transformation, we followed the conversion of MG by purified laccase with HPLC. The dyes were chosen because of its extensive decolorization at high concentration. The HPLC elution profiles of MG before and after laccase treatment show that the dye peak (retention time 3.56 min) disappeared while four new peaks and a shoulder emerged with considerably shorter retention times (2.54, 2.72, 2.91, 3.05 min; Fig. 8) indicating the formation of more polar oxidation products. The major product peak (2.91 min) corresponds to a substance, whose absorption spectrum differs considerably from that of MG. The data clearly demonstrate that laccase is capable of modifying the chemical structure of the dye MG, and also causes a complete degradation (mineralization) which can be confirmed by the accumulated metabolites in the reaction solution.

3.10. Phytotoxicity studies

Despite the fact that, untreated dyeing effluents may cause serious environmental and health hazards, they are being disposed off in water bodies and this water can be used for an agricultural purpose in India [56]. The use of untreated and treated dyeing effluents in agriculture has a direct impact on the fertility of the soil. Thus, it was of concern to assess the phytotoxicity of the dye before and after degradation. The relative sensitivity of *Phaseoulus mungo* and *Triticum aestivum* towards the dye MG and its degradation products were studied. 100% seed germination was observed with water. Whereas, seed germination was inhibited (82%), when seeds were treated with 1000 ppm concentration of MG in *P. mungo*, this indicates the toxic nature of the dyes to plants (Table 5). Parshetti et al. also showed that germination of *T. aestivum* was less with MG treatment compared to its degradation products and distilled water [56]. The phytotoxicity studies show that germination is inhibited to a very small amount, when seeds are treated with degradation products of MG indicates the less toxic nature of the degradation products (Table 5). Hence phytotoxicity studies revealed that biodegradation of the dye by a laccase enzyme, resulted in its detoxification. Thus treated dyes can be used for irrigation.

4. Conclusions

In this present study, we have developed simple, cost-effective and scalable purification procedure like Three Phase Partitioning to purify extracellular laccase from the crude extract of *P. ostreatus*. This has helped us obtain 161% yield with 27.8 fold purity. Using this single step purification method, large volumes of highly active enzyme can be produce at an affordable cost. In comparison to other laccase enzymes, (1) the *P. ostreatus* laccase was able to sustain its activity even at elevated temperatures; (2) that too for extended periods exhibiting high levels of activity. It has been found that the laccase enzyme we use show remarkably high activities and found to be capable of decolorize and degrade different structure of synthetic dyes into non-toxic metabolites without any redox mediators. So the decolorization of dyes by laccase is simple and cheap. The obtained results display that the use of the laccase has an enormous potential to degrade textile dyes. So, this enzyme can be used for treating textile wastewaters, particularly for water recycling.

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