



ORIGINAL ARTICLE

Purification and immobilization of laccase from *Trichoderma harzianum* strain HZN10 and its application in dye decolorization



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Abstract In this study we report the purification of laccase produced by *Trichoderma harzianum* strain HZN10 (using wheat bran under solid state fermentation) and its application in decolorization of synthetic dyes. Extracellular laccase was purified to homogeneity by DEAE-Sepharose and Sephadex G-100 chromatography with specific activity of 162.5 U/mg and 25-fold purification. Purified laccase was immobilized in various entrapments like calcium alginate, copper alginate, calcium alginate–chitosan beads and sol–gel matrix. Optimization results revealed that the laccase immobilized in sol–gel was optimally active in wide pH range (4.0–7.0) and thermo-stable (50–70 °C) than free enzyme which was optimum at 50 °C and pH 6.0. Kinetic analysis showed K_m of 0.5 mM and 2.0 mM and V_{max} of 285 U/mg and 500 U/mg by free laccase and sol–gel immobilized laccase respectively with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS] substrate. Free and immobilized laccase was employed for decolorization of three different synthetic dyes (malachite green, methylene blue and congo red). High performance liquid chromatography (HPLC) analysis results revealed that approximately 100% of malachite green, 90% of methylene blue and 60% of congo red dyes at initial concentration of 200 mg/L were decolorized within 16, 18 and 20 h, respectively by laccase immobilized in sol–gel matrix in the presence of 1-hydroxybenzotriazole (HBT) mediator. During the decolorization all three synthetic dyes showed various peaks on HPLC chromatogram indicating different by-products formation. Finally, phytotoxicity analysis results revealed that the by-products of synthetic dyes (formed during decolorization) showed less toxicity against *Phaseolus mungo* compared to untreated synthetic dyes.

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1. Introduction

Each year the textile industries generate enormous amount of dye effluent during dye manufacturing and 10–15% during dyeing processes which is released in significant amount into

the environment causing severe environmental problems worldwide [1,2]. Around 60–70% of synthetic dyes used in commercial applications are azo dyes which have one or several azo ($-\text{N}=\text{N}-$) bridges linking substituted aromatic structures [3]. Among them sulfonated azo dyes have a serious negative impact on environment [4]. The complex structure of dyes is challenging to decolorize and makes some dyes toxic and potentially carcinogenic. Hence, research on the decolorization of dyes is of much greater interest, and many researchers are working toward the decontamination of dyes in the effluents [5]. The existing physical and/or chemical treatments like adsorption, precipitation, chemical degradation and photo degradation have been found to be expensive, environmentally unattractive and inefficient in synthetic dye decolorization. Hence, an eco-friendly and cost effective approach is biological treatment [6] exploiting microbial system with ligninolytic enzymes. In this view, in recent years intense research has been focused on production of laccases. Laccase with high catalytic efficiency, broad substrate specificity and tolerance to various physical/chemical parameters [6] could be employed for decolorization of synthetic dyes. Commonly, synthetic dyes are decolorized by whole cell. One of the concerns associated with this method is the requirement of longer periods for decolorization. Hence, the use of laccase-mediator systems is an alternative to this conventional method [7]. Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are multicopper oxidases which catalyze the oxidation of wide spectrum of phenolic/non-phenolic lignin-related compounds and recalcitrant environmental pollutants [6]. Due to their wide specificity, laccases are employed in diverse biotechnological applications like paper pulping, bio-bleaching, textile refining, dye decolorization, organic synthesis, juice and wine clarification [8], bioremediation of soil contaminated with insecticides and medical diagnostic tools [9]. Laccases are widely distributed in nature including plants, insects, bacteria and fungi [10] with a molecular mass ranging from 50 to 97 kDa as reported by various researchers [11]. Fungi have been shown to be promising sources, among them, white-rot fungi deserve special attention due of their ability to degrade lignocellulosic biomass by extracellular laccases [12]. Other laccase producers, such as *Trichoderma atroviride*, *Trichoderma harzianum*, and *Trichoderma longibrachiatum* have been also studied [13]. However, few laccases are characterized from *T. harzianum*. Reports have suggested that the use of free enzymes in traditional enzyme applications has many drawbacks which can be overcome by immobilization processes.

Immobilized enzymes have gained popularity due to several advantages like enzyme recovery, increased stability and durability, rapid enzyme separation from the reaction mixture, tolerance to extreme pH, temperatures and high substrate concentrations [14–16]. Various techniques like surface binding, gel entrapment (hydrogel), entrapment in reverse micelles, covalent and cross-linking bonding between an enzyme and matrix have been commonly used for improvement in the operational stability of enzymes [17]. However, the use of biological polymers has added benefits like non-toxicity, economical, and biocompatibility such as alginate, which is commonly employed in immobilization due to its biodegradability property and form gels with divalent cations [18]. Chitosan is a cationic polysaccharide and forms complexes with polyanionic polymers like alginate [19]. However, sol-gels have attracted attention in biotechnology due to high degree of immobiliza-

tion, entrapment of larger amounts of enzymes, retention of activity, enzyme stability, and tolerance to harsh environmental conditions [20]. Because of the potential applicability of laccase in dye decolorization, several studies on utilization of laccases have come to light such as; decolorization of congo red by *Trametes pubescens* [21] and *Cotyledia pannosa* [6] whereas malachite green by *Ganoderma* sp. [22] and *Penicillium ochrochloron* [23]. Methylene blue decolorization by *Protosphaerion variabile* [24] has been also reported. Different capabilities of laccase in dye decolorization from different strains have been recognized.

From this background, in the present work, a systematic study was conducted to purify and characterize laccase produced by *T. harzianum* strain HZN10. Properties investigated in this study included molecular mass, effect of pH, temperature, metal ions, organic solvents on enzyme activity, thermal stability and substrate specificity. In addition, the potential applications of the purified immobilized laccases in the decolorization of synthetic dyes (malachite green, methylene blue and congo red dyes) are discussed.

2. Materials and methods

2.1. Chemicals and substrates

Malachite green, methylene blue and congo red dyes were purchased from Sigma–Aldrich Pvt Ltd. (USA). All the chemicals used were of analytical grade procured from HiMedia (India) and Merck (USA). Wheat bran (WB) was procured from local market.

2.2. Isolation and molecular characterization by 18S rDNA gene sequence analysis of laccase producing fungi

Laccase producing fungal culture was isolated from vermicompost samples. Samples were suspended in sterile distilled water and serially diluted samples were spread on 2% (w/v) malt extract agar (MEA) plates with streptomycin (25 $\mu\text{g}/\text{mL}$) and incubated at 30 °C for 8–10 days. Morphologically different colonies were selected and purified by repeated streaking. Laccase producers were screened on MEA plates containing 0.01% (w/v) guaiacol or 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic acid) [ABTS] as an indicator compound to evaluate the presence of laccase activity. Guaiacol was added before autoclaving and ABTS after autoclaving as sterile filtered solution. The plates were incubated at 30 °C for 8–10 days. The production of an intense brown color under and around the fungal colony in the case of guaiacol supplemented or a deep green color in the case of ABTS supplemented plates was considered as a positive reaction for the presence of laccase activity. A positive isolate HZN10 was selected and characterized based on morphology, reproductive structures and microscopy [25]. The organism's (from mycelia) DNA isolation and 18S rDNA gene sequence analysis were carried out by the methods described previously [26,27]. Further the sequence obtained was uploaded in national center for biotechnology information (NCBI) Blast program and collected closest organisms sequences. Finally, phylogenetic tree (18S rDNA sequence of isolated with closest organisms) was built on the basis of neighbor-joining method by MEGA 6 software [28].

2.3. Fungal growth conditions for production of laccase

The inoculum was prepared by growing *T. harzianum* strain HZN10 in liquid media containing (g/L) glucose 10; (NH₄)₂SO₄ 1; CaCl₂ 0.125; NaH₂PO₄·H₂O 1 and MgSO₄·7H₂O 0.5 [29] on a rotary shaker (150 rpm) at 30 °C for 5 days. The mycelial pellets were harvested and used as inoculum. Laccase production was carried out by solid state fermentation (SSF) according to the methods described previously [30] using wheat bran substrate in above mentioned media. The clear filtrate was used as a source of laccase for purification.

2.4. Enzyme assay and protein determination

Laccase activities were determined by the oxidation of 0.5 mM ABTS in 0.1 M sodium phosphate buffer (pH 6) at 25 °C and the changes in absorbance at 420 nm were measured at one min intervals. The extinction coefficient of ABTS at 420 nm was $\epsilon_{420} = 36,000 \text{ M cm}^{-1}$ at 25 °C. One unit of laccase activity (U) was defined as the amount of laccase that oxidized 1 μM of ABTS per minute under the standard assay conditions [30]. The soluble proteins were determined by BCA protein assay kit [31]. Assays were carried out in triplicate, and the results are presented as mean \pm standard deviation.

2.5. Laccase purification and molecular weight determination by SDS-PAGE

Laccase produced by *T. harzianum* strain HZN10 was subjected to purification according to the procedures described previously [30]. All purification procedures were performed at 4 °C unless otherwise specified. Briefly, the crude enzyme was purified by ammonium sulfate fractionation, ultra-filtration (Amicon, USA) with a 10-kDa cut-off membrane, DEAE-Sepharose and Sephadex G-100 column chromatography using 50 mM sodium phosphate buffer (pH 6.0) as mobile phase. The molecular weight of purified enzyme was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) with protein molecular weight markers [ribonuclease (15.4 kDa), chymotrypsin (25.0 kDa), ovalbumin (43.0 kDa) and bovine serum albumin (67.0 kDa)] according to the method described previously [32]. Protein bands were visualized by staining with coomassie brilliant blue R-250.

2.6. Immobilization and operational stability of laccase

Different enzyme immobilization methods were adopted such as immobilization in calcium alginate beads, copper alginate beads, calcium alginate-chitosan beads and sol-gel matrix. Sodium alginate solution (4%) and enzyme solution (1 mg/mL) was mixed in a ratio of 1:2 (v/v). This mixture was stirred using magnetic stirrer to ensure complete mixing. The mixture was then dropped using a sterile hypodermic syringe needle into CaCl₂ solution (2% w/v) or CuSO₄ (0.15 M) or into fusion solution composed of a chitosan solution (1.5% w/v in 0.1 M HCl) and CaCl₂ solution (2% w/v) with volume ratios of 1:1. The solutions were gently stirred to form the immobilized enzyme beads. The beads were subjected for hardening by storing overnight in the same solution at 4 °C. The immobilized beads were washed repeatedly with distilled

water until there was no detectable protein in the wash out solution and stored at 4 °C until further use [33]. Laccase was also immobilized in sol-gel matrix of trimethoxysilane (TMOS) and propyltetramethoxysilane (PTMS) prepared using 1:5 M ratios. Laccase (1 mg/mL) was mixed with a mixture of polyvinyl alcohol and water. The solution was constantly stirred with the addition of PTMS, followed by TMOS addition. The reaction mixture was vigorously shaken for 2 min on a vortex mixer and then gently shaken till the mixture formed a clear homogenous solution; it was placed in an ice bath until gel formation occurred [34]. The efficiency of immobilization (EF) was calculated using the following relationship [35]:

$$\text{EF (\%)} = \frac{A_i}{A_f} \times 100$$

where A_i is the specific activity of immobilized enzyme = specific activity of free enzyme (A_f) – specific activity of the unbound enzyme.

Storage stability was monitored by measuring the laccase activity of the stored free and immobilized laccase (without buffer) at 4 °C for 8 days. The recycling stability of immobilized laccase was also assessed by determining the laccase activity in each cycle. The immobilized beads were separated after each reaction and washed with sodium phosphate buffer (50 mM, pH 6) repeatedly. Beads were reused for next reaction to oxidize ABTS up to 5 reaction cycles. Laccase activity in first cycle was considered as 100%.

2.7. Biochemical characterization of purified free and immobilized laccase

The optimum pH of the free and immobilized laccases was determined using different buffers mentioned previously [30]. Laccase stability at various pH (4–8) was evaluated by pre-incubating the free and immobilized laccase in respective buffers for 4 h. The optimum temperature for free and immobilized laccase was determined between 20 and 75 °C. The thermo-stability was assessed by pre-incubating the free and immobilized laccase for 4 h at respective temperatures (40–65 °C). The relative activity was considered as 100% at optimum pH and temperature. To assess stability the residual activity was considered as 100% without pre-incubation at respective pH and temperature. The effect of various metal ions (5 mM), additives (5 mM) and organic solvents (20%) on the free laccase activity was determined. The substrate specificity of the purified free laccase was tested using ABTS, 2,6-dimethoxyphenol (DMP), guaiacol, catechol, ferulic acid, syringaldazine and gallic acid. Rates of substrate oxidation were determined by measuring the increase in absorbance at the respective wavelengths. Molar extinction coefficients (ϵ) were obtained from the literature [36,37]. The kinetics parameters K_m and V_{\max} of free and immobilized (sol-gel matrix) laccase were determined with ABTS (0.2–2.4 mM) substrate by Lineweaver-Burk double reciprocal plot.

2.8. Application of purified free and immobilized laccase in dye decolorization

The decolorization of structurally different dyes namely; malachite green (λ_{\max} 620 nm), methylene blue (λ_{\max} 480 nm) and

congo red (λ_{\max} 495 nm) were studied by the purified free and immobilized laccases with and without the addition of 1-hydroxybenzotriazole (HBT), which is a common redox mediator of laccase. Decolorization was determined spectrophotometrically (LABINDIA UV3000, UV/Vis spectrophotometer) by measuring the decrease in the absorbance at maximum wavelength for each dye whereas the formation of metabolites during dye decolorization was monitored by high performance liquid chromatography (HPLC). The reaction mixture for the decolorization contained a final concentration of 200 mg/L of individual dye in 50 mM sodium phosphate buffer (pH 6) and free laccase (50 U) or 5 g of immobilized laccase (52 U) in a total volume of 50 ml with or without 2 mM HBT. All the reactions were incubated at 30 °C under shaking (150 rpm) for 12 h. Control samples were done in parallel with heat denatured laccase. Aliquot samples were withdrawn at different intervals to measure the residual dye. Sol-gel immobilized laccase with redox mediator was reused for 6 cycles at every 24 h for dye decolorization. After each cycle, the liquid phase was drained and the immobilized laccase was washed repeatedly with 50 mM sodium phosphate buffer (pH 6) and supplemented to fresh dye reaction mixture. All the measurements were performed in triplicate. The extent of decolorization was expressed in terms of percentage calculated as follows;

$$\text{Percentage of decolorization (\%)} = \frac{(A_c - A_t)}{A_c} \times 100$$

where, A_c is the absorbance of the control and A_t is the absorbance of the test sample.

2.9. Analytical method

The decolorization of malachite green, methylene blue and congo red dyes at different intervals were extracted with equal volume of dichloromethane. The extracts were evaporated at 40 °C in a rotary evaporator (Billy scientific with stuart thermostat water bath). The extracted residues were dissolved in methanol (HPLC grade) and used for HPLC analysis.

HPLC analysis was performed in an isocratic system (Agilent technologies 1260 Infinity Quaternary Pump VL) equipped with a UV detector. The separation was performed using C_{18} column (4.6 × 100 mm) with methanol:acetonitrile (1:1) as mobile phase at a flow rate of 1 mL/min.

2.10. Phytotoxicity studies

The phytotoxicity of the original dyes (malachite green, methylene blue, and congo red) at a concentration of 200 mg/L and its metabolites extracted with dichloromethane and dissolved in sterilized water were evaluated on the plant seeds like *Phaseolus mungo*. The experiments were carried out at room temperature by placing 10 seeds for germination on a bedded filter paper and 10 mL solutions for respective samples (original dye/metabolite extracts) were irrigated daily. Control set was irrigated with distilled water. The toxicity was assessed in terms of germination (%), plumule (cm) and radicle (cm) lengths after 7 days [38].

3. Results and discussion

3.1. Isolation and molecular characterization by 18S rDNA gene sequence analysis

Different fungal strains were isolated and among them HZN10 strain demonstrated to be a laccase producer showing a positive reaction when subjected to primary screening with different chromogenic substrates like ABTS and guaiacol. On the basis of molecular identification (18S rDNA sequence analysis), the pure fungal strain HZN10 belonged to *T. harzianum* species. The phylogenetic tree was constructed with closest organisms (18S rDNA sequences) by the neighbor joining method as shown in Fig. 1. The fungi, *T. harzianum* strain HZN10 ITS sequence has been deposited in NCBI GenBank with the accession number KP050785. Laccase potential of *T. harzianum* strain HZN10 was assessed based on its growth and secretion of laccase. Morphologically, the strain looked whitish at its mycelial stage and changed to green upon sporulation. *T. harzianum* strain HZN10 was observed to possess globose to sub-globose conidia and flask shaped phialides after staining with lactophenol cotton blue. Several researchers have reported the isolation of laccase producing strains like *Tricholoma giganteum* AGHP [39], *Trametes* sp. [36], *T. harzianum* M06 [40] and *Trichoderma viride* Pers. NFFCI-2745 [41]. Few laccases are reported from *T. harzianum* strain.

3.2. Purification of laccase and molecular mass determination

Laccase produced from *T. harzianum* strain HZN10 was purified to homogeneity using different steps like $(NH_4)_2SO_4$ precipitation (70%), ultra-filtration, DEAE-Sepharose and Sephadex G-100 chromatography. In the course of ultra-filtration maximum laccase activity was detected in the retentate and used for subsequent purification. Chromatography results (on DEAE-Sepharose and Sephadex G-100) revealed a single major peak showing laccase activity indicating no multiple isoforms of enzyme produced as shown in Fig. 2. The yield and fold purification of laccase were 7% and 25, respectively with specific activity of 163 U/mg protein as summarized in Table 1. The purified laccase showed a single protein band on SDS-PAGE with molecular weight ~56 kDa (Fig. 3) as visualized by coomassie brilliant blue staining and was found to be a monomeric protein from native gel. There are reports of diverse molecular mass of laccase from various organisms, for example, 79 kDa from *T. harzianum* WL1 [42] and 38 kDa from *Leptosphaerulina chartarum* [43]. Multiple laccase forms with 68 and 66 kDa from *Pycnoporus sanguineus* have been reported [12].

3.3. Laccase immobilization, storage and operational stability

Laccase immobilized in sol-gel matrix showed higher immobilization efficiency (93%) than the other matrices. Hence, laccase entrapment in sol-gels could be a better immobilization method as reported by other workers [34]. Ca-alginate beads coated with chitosan showed a reasonable efficiency (86%). Chitosan usually forms a polyelectrolyte complex with alginate thereby increasing the mechanical properties [33]. In comparison to Ca-alginate, Cu-alginate had better efficiency probably

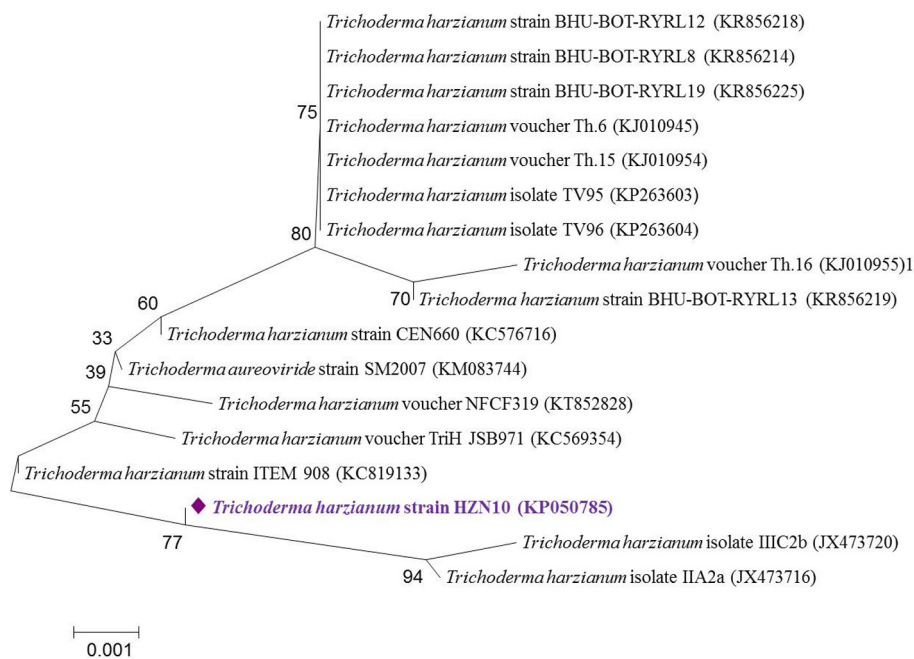


Figure 1 Phylogenetic tree was constructed with 18S rDNA sequence of isolated fungal culture. [*Trichoderma harzianum* strain HZN10 (KP050785)] and related organisms using neighbor-joining method (MEGA 6 software). Numbers at branches are bootstrap values of 1,000 replications.

due to high affinity of copper for alginates and moreover the enzyme leaching is higher from Ca-alginate beads [44].

Free enzyme (laccase) lost 45% of activity after 8 days of storage at 4 °C whereas the stability of immobilized laccase was enhanced. Sol-gel matrix immobilized laccase was found to be the most stable with a mere loss of 2% among the Ca-alginate-chitosan, Cu-alginate and Ca-alginate beads with a loss of 10%, 17% and 25%, respectively. Daniele et al. [35] reported improved storage stability with amberlite-laccase system. For economic feasibility, the reuse of immobilized laccase for 6 cycles showed a reduction in operational stability as the capacity of the binding between matrix and enzyme is weakened and also the catalytic efficiency is lowered. Ca-alginate, Cu-alginate, Ca-alginate-chitosan and sol-gel immobilized laccase showed an operational stability of 36%, 51%, 66% and 82%, respectively after 6 cycles. Reduced operational stability was also reported by other immobilization systems like amberlite-laccase [35]. Immobilized laccases are more durable, vigorous and resistant toward alterations in the environment. It also helps in easy recovery and recycling [11].

3.4. Characterization of purified free and immobilized laccases

3.4.1. Effect of pH and temperature

The effect of pH (3.0–11.0) on purified free and immobilized laccases was evaluated (Fig. 4A). Free and calcium alginate immobilized laccase showed pH 6 as optimum, copper alginate and calcium alginate-chitosan immobilized laccase showed optimum activity at pH (5–6) and sol-gel immobilized laccases was found to be optimum in wide pH range (4–7). The entrapment of laccase in copper alginate, calcium alginate-chitosan and sol-gel caused a shift of pH to a wide range. This could be because of charged support, which attracts or repels the

substrate and product. The decrease in the activity of both free and immobilized laccases at higher pH could be due to the change in the ionic form of the enzyme active site and also due to variations in folding of the three-dimensional structure of the protein [45]. Free and immobilized laccases were incubated at pH (4–8) for 4 h to monitor the pH stability (Fig. 4B). The results revealed higher stability in sol-gel immobilized laccase retaining 98% (pH 4), 100% (pH 5–6), 96% (pH 7) and 92% (pH 8) of residual activity. However, in comparison to free laccase better stability was observed in the order, sol-gel matrix > calcium alginate-chitosan > copper alginate > calcium alginate. The binding of enzyme on the support gives it a lower probability to suffer pH induced conformational changes [45]. Similar results of pH shifts and stability have been reported in case of sol-gel matrix [34] and hydrogels [46] immobilized laccase.

The optimum temperature was found to be 50 °C for both free and immobilized laccases (Fig. 4C). All the forms of immobilized laccases showed >90% relative activity at higher temperature of 55 °C and 60 °C. Sol-gel immobilized laccase showed 97% (65 °C) and 94% (70 °C) relative activity at higher temperatures. Free enzyme lost maximum activity above 55 °C. Higher relative activities of immobilized laccases may be attributed to the restricted conformational changes of laccase after immobilization. The temperature stability of free and immobilized laccases studied in the range of 40–65 °C for 4 h is depicted in Fig. 4D. Both free and immobilized laccases retained more than 90% activity at 50 °C. Copper alginate showed better stability than calcium alginate immobilized laccase. Calcium alginate-chitosan immobilized laccase retained 91% activity at 55 °C whereas, sol-gel immobilized laccase retained 90% activity even at 65 °C indicating higher thermo-stability in sol-gels. Increased thermo-stability in

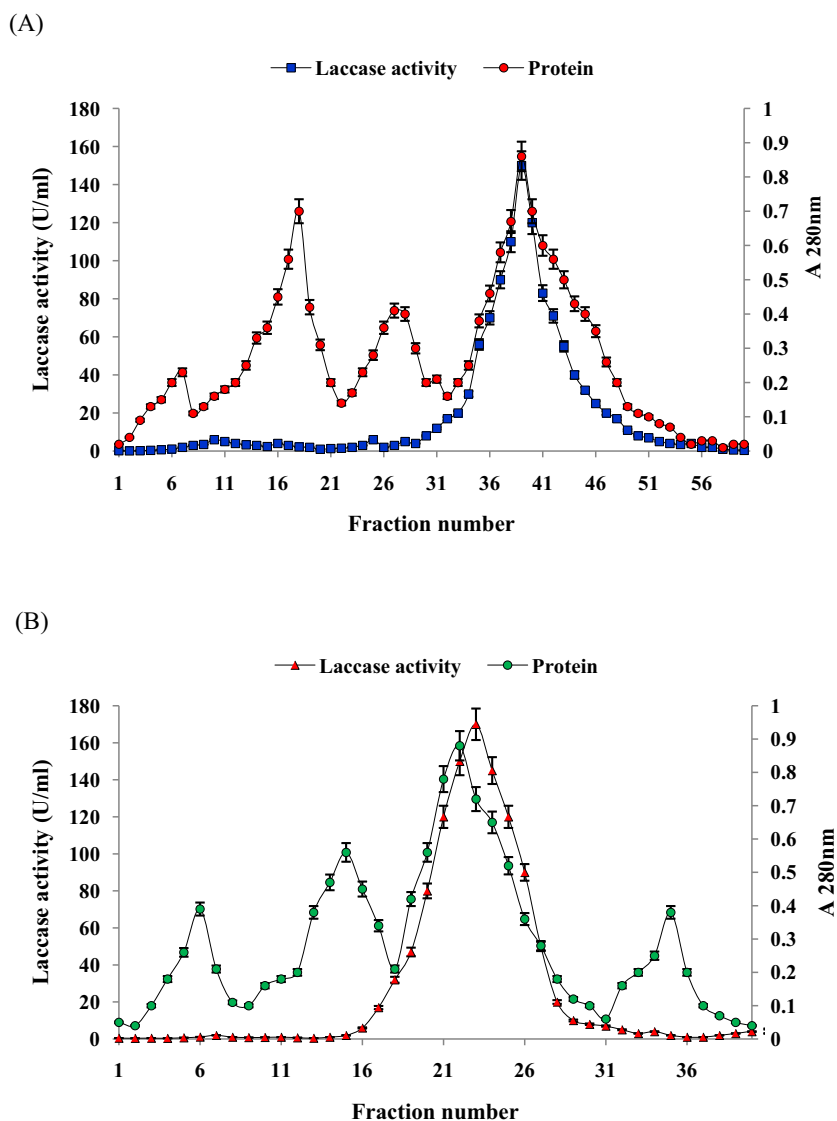


Figure 2 Purification of laccase from *Trichoderma harzianum* strain HZN10 by DEAE-Sepharose (A) and Sephadex G-100 (B) chromatography. Data values represent average of triplicates and error bars represent standard deviation.

Table 1 Purification summary of laccase from *Trichoderma harzianum* strain HZN10.

Purification steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold Purification
Crude extract	1200	7800	7	100	1
Ammonium sulfate	263	2818	11	36	2
Fractionation (70%)					
Ultra filtration (10-kDa cut-off)	133	2184	16	28	3
DEAE-Sepharose	21	922	44	12	7
Sephadex G-100	3	520	163	7	25

sol-gels may be due to protection to the enzyme within the gel. Improved thermo-stability of laccase was also demonstrated in sol-gels [34] and hydrogels [46]. Stability at 55–60 °C in calcium alginate-chitosan immobilized alcalase and trypsin is reported [33]. The wide pH and thermo-stability attributes of immobilized laccase make them more suitable for environmental applications.

3.4.2. Effect of metal ions, additives and organic solvents

The effects of various metal ions (5 mM) on free laccase activity are summarized in Table 2. Cu^{2+} was observed to significantly enhance the activity. Metal ions such as Ca^{2+} , Mg^{2+} and Mn^{2+} activated the enzyme whereas Fe^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} , Al^{3+} and Cd^{2+} strongly inhibited. The results were similar to that seen for *Shiraia* sp. SUPER-H168 [47].

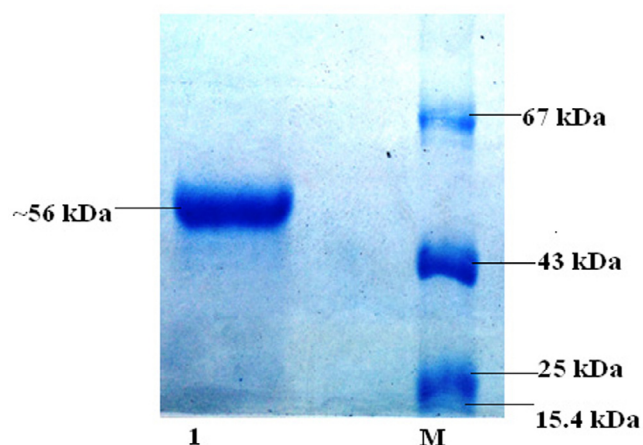


Figure 3 SDS-PAGE analysis with Lane 1: purified laccase on Sephadex G-100, Lane M: molecular weight markers [ribonuclease (15.4 kDa), chymotrypsin (25.0 kDa), ovalbumin (43.0 kDa) and bovine serum albumin (67.0 kDa)].

The role of copper in the enhancement of laccase activity has been also demonstrated by other workers [48]. The presence of β -mercaptoethanol, EDTA and sodium dodecyl sulfate (SDS) reduced the enzyme activity. Sulfhydryl compounds like DTT and L-cysteine inactivated laccase. Urea did not show any significant effect (Table 2). Similar findings have been reported from *Thermobifida fusca* [48]. Surfactants like tween-40 enhanced the activity and triton X-100 did not show any effect. Free laccase showed >80% relative activity in organic solvents like ethanol, methanol, toluene and acetonitrile. Glycerol was found to enhance laccase activity whereas; acetone and isopropanol reduced the activity (Table 3). Similar solvent effect on laccase from *Pycnoporus sanguineus* has been reported [12].

3.4.3. Substrate oxidation and enzyme kinetics

The ability of free laccase from *T. harzianum* strain HZN10 to oxidize different substrates is shown in Table 4. Laccase was able to oxidize all the assayed substrates; the highest activity was demonstrated toward syringaldazine followed by ABTS. Laccase from *Trametes* sp., have reported high activity toward ABTS [36] whereas, from *Lentinus squarrosulus* MR13 toward syringaldazine [37].

Kinetics of laccase revealed a K_m of 0.5 mM and 2.0 mM and V_{max} of 285 U/mg and 500 U/mg by free and sol-gel immobilized laccase, respectively. K_m and V_{max} values of immobilized laccase was higher as compared to free enzyme indicating lower substrate affinity possibly due to steric hindrance of enzyme active site possibly due to diffusion limitations of the substrate into the gel matrix and decreased protein flexibility. The data are substantially in agreement with the kinetic parameters of immobilized laccase in sol-gel [34]. The increase in K_m parameter upon immobilization is commonly suggested in literature [46,49].

3.5. Decolorization of dyes by free and immobilized laccases

The decolorization of synthetic dyes (malachite green, methylene blue and congo red) at 200 mg/L by purified free and immobilized laccases from *T. harzianum* strain HZN10 and

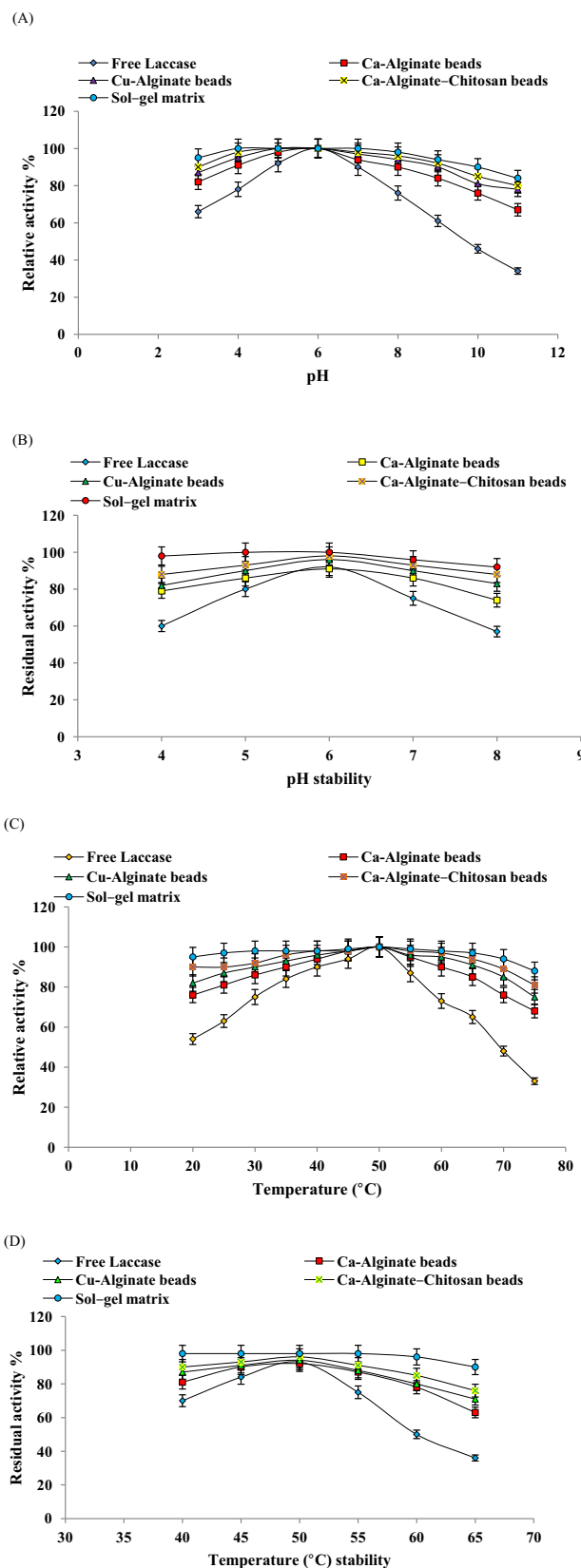


Figure 4 Effect of pH (A), pH stability (B), effect of temperature (C) and temperature stability (D) of free and immobilized laccases.

Table 2 Effect of metal ions and additives on activity of purified laccase.

Metal ions and additives	^a Relative activity (%)
None	100
Mn ²⁺	108 ± 0.8
Mg ²⁺	115 ± 0.3
Ca ²⁺	118 ± 0.4
Zn ²⁺	100 ± 0.6
Cu ²⁺	185 ± 0.4
Fe ²⁺	40 ± 0.4
Cd ²⁺	22 ± 0.5
K ⁺	100 ± 0.3
Ni ²⁺	65 ± 0.4
Co ²⁺	33 ± 0.2
Al ³⁺	40 ± 0.7
Hg ²⁺	10 ± 0.3
L-Cysteine	80 ± 0.5
EDTA	30 ± 0.7
β-mercaptoethanol	85 ± 0.6
DTT	35 ± 0.4
Urea	98 ± 0.5
SDS (0.5%)	40 ± 0.6
Tween 40 (0.5%)	110 ± 0.2
Triton × 100 (0.5%)	100 ± 0.5

Each data value represents Mean ± SD.

^a The activity was assayed in the absence of any metal ions or additives were considered as 100%.

Table 3 Effect of organic solvents on activity of purified laccase.

Organic solvent	^a Relative activity (%)
Glycerol	115 ± 1.2
Ethanol	85 ± 1.1
Methanol	80 ± 1.1
Acetone	58 ± 0.9
Isopropanol	70 ± 0.9
Toluene	82 ± 1.2
Acetonitrile	98 ± 0.7

Each data value represents Mean ± SD.

^a The activity was assayed in the absence of any organic solvents was considered as 100%.

effect of HBT redox mediator was investigated. Initially dye decolorization in a concentration range of 50–200 mg/L was carried out and a final concentration of 200 mg/L was used in further experiments. Table 5 illustrates the decolorization of dyes in 24 h, enhanced decolorization of dyes was observed in the presence of HBT redox mediator. Sol-gel + HBT immobilized laccase proved to be a better system for dye decolorization among the other immobilization methods. 100% (16 h), 90% (18 h) and 60% (20 h) of decolorization of malachite green, methylene blue and congo red was achieved with sol-gel + HBT matrix immobilized laccase in comparison to free laccase which showed 48%, 30% and 22% decolorization of malachite green, methylene blue and congo red in 24 h. Laccase from *P. variabile* has been reported to decolorize congo red (18.5%) and methylene blue (21.3%) after 3 h incubation in the presence of 5 mM HBT [24]. Immobilization strategies enhance the decolorization capabilities of laccase and hence

Table 4 Substrate oxidizing activity of purified laccase.

Substrates	λ_{\max}	ϵ (M ⁻¹ cm ⁻¹)	^a Enzyme activity (U/ml)
ABTS	420	36,000	104 ± 0.6
2,6 DMP	470	49,600	96 ± 0.5
Guaiacol	436	6,400	83 ± 0.7
Catechol	450	2,211	44 ± 0.5
Ferulic acid	287	12,483	24 ± 0.7
Syringaldazine	525	65,000	148 ± 0.6
Gallic acid	250	4,910	54 ± 0.7

^a Each data value represents Mean ± SD.

Table 5 Decolorization of synthetic dyes with purified free and immobilized laccase and effect of HBT mediator.

Treatment	Decolorization (%)		
	Malachite green (λ_{\max} 620 nm)	Methylene blue (λ_{\max} 480 nm)	Congo red (λ_{\max} 495 nm)
Free laccase	48 (24 h)	30 (24 h)	22 (24 h)
Free laccase + HBT	54 (24 h)	36 (24 h)	28 (24 h)
Ca-alginate beads	61 (20 h)	41 (20 h)	33 (20 h)
Ca-alginate beads + HBT	66 (20 h)	48 (20 h)	40 (20 h)
Cu-alginate beads	70 (18 h)	52 (20 h)	45 (20 h)
Cu-alginate beads + HBT	75 (18 h)	59 (20 h)	50 (20 h)
Ca-alginate-chitosan beads	78 (16 h)	60 (20 h)	50 (20 h)
Ca-alginate-chitosan beads + HBT	85 (16 h)	66 (20 h)	54 (20 h)
Sol-gel matrix	90 (16 h)	87 (18 h)	53 (20 h)
Sol-gel matrix + HBT	100 (16 h)	90 (18 h)	60 (20 h)

2 mM HBT used as redox mediator; Control samples were with heat denatured laccase.

immobilized laccases are regarded as robust dye decolorizers. The efficiency of sol-gel matrix-entrapped laccase has been demonstrated previously [34]. Among the dyes, malachite green showed highest decolorization by laccase from *T. harzianum* strain HZN10. This could be attributed to the complexities in the structure and size of the dyes. Dyes with less number of aromatic rings and with simple structure are decolorized more rapidly than the complex molecule. Congo red dye (azo dye) has a high molecular mass of 696.67 g/mol with 6 aromatic rings making it more difficult for decolorization. The need for a mediator to achieve the oxidation of synthetic dyes has been reported [50]. The effect of HBT redox mediator on synthetic dye decolorization by laccase was reported in previous studies [38]. Moreover, the decolorization by immobilized enzyme is the result of both enzymatic catalyzation and support adsorption. Previous dye decolorization studies with immobilized systems have shown the participation of the support in color removal [49]. The reuse of sol-gel + HBT immobilized laccase system for continuous dye decolorization up to

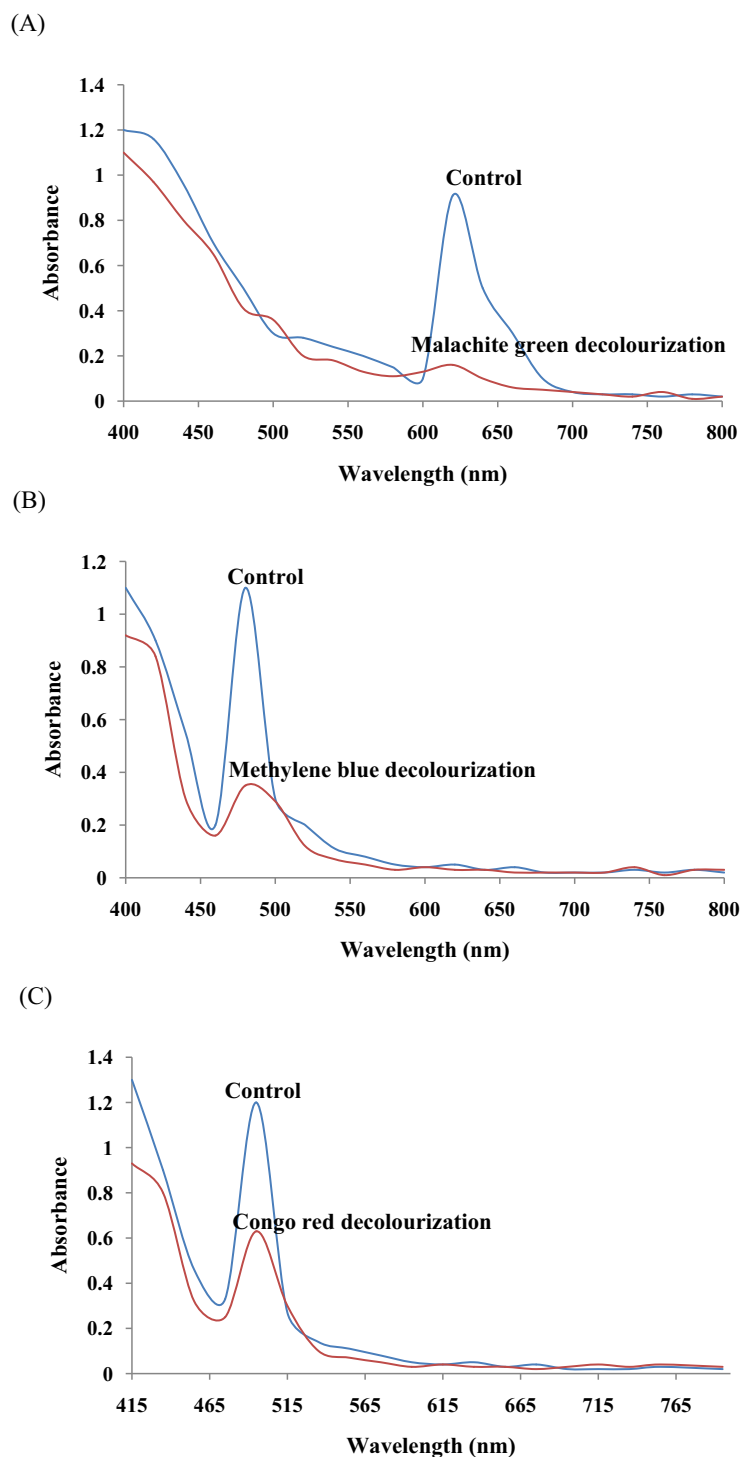


Figure 5 UV-Vis scanning spectra of Malachite green at 0th h and its decolorization at 16th h (A), Methylene blue at 0th h and its decolorization at 18th h (B) and Congo red at 0th h and its decolorization at 20th h (C).

6 cycles showed a reduction in decolorization %. In the 6th cycle 54%, 46% and 26% of malachite green, methylene blue and congo red were decolorized respectively. The decrease in decolorization efficiency may be co-related to inactivation of enzyme and diffusion issues associated to support materials. The reduction in decolorization efficiency with hydrogels has

been reported [46]. The UV-visible spectrum of malachite green (λ_{\max} 620 nm) (Fig. 5A), methylene blue (λ_{\max} 480 nm) (Fig. 5B) and congo red (λ_{\max} 495 nm)(Fig. 5C) dyes was performed. The laccase treated decolorized samples showed a decrease in absorbance at respective wavelengths without peak shifting.

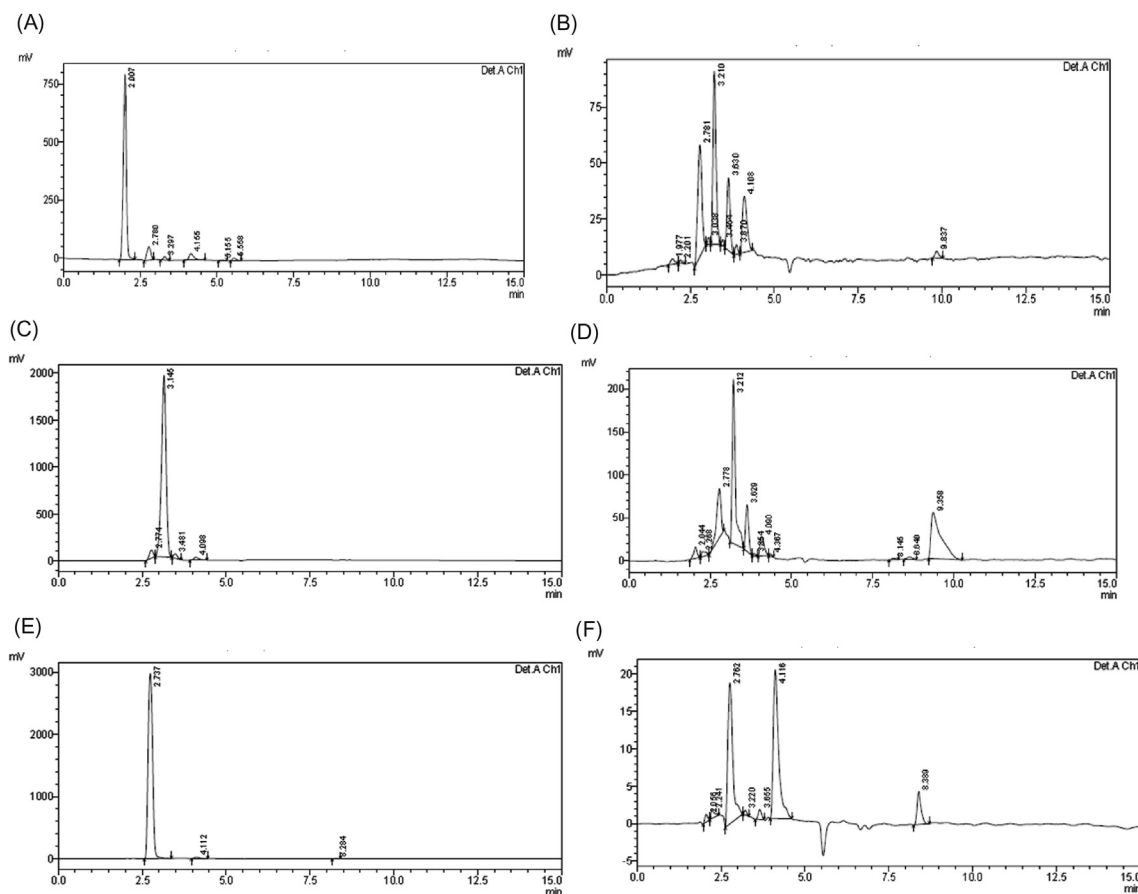


Figure 6 HPLC analysis of Malachite green control (A), metabolites from Malachite green decolorization (B), Methylene blue control (C), metabolites from Methylene blue decolorization (D), Congo red control (E), metabolites from Congo red decolorization (F).

Table 6 Phytotoxicity assessment of malachite green, methylene blue, congo red and their metabolites on *Phaseolus mungo*.

Parameters	<i>Phaseolus mungo</i>						
	Control	MG	Products	MB	Products	CR	Products
Germination (%)	100 ± 0.4	70 ± 0.4	95 ± 0.5	60 ± 0.4	90 ± 0.4	40 ± 0.2	70 ± 0.4
Plumule (cm)	12.2 ± 0.3	6.8 ± 0.5	11.3 ± 0.4	5.4 ± 0.3	10.6 ± 0.3	3.5 ± 0.3	7.5 ± 0.6
Radicle (cm)	2.3 ± 0.5	0.5 ± 0.3	1.8 ± 0.6	0.4 ± 0.2	1.4 ± 0.5	0.6 ± 0.6	0.9 ± 0.5

MG: Malachite Green; MB: Methylene Blue; CR: Congo Red. Values are mean ± SD of ten germinated seeds in three sets.

3.6. HPLC analysis

HPLC analysis of malachite green (Fig. 6A) displayed a peak at 2.007 min, whereas that of the extracted metabolites after decolorization (Fig. 6B) by laccase displayed detectable peaks at retention time 2.781, 3.210, 3.630, 4.108 and 9.837 min. HPLC elution profile of methylene blue decolorization (Fig. 6D) showed prominent peaks at retention time of 2.778, 3.212, 3.629, 4.090 and 9.338 min when compared to control (Fig. 6C) peak with retention time at 3.145 min. Similarly, the HPLC profile of congo red dye decolorization (Fig. 6F) demonstrated peaks with retention time of 2.762, 4.116 and 8.389 min when compared to control (Fig. 6E) peak at 2.737 min. The analysis showed the occurrence of new peaks with disappearance of the control peaks in malachite green and

methylene blue confirming effective decolorization by laccase whereas in case of congo red decolorization, appearance of new peaks along with detainment of control peak indicate slow and incomplete decolorization. The dye decolorization was supported with the help of HPLC analysis by various researchers earlier [23,51,52]. However, further study on identification of metabolites formed during dye decolorization is necessary.

3.7. Phytotoxicity assessment

Phytotoxicity assessment of malachite green, methylene blue, congo red and metabolites on *P. mungo* is shown in Table 6. Germination of *P. mungo* seeds were significantly inhibited by original dyes when compared to metabolites obtained after decolorization and control (sterilized water). Results illustrate

higher toxicity effect of congo red dye indicating high level inhibitory effect of azo dyes. However, seeds irrigated with metabolites sample showed decreased growth in comparison to control. Comparatively decreased lengths of plumule and radical are evidenced with metabolites of congo red dye decolorized sample indicating the complex structure of azo dyes and resistant nature. Further, these results confirm that non-toxic metabolites were formed during dye decolorization by laccase from *T. harzianum* strain HZN10 which results in detoxification of the dyes. Reduction in the toxic nature of dye molecules has also been reported previously in the literature [23,38].

4. Conclusions

In the present study, *T. harzianum* strain HZN10 isolated from vermicompost produced laccase using wheat bran agro-waste residue. A ~56 kDa laccase was purified to homogeneity. Among the different immobilization strategies, sol-gel immobilized laccase showed enhanced thermo-stability, increased K_m and V_{max} parameters in comparison to free enzyme. Laccase (free and immobilized) were employed for synthetic dye (malachite green, methylene blue, and congo red) decolorization. Decolorization and detoxification was confirmed by HPLC and phytotoxicity analysis respectively. The decolorization of synthetic dyes by laccase from *T. harzianum* strain HZN10 makes it a suitable candidate for the treatment of wastewater from industrial effluents.

Author contributions

ZB, SM and HN have contributed in designing of experiments. ZB and SM have performed experimental works. ZB, SM, and HN analyzed results and wrote the manuscript. All authors agreed with the contents of the manuscript.

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