RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Textile dye degradation potential of plant laccase significantly enhances upon augmentation with redox mediators

Anuradha N. Kagalkar[a], Rahul V. Khandare[b] and Sanjay P. Govindwar[a]*

[a]*Department of Biochemistry, Shivaji University, Kolhapur-416004, India*

[b]*Department of Biotechnology, Shivaji University, Kolhapur-416004, India*

***Address for correspondence**

Prof. S. P. Govindwar

Department of Biochemistry,

Shivaji University, Kolhapur- 416 004, India

Email: spg_biochem@unishivaji.ac.in

Tel: +91-231-2609152

Fax: +91-231-2691533

Abstract

Cell suspension cultures of *Blumea malcolmii* Hook. exhibited 98% decolorization of a textile dye Brilliant Blue R (BBR) at a concentration of 40 mg L^{-1} within 24 h. A significant induction in the intracellular laccase activity (607%) was observed during decolorization of BBR. Twelve different redox mediators showed noteworthy degradation of BBR when added independently to cell cultures. Nevertheless, augmentation of 2, 2' Azino-bis 3-ethylbenzothiazoline 6-sulfonic acid (ABTS) achieved 100% decolorization within 30 min. Purified laccase from *B. malcolmii* was revealed to have a molecular weight of 40 kD. Thirteen different phenolic and non-phenolic substrates were also oxidized by the purified enzyme. Purified enzyme was found to degrade five structurally different textile dyes in presence of ABTS. The enzyme took 12 h to completely remove BBR from the solution, however, addition of ABTS tuned up the catalytic action of enzymes achieving up to 96% decolorization within 5 min. Degradation of BBR was confirmed by high performance liquid chromatography and gas chromatography-mass spectroscopy. Precise role of laccase in phytodegradation of BBR was further proposed in a schematic pathway. Phytotoxicity studies revealed decrease in toxicity of degradation metabolites of the parent dye.

Keywords: Decolorization; phytoremediation; cell suspension cultures; *Blumea malcolmii* Hook.; Brilliant Blue R; purified laccase

1. Introduction

The extraordinary pollutant degrading abilities of plants directly rely on their complex mechanisms of metabolism.[1] Plants are endowed with efficient enzymatic machineries which are known to treat a variety of xenobiotic compounds covering heavy metals, polyaromatic hydrocarbons, pesticides, munitions, petroleum products and crude oils.[2] Out of the different types of pollutants that have been contaminating our natural resources, dyes which are released by textile, dyestuff and dyeing industries constitute heavily recalcitrant pollutants. Many of the textile dyes are reported to have carcinogenic and mutagenic effects with a potential toxicity to all life forms.[3] Textile dyes generally have very complex chemical structures which craft them difficult to be degraded by physico-chemical methods. For instance, in sulfonated dyes, the organosulfonate group plays an important role in altering the solubility and dispersion properties of the dye molecule. It increases recalcitrance to normal environmental breakdown because of the thermodynamically stable carbon-sulfur bond.[4] Plant enzymes have been shown to carry out the breakdown the dye structures through oxidation, desulfonation, dehalogenation, denitrification and can even cleave the aromatic structures.[5-8]

Phytodegradation research still lacks the knowledge regarding the basic mechanisms with respect to enzymatic involvement for the removal of dyes and their products formed after degradation. This sort of basic research is an essential prerequisite that would lay the foundation for *in situ* application of these phytotechnologies. In the last decade, a number of reports on plant based removal of textile dyes have focused on enzymatic degradation. Various enzymes like lignin peroxidase, tyrosinase, azo reductase, laccase, dichlorophenol indophenol reductase and riboflavin reductase from *Blumea*

malcomii, Typhonium flagelliforme, Aster amellus, Glandularia pulchella, Petunia grandiflora, Zinnia angustifolia, Portulaca grandiflora etc. have been shown to play a vital role in breaking the complex structures of textile dyes to form different products.[9-15] All these studied enzymes were used from crude extracts of plant tissues, therefore the roles of individual enzymes could not be predicted. Because of this lacuna in earlier works, the underlying cellular and metabolic mechanisms during enzymatic degradation of dyes were partially proposed or are unclear.

Enzymatic processes for bioremediation are characterized by high reaction rates and stoichiometric efficiencies.[16] Laccases have always been endorsed as key enzymes for the biodegradation of xenobiotics. Most of the isolated laccases are from fungal sources and there are fewer reports on purification of the enzyme from plant sources.[17] A broad substrate specificity of laccases facilitate their application in the degradation of textile dyes. In an earlier study, *in vitro* grown suspension cultures of *B. malcolmii* were proposed to metabolize Malachite Green dye showing a prominent involvement of laccase during the degradation process.[18] In the present work, *B. malcolmii* cell suspension was used for the degradation of dyes with different structures and Brilliant Blue R (BBR) was taken further for detailed mechanistic studies with purified laccase. Phytoremediation technologies are generally slower when compared to other physico-chemical and even biological treatment methods. In this study, various redox mediators were therefore explored to enhance the degradation rate of BBR. The biochemical mechanism of metabolism of BBR by purified laccase is also proposed.

2. Materials and Methods

2.1. Chemicals and dyes

2, 2' Azino-bis 3-ethylbenzothiazoline 6-sulfonic acid (ABTS) was purchased from Sigma Aldrich (St Louis, MO, USA). Tartaric acid, dichlorophenol indophenol (DCIP), *n*-propanol, *o*-tolidine, hydroquinone, pyrogallol, guaiacol, catechol, DMP, *o*-danisidine, DAB, L-DOPA, $MgCl_2$, $MnSO_4$, $CaCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, sodium azide, L-cysteine, EDTA, NADH (nicotinamide adenine dinucleotide reduced disodium salt) and coomassie brilliant blue R-250 were obtained from Sisco Research Laboratories, India. DEAE-cellulose, 3,4-dimethoxy benzyl alcohol (veratryl alcohol), syringic acid, catechol, N,N'-dimethyl phenylenediamine, caffeic acid, Murashige and Skoog medium and clarigel were obtained from Hi-Media, India. Protein markers were obtained from Bangalore Genei Pvt. Ltd. All chemicals used were of the highest purity available and of analytical grade. Malachite Green used was from Himedia, India and Methyl Orange was obtained from Merck Limited, Mumbai, India. Other textile dyes and the effluent were obtained from Manpasand textile industry, Ichalkaranji, India.

2.2. Suspension cultures of *B. malcolmii malcolmii* Hook.

Cell suspension cultures of *B. malcolmii* were a kind gift from Prof. V. A. Bapat, Department of Biotechnology, Shivaji University, Kolhapur, India. The suspension cultures were maintained on Murahige and Skoog's medium supplemented with 2,4-Dichlorophenoxyacetic acid (5 mg L^{-1}), glutamine (100 mg L^{-1}), sucrose (3%) and coconut milk (20%). The pH of the medium was adjusted to 5.8±0.05 and was autoclaved at 15 psi and 120 ^{o}C for 20 min. The cultures were maintained at 25±2 ^{o}C with 16 h light and 8 h darkness with continuous shaking at 100 rpm which facilitated a uniform dispersion of cells. A 10 mL of the inoculum was further transferred into 250 mL Erlenmeyer flasks

114 containing 40 mL of autoclaved medium. The cells were cultured regularly after every 10
115 days and were used for phytoremediation studies.

116 **2.3. Decolorization experiments with suspension cultures of *B. malcolmii***

117 To determine decolorization ability of the suspension culture of *B. malcolmii,* BBR at
118 the concentration of 20 mg L^{-1} was added into Erlenmeyer flasks containing 40 mL of the
119 medium. The dye containing medium was autoclaved and then inoculated with 10 mL of
120 inoculum of the suspension culture which was grown for 10 days. The flasks were exposed
121 to continuous shaking at 100 rpm at 25 ± 2 ^{o}C. Aliquots of the sample were withdrawn at
122 regular intervals. The samples were filtered through Whatmann filter paper. The clear
123 filtrate was used to determine the absorbance at the respective absorption maxima of the
124 dyes used. Following dyes namely BBR, Reactive Red 2, Direct Red 5B, Malachite Green and
125 Methyl Orange with absorbance maxima of 560, 530, 520, 620 and 540, respectively were
126 used in this study. Abiotic controls consisted of the dye containing medium without the
127 inocula and biotic controls comprised of the medium inoculated with the suspension
128 culture and devoid of the dye.

129 Decolorization percentage for the respective dyes in all the experiments was
130 calculated using the equation 1,

$$\text{Decolorization (\%)} = \frac{\text{Initial Absorbance} - \text{Observed absorabance}}{\text{Initial absorbance}}$$

131 ……Equation (1)

132 Based on the screening experiments, BBR was selected as the model dye for all the
133 further experiments.

For studying the effect of varying dye concentrations on decolorization performance of suspension cultures of *B. malcolmii,* the cells were subjected to 20, 40, 80, 160 and 320 mg L^{-1} concentrations of BBR. Absorbances were noted after every 12 h. To study the effect of increasing biomass on the removal of BBR, 5, 10, 15 and 20 ml of 10 day old inocula of the suspension culture of *B. malcolmii* were added into different flasks containing 40 mg L^{-1} of the dye BBR. The flasks were exposed to continuous shaking at 20 ± 2 ^{o}C and the absorbance was noted after every 6 h.

2.4. Preparation of cell free extract

Cell suspension cultures of *B. malcolmii* before and after decolorization of BBR (40 mg L^{-1}) were used for the preparation of cell free extracts. Cells were separated from the medium by filtration through a Whatmann filter paper. The clear filtrates obtained were used as sources of extracellular enzymes. The collected cells were suspended in ice cold 50 mM potassium phosphate buffer (pH 7.4), ground finely in a mortar and pestle and then homogenized in a glass homogenizer with intermittent cooling and the extract was further centrifuged at 8000 rpm for 20 min at 4 ^{o}C. The supernatant thus obtained after centrifugation was used as a source of intracellular enzymes.

2.5. Enzyme assays

Activities of lignin peroxidase, laccase, tyrosinase, veratryl alcohol oxidase, NADH-DCIP reductase, azo reductase and riboflavin reductase were assayed spectrophotometrically in cell free extract of test as well as in the control supernatant. Lignin peroxidase activity was calculated by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 mL containing 100 mM n-propanol, 250 mM tartaric

acid, 10 mM H_2O_2.[19] Laccase activity was determined in a reaction mixture of 2mL containing 10% ABTS in 0.1M acetate buffer (pH 4.9), and increase in optical density was measured at 420 nm.[20] For tyrosinase activity, the final assay concentration in 3 ml reaction mixture with 50 mM potassium phosphate (pH 7.4), 0.17 mM catechol, 0.070 mM L-ascorbic acid equilibrated to 30 ^oC. The absorbance monitored at 265 nm. A 0.1 mL of the enzyme sample was added and decrease in the absorbance was recorded for 1 min.[21] Veratryl alcohol oxidase activity was determined using veratryl alcohol as a substrate. The reaction mixture of volume 2 mL containing 4 mM veratryl alcohol, in 0.05 M citrate phosphate buffer (pH 3.0), and 6.8 mg of enzyme was prepared and oxidation of the substrate at room temperature was monitored by an absorbance increase at 310 nm due to the formation of veratraldehyde.[22] NADH-DCIP reductase activity was measured in cell-free extract. DCIP reduction was monitored at 620 nm, and calculated using an extinction coefficient 19 mM cm^{-1}. The reaction mixture (5.0 mL) contained 50 mM substrate (DCIP) in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 mL enzyme. From this, 2.0 mL reaction mixture was assayed at 620 nm by addition of freshly prepared 50 mM NADH.[23] Azo reductase activity was measured in cell free extract by taking 2.0 mL reaction mixture contained 25 mM of Methyl Red, 50 mM NADH, 1.2 mL of potassium phosphate buffer of 50 mM concentration (pH 7.4). Substrate Methyl Red reduction was observed at 430 nm at room temperature and determined using an extinction coefficient 23 mM cm^{-1}.[24] Riboflavin reductase NAD(P)H:flavin oxidoreductase was measured by monitoring the decrease in absorbance at 340 nm. Cell free extract was added to a solution (final volume 2 mL) containing 100 mM of Tris-HCl (pH 7.4), 25 mM of NADPH and 10 mM of riboflavin. Reaction rates were calculated by using a molar extinction coefficient of 0.0063 mM^{-1} cm^{-}

[1,25] All enzymes assayed for test sample and biotic control sample were carried out in triplicates.

2.6. Phytotoxicity studies

Phytotoxicity studies with 1000 ppm concentration of BBR and its extracted products for two common edible plants viz. *Phaseolus mungo* and *Triticum aestivum* seeds. Ten seeds of each plant were independently taken in petridishes were watered with dye and metabolite solutions (5 mL) for 8 d. A control set was also kept supplying distilled water to it. Germination percentages and lengths of plumules and radicles were recorded on 8 d.

2.7. Mediator studies with cell suspension cultures

With a view of enhancing the decolorization potential of suspension cultures of *B. malcolmii* for the dye BBR, different known redox mediators [ABTS (0.4 μM), 1-hydroxybenzotriazole (HBT), acetosyringone (ACS), syringic acid (SA), vanillin (VAN), hydroquinone (HQ), 2,6-dimethoxy phenol (DMP) and pyrogallol (PG) (2 mM)] were added into the suspension culture medium in earlier mentioned section 2.2. The suspension culture medium was autoclaved with the dye BBR (40 mg L^{-1}) and sterilized solutions of the different mediators were aseptically added into each of the respective flasks followed by inoculating them with 10 day old suspension cultures and decolorization percentage was calculated as mentioned above. The mediators were selected looking at chemical structures and ability to undergo repeated oxidation-reduction cycles.

2.8. Purification and characterization of laccase

Ten day old cultures of *B. malcolmii* were used for purification studies. The cell cultures were filtered with a Whatmann filter paper No. 1 under cold conditions (4 °C). The filtrate was used as the crude source of extracellular laccase. The enzyme was dialyzed in a dialysis bag against sodium phosphate buffer (pH 7.4) for 4 h at 4 °C to remove media components. DEAE-anion exchange chromatography was further carried out using automated Econo purification system (Bio-Rad). The dialyzed enzyme was directly applied on the DEAE-cellulose anion exchange column (cylindrical glass column with 15 cm height and 1 cm diameter) equilibrated with 0.2 M potassium phosphate buffer (pH 8.0) at a flow rate of 0.88 mL min^{-1}. The retained proteins were eluted with a linear NaCl gradient former (0 to 1 M).

Native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 5% stacking gel and 11% resolving gel using a Genei vertical electrophoresis system (Bangalore Genei, Pvt. Ltd., India). Activity staining was carried out using non-denaturing PAGE using L-DOPA as the substrate. Molecular mass of the purified enzyme was determined by SDS-PAGE using high molecular mass-standards such as phosphorylase b (98 kD), bovine serum albumin (66.0 kD), ovalbumin (43.0 kD), carbonic anhydrase (29.0 kD), lactoglobulin (18.4 kD) and aprotinin (6.5 kD). Protein bands were visualized using silver staining method.[26]

2.9. Substrate specificity, kinetic constants and spectral character of the purified enzyme

Substrate specificity of the purified enzyme was studied by using several phenolic and nonphenolic compounds, such as 3,4-dimethoxy benzyl alcohol, guaiacol, 2,6-dimethoxy phenol, L-DOPA, *o*-tolidine, hydroquinone, catechol, ABTS, caffeic acid,

pyrogallol, *p*-cresol, syringic acid, *o*-dianisidine and syringaldazine. The reaction mixture (2.0 mL) contained 1 mM substrate, 35 µM ABTS and 20 mM sodium acetate buffer. The reaction was started by adding 0.2 mL of enzyme solution.

Kinetics of the purified enzyme was studied by observing the oxidation of increased concentration of ABTS at optimum condition. Michaelis constant (K_m) and maximum rates (V_{max}) were determined by using ABTS in the range of concentrations 0.125-1.25 µM at pH 4.8 and 30°C. The reaction was followed in a spectrophotometer (Hitachi U-2800) and data were plotted according to Lineweaver-Burk. Sixty µg of laccase in potassium phosphate buffer (pH 8.0) was subjected to wavelength scan (200 to 800 nm) on UV-visible spectrophotometer (Hitachi UV 2800).

2.10. Effect of pH, temperature, metal salts and inhibitors on laccase activity

The effect of pH on laccase activity was determined by observing the oxidation of ABTS within the pH range of 2.0 to 10.0 at room temperature (30 °C). Optimum temperature for purified laccase was examined over the temperature range of 0-100°C with ABTS as a substrate at optimal pH. Salt concentration was standardized before application with varying concentrations (0.01-1.5 nM). The effect of different metal salts (1 mM; $MgSO_4$, $CaCl_2$, $MnCl_2$, $MnSO_4$, $ZnSO_4$, $CoCl_2$, and $CuSO_4$) and inhibitors viz. sodium azide, EDTA and L-cysteine (5 mM) on the activity of purified enzyme was also studies. The enzyme activity was determined by using ABTS as substrate. Heat inactivated enzyme was used as a control. All the experiments were run in triplicates and average values were calculated.

2.11. Decolorization of the textile dyes and analyses of decolorization products using purified laccase

Five structurally different dyes viz. BBR, Malachite Green, Reactive Red 2, Direct Red 5B and Methyl Orange were studied for their decolorization by using purified laccase. The reaction mixture for the degradation of textile dyes using purified laccase contained the respective dyes (40 mg L^{-1}), 0.2 M sodium acetate buffer (pH 4.8) and 0.5 mL enzyme and 35 µM ABTS. The reaction mixture was incubated at 30 °C at static as well as shaking conditions at 100 rpm.

Decolorization of all the individual dyes in culture was monitored qualitatively using UV-visible spectrophotometer (Hitachi U-2800; Hitachi, Tokyo, Japan) at the respective wavelengths of maximum absorption of dyes used as mentioned earlier in section 2.3, while HPLC was used for the confirmation and analysis of phytotransformation. GC-MS analysis was carried out for the identification of metabolites produced. The cells were separated from the liquid medium after 48 h of their exposure to BBR by filtration through a Whatmann filter paper No. 1 and the clear filtrate was used for the extraction of the products formed after the degradation of BBR. In case of studies with the purified enzyme, the reaction mixture after complete decolorization was used for the extraction of the products formed owing to the action of the enzyme.

The dye and its products were extracted using equal volume of ethyl acetate and the extract was then evaporated over anhydrous Na_2SO_4 in vacuum and later dried.[14] HPLC analysis was carried out (Waters model no. 2690; Waters Corp., Milford, MA) on C18 column (symmetry, 4.6 mm x 250 mm) using methanol with flow rate of 1 mL min^{-1} for 10 min and UV detector at 254 nm.[11] Gas Chromatography Mass Spectroscopy (GC-MS)

analysis of the metabolites was carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with an HP1 column (60 m long, 0.25 mm i.d., nonpolar). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injector temperature was maintained at 280 ^{o}C with oven conditions as: 80 ^{o}C kept constant for 2 min-increased up to 200 ^{o}C with 10 ^{o}C min^{-1}, raised up to 280 ^{o}C with 20 ^{o}C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the National Institute of Standards and Technology (NIST) library.[11]

2.12. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. Readings were considered significant when $P<0.05$.

3. Results and discussion

3.1. Decolorization of the dye Brilliant Blue R by suspension cultures of *B. malcolmii*

The cell cultures were found to be efficient in decolorizing a wide variety of dyes belonging to structurally diverse groups but the maximum decolorization was observed in case of the dye BBR (98% within 24 h and more than 90% within 12 h). BBR was therefore selected as the model dye for further studies. UV-visible spectrophotometric analysis (400-800 nm) of the untreated dye showed a distinct peak at 600 nm, which was completely lost after treatment of the dye with the cell culture indicating the decolorization of BBR (Fig. 1a). *B. malcolmii* suspension cells have earlier been reported to degrade toxic paint preservatives called Troysan S-89.[27] *Nopalea cochenillifera* cell cultures have also shown degradation of a textile dye Red HE7B at a concentration of 1000 ppm within 7 d.[5] The efficacy *B. malcolmii* cell to decolorize most of the dyes taken in this study was found to be

similar to or even more efficient than many microbial systems. These findings with the use of suspension cell cultures challenges the previous observations on phytoremediation of dyes which reported a sluggish removal rate of pollutants.

While studying the effect of various dye concentrations starting from 20 mg L^{-1} to 320 mg L^{-1} on the decolorization of BBR mediated by cell cultures, the percentage decolorization was found to decrease and the highest percentage decolorization values were obtained for 20 mg L^{-1} concentration of the dye. Increase in the concentration of dyes from 20 to 80 mg L^{-1} quite insignificantly reduced the percentage decolorization values while the dye concentrations of 160 and 320 mg L^{-1} showed a drastic reduction in decolorization percentages (Fig. 1b). A drastic reduction in decolorization at 160 and 320 mg L^{-1} could be attributed to the toxicity of BBR at these concentrations. SEM images of the cells subjected to increasing dye concentrations showed no apparent damage to cells (Fig. 1c). Since almost similar percentage decolorization values were obtained for the dye concentrations of 20 and 40 mg L^{-1}, the concentrations selected for further studies was 40 mg L^{-1}. Inability to tolerate higher concentrations of pollutants is said to be one of the major disadvantages of phytoremediation technologies and has limited their applications.[28] However, *B. malcolmii* cell cultures have shown the capacity to tolerate and decolorize higher dye concentrations. In case of decolorization of Malachite Green by *Kocuria rosea*, only 13 and 6% decolorization was observed at 70 and 100 mg L^{-1} dye concentrations, respectively.[29] While, *B. malcolmii* cell cultures showed 52 and 42% decolorization even at 160 and 320 mg L^{-1} concentrations which proved them to be more efficient than many microbial dye degrading systems.

3.2. Enzymatic analysis of degradation by *B. malcolmii* cell cultures

B. malcolmii cell cultures have shown to be metabolically highly active tissues with a rich source of degradative enzymes. The cell suspension cultures showed significant inductions in intracellular and extracellular activities of laccase by 607 and 28%, respectively during decolorization of BBR. Inductions in the intracellular activities of DCIP reductase (8%) and veratryl alcohol oxidase (167%) were also observed during decolorization. Interestingly, azoreductase seems to be repressed after the degradation of dye which may have occurred due to its toxicity. Similarly, tyrosinase was also found to be repressed. A noteworthy induction in the laccase activity recommended its importance in the dye biodegradation process (Table 1). This enzyme was therefore targeted for further studies. *B. malcolmii* root tissues showed the absence of laccase[9] while treating Direct Red 5B. The cell suspensions however revealed a clear activity of laccase during BBR degradation in this report. These observations ultimately lead to purification of the enzyme from *B. malcolmii* suspension cultures.

Marigold hairy roots have shown complete absence of laccase activity in control tissues that were unexposed to the dye Reactive Red 198 while activity of the enzyme was detected in root tissues that were exposed to the dye.[35] Similar observation were also made with studies on hairy root cultures of *B. juncea*. Methyl Orange exposed hairy roots revealed an enhanced activity of laccase, however, it was completely absent in the dye unexposed roots.[36] In this study, activity of laccase was found to be induced during the decolorization of BBR which helped to speculate that laccase could be the principal enzyme from *B. malcolmii* suspension cultures that imparts it to possess dye degrading properties. Thus, to determine the exact role of laccase in decolorization of textile dyes, further attempts were made to purify the enzyme.

3.3. Phytotoxicity studies

1000 ppm concentration of BBR was found to cause total (100%) inhibition of seed germination of *Phaseolus mungo* while 60% germination in case of *Triticum aesitivum*. Germination percentage was found to be slightly higher in case of *T. aestivum* seeds treated with products than those treated with the parent dye. In case of *P. mungo*, no difference in germination percentage was observed in distilled water and dye metabolites. Lengths of plumules and radicles of both *T. aestivum* and *P. mungo* were observed to be superior in the solutions of dye metabolites than those germinated in BBR. These studies showed that the products of BBR after treatment with *B. malcolmii* cell cultures were with reduced toxicity as compared to BBR and thus could be proposed to be effective to render textile waste waters safer for the environment (Table 2).

3.4. Effect of redox mediators on the decolorization of BBR

Out of eight different mediators used, ABTS was observed to be the most efficient mediators which led to complete decolorization of the dye within 30 min whereas control set (those without any mediator) took 24 h. HBT was found to mediate almost 85% decolorization within 30 min while in absence it required more than 12 h. DMP was found to give 95.21% decolorization at the end of 30 min. However, it was surprising to find lesser decolorization in subsequent hours. This might have taken place due to formation of some other color intermediates which showed the absorbance at same wavelengths. In case of other mediators tested viz. VAN, ACS, PG, SA and HQ, the absorbance measured at 24 h showed no significant loss of color (Fig. 2). The redox mediators showed varying performances owing to their redox potentials. This work is the first report where redox

mediators have been used to enhance the decolorization of dyes mediated by plant suspension cultures thereby facilitating such a rapid dye removal.

Redox mediators speed up the reaction rate by shuttling electrons from biological oxidation of primary electron donors or from bulk electron donors to the electron-accepting organic compounds.[30] A mediator goes through many oxidation reduction cycles. Oxidized form of the mediator further gets reduced because of the oxidation of dye substrates. Lesser decolorization of BBR (as compared to controls) in presence of certain mediators such as VA, ACS, PG, SA and HQ could have taken place because of the inhibition of laccase caused by radicals generated in the process (Fig. 2). Prediction of prospective of a redox mediator is difficult as there are a number of factors such as redox potential between laccase and the mediator, and type and position of substituent in the mediator. Properties of the oxidized form of mediator such as stability, inactivation and substrate affinity also affect the redox process.[31-33] The negative effect of HBT on laccase has been studied where it has been known to attack aromatic amino acids such as tyrosine and tryptophan.[33] Inactivation of laccase by HBT during degradation of an indigo dye depended on the presence of substrates oxidizable by HBT radical, since this factor avoids the enzyme inactivation reaction.[34] In our experiments, dyes were the oxidizable substrates by this mediator and have a positive influence on the stability of laccase. Despite great promise, slow removal rate of pollutants has limited the application of phytoremediation technologies. Such enhanced decolorization by using mediators and finding natural and less toxic mediators could help us take a step forward in overcoming the hurdles in the application of phytoremediation technologies.

3.5. Purification of laccase from *B. malcolmii* cell suspension cultures

The DEAE-cellulose anion exchange chromatography elution profile of proteins is shown in Fig. 3a. The enzyme was eluted with 0.4 M NaCl concentration from DEAE cellulose anion exchange column. The procedure yielded 0.72 mg mL^{-1} of pure protein. The recovery of laccase activity was 62.4% with a purification fold of 7.8 (Table 3).

UV-Visible spectrum of purified *B. malcolmii* laccase showed peaks at 280 nm and 360 nm whereas no absorbance was detected at 610 nm (Fig. 3b). Similar observation was also made by Madhavi and Lele (2009).[37] Laccase contains four copper atoms that have been classified according to their electron paramagnetic resonance (EPR) features. They are classified as Type 1 or blue, Type 2 or normal and Type 3 or coupled binuclear copper site. Type 1 enzyme is associated with an intense optical absorption band near to 610 nm. In addition, Type 3 non paramagnetic is supposed to be associated with a cooperative two-electron acceptor, which show a strong optical absorption at about 340 nm in the oxidized state.[38] These observations helped to infer that the enzyme lacks Type 1 copper which is found to be present in blue laccases. The absorbance shown by the enzyme at 360 nm could be a characteristic of Type 3 copper (Fig. 3b).

The purified laccase appeared as a single protein band on SDS-PAGE (Fig. 4). The molecular weight of purified laccase was observed to be 40 kD. The molecular weight of purified *B. malcolmii* laccase was found to be in correspondence with the molecular weight of plant laccases. Fungal laccases have been reported to have a molecular weight between 40-80 kD.[39] The protein size of PPOs depends on plant species and varies from 39 kD in snapdragon to 73 kD in spinach.[40] Laccases from spent *Lentinus polychrous* Lev. Mushroom compost was found to have the molecular weight of 32 kD.[41] *Botrytis cinerea* laccase was previously reported to be as small as 38 and 36 kD.[42]

3.6. The effect of pH, temperature, metal ions and inhibitors on laccase activity

The purified laccase exhibited the oxidation of ABTS within a wide range of pH at 30 ^{o}C. The highest activity of enzyme was demonstrated at pH 3.0 (Fig. 5a). The activities of enzyme at pH 4.0, 5.0 and 6.0 did not show any drastic changes and the values of activities plotted against pH showed almost a plateau on the graph within this range. The pH optima of laccase from *Rhus vernicifera* for ABTS as the substrate, has been found to be close to 4.0.[43] Laccase from fungus *Ceriporiopsis subvermispora* also showed an optimum pH value of 3.0 when ABTS was used as a substrate.[44]

Although the optimum temperature of purified enzyme was found to be 30 ^{o}C, the enzyme could remain active within a broad temperature range (Fig. 5b). Extracellular laccase from *Pseudomonas* sp. LBC showed optimum activity at 40 ^{o}C temperature.[12] Characterization of laccase from tuberous roots of *Amorphophallus campanulatus* showed that the enzyme underwent rapid inactivation when pre-incubated for 5 min at temperatures greater than 40 ^{o}C.[45] *B. malcolmii* laccase thus was found to oxidize ABTS even when the reaction mixture was pre incubated for 10 min at high temperatures.

When the effect of different metal ions on laccase activity was studied, it was found that $ZnSO_4$ led to marginal increase in enzyme activity. The effect of metal ions on activities of laccase from a white rot fungus *Ganoderma lucidum* also showed that at low concentrations, $ZnSO_4$ was found to enhance the activity of laccase.[44] Activity of laccase was also enhanced by about 8% in the presence of $CuSO_4$. Since, copper is a component of active site of laccases. Previous reports also confirm the enhancement of laccase activity in the presence of $CuSO_4$. This might have happened because of the filling of Type 2 copper binding sites with copper ions.[44] The highest inhibition of laccase was observed in the

presence $MnSO_4$ which about 13%. Other metal salts viz. $MgSO_4$, $CaCl_2$ and $MnSO_4$ were also found to inhibit laccase activity to various extents (Fig. 5c). The results obtained again were similar to those obtained with *Ganoderma lucidum* where Mn was again found to show maximum inhibition of laccase activity.[44]

3.7. Substrate specificity and kinetics of purified laccase

Besides ABTS, the purified enzyme was found to oxidize a wide range of phenolic and non-phenolic substrates including guaicol, *o*-tolidine, pyrogallol, syringaldazine, L-DOPA, *o*-danisidine and 2,6-dimethoxyphenol in presence of ABTS as the redox mediator (Table S1). The Km for *B. malcolmii* laccase was found to be 20 µM while V_{max} was found to be 5.04 moles L-1 (Fig. 6). *Trametes hirusita* laccase has been reported to have the Km of 41 µM for ABTS.[43] Generally, lower Km values have been observed for syringaldazine as the substrate while comparatively higher Km values were observed for ABTS. A low Km value for *B. malcolmii* laccase indicated that the enzyme possessed a very high affinity for ABTS as the substrate.

B. malcolmii laccase showed the ability to oxidize polyphenols, methoxy substituted phenols, diamines etc. in the presence of ABTS. The enzyme seemed to show higher activity values for methyl and methoxy substituted substrates and diamines. Introduction of OH, OCH3 or CH3 groups into aromatic system renders the compound easily oxidizable by laccase. Methoxy groups can donate an electron easily to introduce one-electron oxidation.[46] The highest activity was observed for *o*-tolidine which has methyl and amino groups as substituents. DMP which has methoxy substituent and *o*-danisidine which has additional amino substituent were also significantly oxidized. Guaicol and syringaldazine were oxidized considerably though syringic acid and veratryl alcohol were not oxidized

449 despite of having methoxy substituents and pyrogallol. A polyphenol was also oxidized
450 despite the absence of methyl, methoxy or amino substituent. The enzyme however failed
451 to oxidize substrates which were diphenols. The ability to oxidize syringaldazine is a
452 distinctive characteristic of laccase (Table S1). A lower redox potential of substrates or
453 higher redox potential of laccase results into higher oxidation rates of substrates.[37] Redox
454 mediators allow laccases to oxidize non-phenolic compounds, thereby vastly expanding the
455 range of substrates that can be oxidized by this enzyme.[47] As the catalyzed reactions
456 depend on the difference of redox potential between laccase and substrate.[33, 47] The organic
457 compound best fitting the term "redox mediator" was found to be ABTS as evident with the
458 outcome of this work.

459 When different known inhibitors at 5 mM concentrations were used to detect their
460 effect on laccase activity, no inhibition was observed in the presence of EDTA whereas,
461 complete inhibition was observed in the presence of L-cysteine and sodium azide.

462 **3.8. Decolorization of dyes by purified laccase**

463 Purified laccase from suspension cultures of *B. malcolmii* showed the ability to
464 decolorize a variety of different dyes like BBR, Malachite Green, Reactive Red 2, Methyl
465 Orange and Direct Red 5B at 40 mg L^{-1} concentrations. The model dye BBR was found to be
466 completely removed by purified laccase within 12 h. This performance was tuned upon
467 addition of ABTS as the redox mediator. Addition of ABTS achieved a decolorization of 96%
468 just within 5 min at static conditions. Other dyes namely Reactive Red 2 and Direct Red 5B
469 were also decolorized up to 95 and 77% within 30 min of addition of the enzyme. While
470 Malachite Green and Methyl Orange were decolorized up to 80 and 49%, respectively
471 within 2 h in presence of ABTS. UV-Visible spectra of all the dyes screened showed a

decrease in the absorbances measured at the characteristic wavelength maxima of dyes (Table S2). Fungal laccases have been known to be responsible for the degradation of recalcitrant compounds like phenols while plant laccases are known to be involved in synthetic processes such as lignin formation.[37] There are only a few reports where purified plant laccases have been used for the degradation of dyes. Decolorization studies with cell suspension cultures of *B. malcolmii* have shown the prominent role of laccase in the decolorization of the dye BBR. Moreover, ABTS was found to be the best mediator that gave the most rapid degradation of the dye. These results led to the use of ABTS as the mediator for the degradation of the different textile dyes using purified *B. malcolmii* laccase. The decolorization of Remazol Brilliant Blue R by a commercial laccase formulation with a nonionic surfactant as the redox mediator was also reported.[30]

3.9. Degradation analysis of BBR

HPLC analysis was performed in order to confirm the degradation of the BBR. HPLC profile of the untreated dye showed peaks at 1.915, 2.236 and 3.106 min while, the products formed with whole cell cultures of *B. malcolmii* showed peaks at 2.899, 3.183, 3.517, 3.676, 5.824, 9.698 min and the products formed after 5 min of degradation of the dye by purified *B. malcolmii* laccase showed peaks at 2.662, 2.858, 3.359, 5.505 and 9.285 min. Differences in the HPLC profiles of BBR and the metabolites formed confirm the dye degradation. HPLC profile of the control sample (extracts of the buffer, ABTS and the enzyme) and that of the metabolites formed after the degradation of BBR by purified laccase showed just one peak in common (9.285 min) which indicated that all the other peaks in the HPLC profile of the metabolites were a consequence of degradation of the dye by *B. malcolmii* laccase (Fig. 7).

To detect the products of metabolism of BBR with whole cell cultures of *B. malcolmii*, GC-MS of control samples (extracts of the medium inoculated with *B. malcolmii* suspension cultures and devoid of the dye) and test samples (extracts of the products formed due to the metabolism of the dye) was done. A number of peaks were detected which were common in test and the control samples while there was only one peak which was eluted at the retention time of 22.598 min which was not present in the control sample. The GC-MS analysis of the products formed with the purified enzyme also showed a peak at the retention time of 22.598 min along with an additional peak at 18.684 min. Moreover, the mass spectrum of products formed both in case of degradation of dye with purified enzyme and with whole cell culture, eluted at the retention time of 22.598 min was found to be exactly similar. The pathway predicted for degradation of BBR by whole cell cultures involved a asymmetric cleavage of BBR followed by demethylation with laccase to give *N*-ethyl-4-4-{(*Z*)-[(4*Z*)-4-(methylimino)cyclohexa-2,5-dien-1-ylidene]methyl}aniline; (MW = 238) (Fig. 8). Strikingly similar fate of metabolism seems to be carried out by purified laccase where only difference was the detection of another compound [3-methylbenzenesulfonic acid (MW =172)] in the GC-MS analysis. This compound was thought to be formed after the first step where the enzyme is suspected to bring about the asymmetric cleavage of BBR. No other products were detected with whole cell cultures which could be attributed to a complete mineralization of the products with the help of the other enzymes present in the suspension cultures of *B. malcolmii*. The GC-MS analysis revealed a remarkable similarity in the nature of products formed due to the decolorization of BBR by crude enzyme source and that of purified laccase both of which show a peak at 22.598 min (Fig. 8). The present study clearly helped to determine the role of laccase in

degradation of BBR and gave confirmatory evidences to elucidate the mechanism of action of laccase. This also reveled that the enzyme catalyzes exactly the same reaction when present in whole cell and when present in its purified form. Laccase thus seems to dominate the degradation process even when there are a couple of other enzymes that can compete with laccase for degradation of BBR.

4. Conclusions

The present study explored the potential of *B. malcolmii* suspension cultures to decolorize structurally different textile dyes along with unraveling the detailed mechanistic basis underlying degradation of BBR dye by whole cell cultures and purified laccase. The studies also demonstrated an interesting approach to enhance degradation process with the use of mediators and the ABTS-laccase mediator system was found to prove to be highly efficient. The purified enzyme also showed versatility in degrading structurally dissimilar dyes and many other laccase substrates. A schematic pathway of degradation by purified laccase was deciphered to understand the fate of metabolism of dye. Novel insights towards understanding the phytoremediation mechanisms behind removal of textile dyes with purified laccase lays the foundation for future advances. Enhancement and application of these technologies will help in broadening the horizons of phytoremediation research.

Acknowledgements

Authors are thankful to Prof. V.A. Bapat, Department of Biotechnology, Shivaji University, Kolhapur for the kind gift of *B. malcolmii* cell culture. Thanks are also due to the Department of Biotechnology, Government of India, New Delhi for funding under

Interdisciplinary Program for Life Science to Shivaji University, Kolhapur, India (Grant No. BT/PR4572/INF/22/147/2012).

References

1 S. Govindwar and A. Kagalkar, Nova Science Publishers, New York, USA. 2010.

2 E. Pilon-Smits, *Annu. Rev. Plant Biol.*, 2005, 56, 15–39.

3 A. Watharkar, R. Khandare, P. Waghmare, A. Jagadale, S. Govindwar and J. Jadhav, *J. Hazard. Mater.*, 2015, 283, 698–704.

4 R. Duc, T. Vanek, P. Soudek and J. Schwitzguebel, *Int. J. Phytorem*. 1999, 1, 255-271.

5 V. Adki, J. Jadhav and A. Bapat, *Int. J. Phytorem.*, 2012, 14, 554–569.

6 A. Watharkar, R. Khandare, A. Kamble, A. Mulla, S. Govindwar and J. Jadhav, *Environ. Sci. Pollut. Res.*, 2013, 20, 939–949.

7 N. Rane, V. Chandanshive, R. Khandare, A. Gholave, S. Yadav and S. Govindwar, *RSC Adv.*, 2014, 4, 36623-36632.

8 N. Rane, V. Chandanshive, A. Watharkar, R. Khandare, S. Patil, P. Pawar and S Govindwar, *Water Res*., 2015, 83, 271-281.

9 A. Kagalkar, U. Jagatap, J. Jadhav, V. Bapat and S. Govindwar, *Bioresour. Technol*., 2009, 100, 4104-4110.

10 A. Kagalkar, U. Jagatap, J. Jadhav, S. Govindwar and V. Bapat, *Planta*, 2010, 232, 271-285.

11 A. Kabra, R. Khandare and S. Govindwar, *Water Res.*, 2013, 47, 1035–1048.

12 R. Khandare, A. Kabra, M. Kurade and S. Govindwar, *Bioresour. Technol.*, 2011, 102, 533 6774–7.

13 R. Khandare, A. Kabra, D. Tamboli and S. Govindwar, *Chemosphere* 2011, 82, 1147– 535 1154.

562 14 R. Khandare, N. Rane, T. Waghmode and S. Govindwar, *Environ. Sci. Pollut. Res.*, 2012,
563 19, 1709–1718.
564 15 A. Watharkar, N. Rane, S. Patil, R. Khandare and J. Jadhav, *Bioresour. Technol.*, 2013, 142,
565 246–54.
566 16 Telke, D. Kalyani, U. Jadhav, G. Parshetti and S. Govindwar, *J. Mol. Catal. Enz.*, 2009, 61,
567 252-260.
568 17 R. Sterjiades, J. Dean and K. Eriksson, *Plant Physiol*., 1992, 99, 1162-1168.
569 18 A. Kagalkar, M. Jadhav, V. Bapat and S. Govindwar, *Bioresour. Technol*., 2011, 102,
570 10312–10318.
571 19 V. Shanmugam, M. Kumari and K. Yadav, *Indian J. Biochem. Biophys*., 1999, 36,39–43.
572 20 N. Hatvani and I. Mecs, *Process Biochem*., 2001, 37, 491–496.
573 21 S. Surwase and J. Jadhav, *Amino Acid*., 2011, 41, 495-506
574 22 R. Bourbonnais and M. Paice, *Biochem. J*., 1988, 255, 445– 450.
575 23 M. Salokhe and S. Govindwar, *World J. Microbiol. Biotechnol*., 1999, 1, 229–232.
576 24 S. Kalme, G. Parshetti, S. Jadhav and Govindwar, *Bioresour. Technol*., 2007, 98, 1405–10.
577 25 R. Russ, J. Rau and A. Stolz, *Appl. Environ. Microbiol*., 2000, 66,1429–1434.
578 26 R. Switzer, C. Merril and S. Shifrin, *Anal. Biochem.*, 1979, 98, 231-237.
579 27 V. Adki, U. Shedbalkar, U. Jagtap, J. Jadhav and V. Bapat, *J. Hazard. Mater.*, 2011, 191,
580 150–157.
581 28 Environmental Protection Agency (EPA), Cincinnati, OH, USA, 2000.
582 29 G. Parshetti, S. Kalme, G. Saratale and S. Govindwar, *Acta. Chim. Slov*., 2006, 53, 492–498.
583 30 M. Hussain, Q. Hussain, *Crit. Rev. Environ. Sci. Tech*., 2008, 38, 1-42.

584 31 F. Xu, J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H. Deussen, E. Abbate, V. Galinyte and P.
585 Schneider, *Appl. Environ. Microbiol*., 2000, 66, 2052-2056.
586 32 Johannes and A. Majcherczyk, *Appl. Environ. Microbiol*., 2000, 66, 524-528.
587 33 Moldes, and M. Sanroma´n, *World J. Microbiol. Biotechnol*., 2006, 22, 1197-1204.
588 34 K. Li, F. Xu and K. Eriksson, *Appl. Environ. Microbiol*., 1999, 65, 2654-2660.
589 35 P. Patil, N. Desai, S. Govindwar, J. Jadhav and V. Bapat, *Planta*, 2009, 230, 725-735.
590 36 A. Telke, A. Kagalkar, U. Jagtap, N. Desai, V. Bapat and S. Govindwar, *Planta*, 2011, 234,
591 1137–1149.
592 37 V. Madhavi and S. Lele, *Bioresources*, 2009, 4, 1694-1717.
593 38 L. Octavio, P. Irma, B. Ricardo and V. Francisco, V.O., Trivandrum, India, 2006. pp. 323-
594 340,
595 39 Y. Kim, N. Cho, T. Eom and W. Shin, *Bull. Korean. Chem.*, 2002, 23, 985-989.
596 40 T. Aniszewski, R. Lieberei and G. Krzysztof, *Acta. Biol. Cracov. Bot.*, 2008, 50, 7-18.
597 41 S. Khammuang and R. Sarnthima, *Biotechnology*, 2007, 6, 408-413.
598 42 V. Gochev and A. Krastanov, *Bulg. J. Agric. Sci.*, 2007, 13, 75-83.
599 43 M. Frasconi, G. Favero, H. Boer, A. Koivula and F. Mazzei, *Biochim. Biophys. Acta*., 2010,
600 1804, 899-908.
601 44 K. Murugesan, Y. Kim, J. Jeon and Y. Chang, *J. Hazard. Mater.*, 2009, 168, 523-529.
602 45 Y. Fukushima and T. Kirk, *Appl. Environ. Microbiol*., 1995, 61, 872-876.
603 46 K. Min, Y. Kim, Y. Kim, H. Jung and Y. Hah, *Arch. Biochem. Biophys.*, 2001, 392, 279-286.
604 47 R. Bourbonnais and M. Paice, *FEBS Lett.*, 1990, 267, 99-102.
605
606

Figure captions

Fig. 1. a). UV-Visible spectra of Brilliant Blue R before and after decolorization, b) Effect of increasing concentrations of BBR on the decolorization performance of *B. malcolmii* cell cultures and c) SEM images of *B. malcolmii* cell cultures unexposed to and those subjected to increasing concentrations of BBR.

Fig. 2. The effect of different mediators on the decolorization of Brilliant Blue R measured at 0 h (▧) and after 0.5 (⊡), 4 (▤ 8 (▨and 12 ( )□ours of decolorization.

Fig. 3. a) DEAE cellulose anion exchange elution profile of purified enzyme showing absorbance (■) at 280 nm and enzyme activity in U (▲) which was assayed in each fraction with ABTS as substrate. The samples (200 µl) from each fraction were added in reaction mixture containing 1.7 mL acetate buffer (pH 4.8). The formation of oxidized product was measured at 420 nm and b) UV-Visible spectrum of purified *B. malcolmii* laccase

Fig. 4. SDS-PAGE of proteins obtained after purification of enzyme. The lanes A and B represent the activity staining bands of crude and purified laccase, respectively on PAGE. The lanes C and D represent the protein staining bands of crude and purified laccase, respectively and the lane E represents molecular weight markers on SDS PAGE.

Fig. 5 a) Optimum pH for purified *B. malcolmii* laccase, b) Optimum temperature for purified *B. malcolmii* laccase and c) The effect of metal salts on the activity of purified *B. malcolmii* laccase

Fig. 6. Km of purified laccase from *B. malcolmii.*

627 **Fig. 7.** HPLC profile of a) Brilliant Blue R, b) products formed after the degradation of
628 Brilliant Blue R by whole cell cultures of *B. malcolmii malcolmii*, c) the control sample after
629 12 h of inoculation of cells in the medium devoid of the dye, d) Products formed after the
630 degradation of BBR by purified *B. malcolmii* laccase and e) control sample containing the
631 buffer, ABTS and the enzyme.

632 **Fig. 8.** Proposed pathway for the degradation of the dye BBR by whole cell cultures and
633 purified laccase from *B. malcolmii* on the basis of GC-MS data showing the detected
634 metabolites.

635 **Table 1** Enzyme activities in *B. malcolmii* cells at before and after 12 h of exposure to the
636 dye BBR

Enzyme	Control		After decolorization of BBR	
	I	E	I	E
Peroxidase[a]	0.206±0.002	NA	0.217±0.001	NA
Laccase[a]	0.095±0.001	0.716±0.007	0.672±0.034**	0.918± 0.004*
Tyrosinase[a]	0.113±0.008	NA	0.073±0.008	NA
Veratryl alcohol oxidase[a]	0.464±0.008	NA	1.24±0.050*	NA
DCIP Reductase[b]	182.78±0.79	NA	197.37± 0.082*	NA
Azo reductase[c]	6.79±0.129	NA	3.55±0.030	NA
Riboflavin reductase[d]	19.64±0.201	NA	19.79±0.150	NA

637

638 Values are a mean of three experiments ± SEM, significantly different from control (0 h) at
639 *P< 0.001 by one-way ANOVA with Tukey Kramer comparison test.

640 [a] Activity in units min^{-1} mg^{-1}.

641 [b] µg of DCIP reduced min^{-1} mg $protein^{-1}$.

642 [c] µM of MR reduced min^{-1}mg $protein^{-1}$.

643 [d] µg of riboflavin reduced min^{-1} mg $protein^{-1}$.

644 NA : No Activity.

645 I – Intracellular, E - Extracellular

Table 2 Phytotoxicity studies of Brilliant Blue R and its degradation products

Parameters	*Triticum aesitivum*			*Phaseolus mungo*		
	Water	**Brilliant Blue R**	**Extracted metabolite**	**Water**	**Brilliant Blue R**	**Extracted metabolite**
Germination (%)	80	60	70	70	0.00	70
Plumule (cm)	13.50 ± 0.50	3.50 ± 0.50**	12.32 ± 0.34$^{\$\$}$	6.10 ± 0.50	0.00**	6.00 ± 0.39$^{\$\$}$
Radicle (cm)	4.75 ± 0.25	1.35 ± 0.15*	4.23 ± 0.50$^{\$}$	4.30 ± 0.40	0.00**	3.98 ± 1.0$^{\$\$}$

Values are a mean of three experiments ± SEM. Root and shoot lengths of plants grown in BBR are significantly different from those of plants grown in water by *P<0.05 and **P<0.001.

Root and shoot lengths of plants grown in the extracted metabolites is significantly different from that of plants grown in BBR by $^{\$}P$<0.05 and $^{\$\$}P$<0.001.

659 **Table 3** Summary of purification of laccase from cell suspension cultures of *B. malcolmii*

Purification steps	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U mg^{-1})**	**Purification fold**	**Yield (%)**
Crude culture	11.25	9	1.25	-	100
DEAE-anion exchanger	7.02	0.72	9.75	7.8	62.4

660

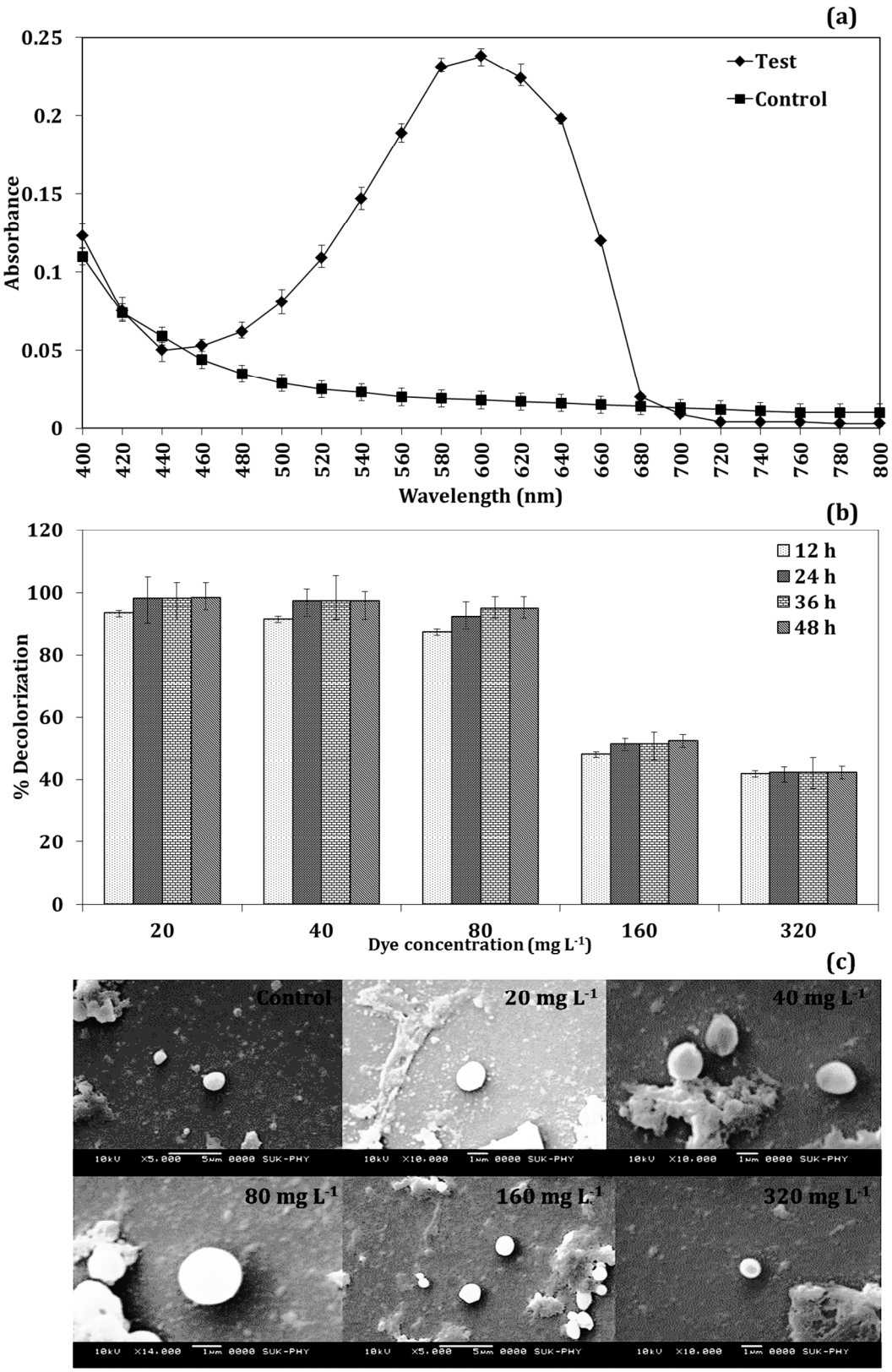


Fig. 1

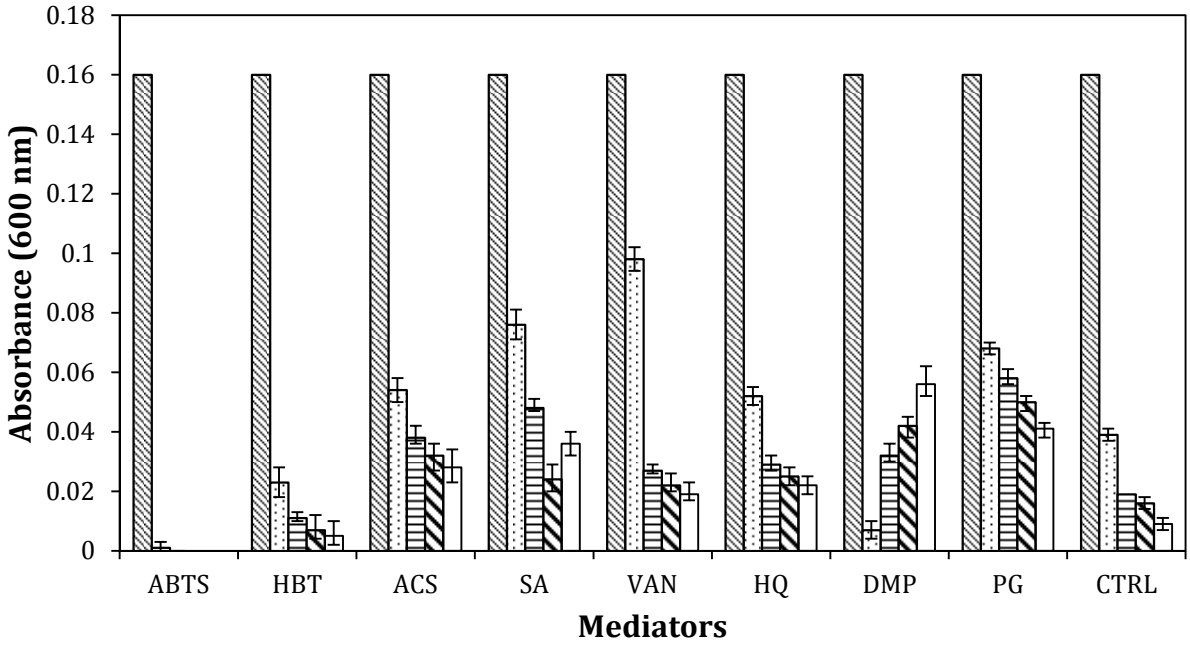


Fig. 2

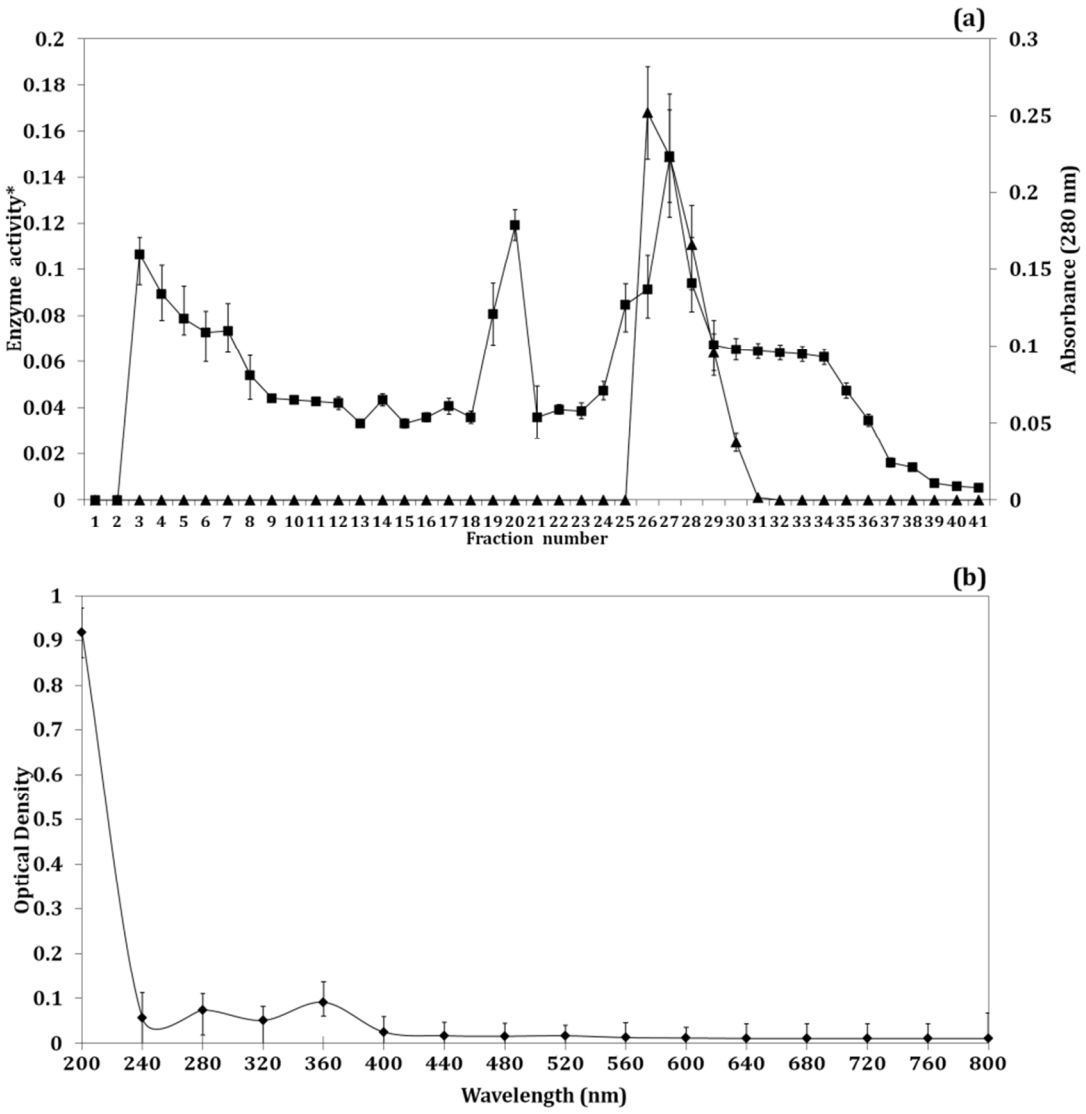


Fig. 3

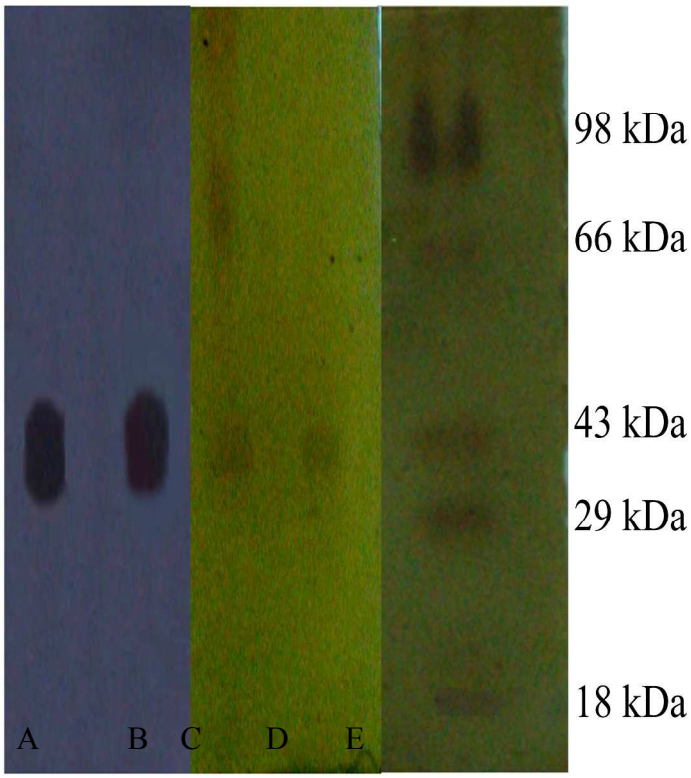


Fig. 4

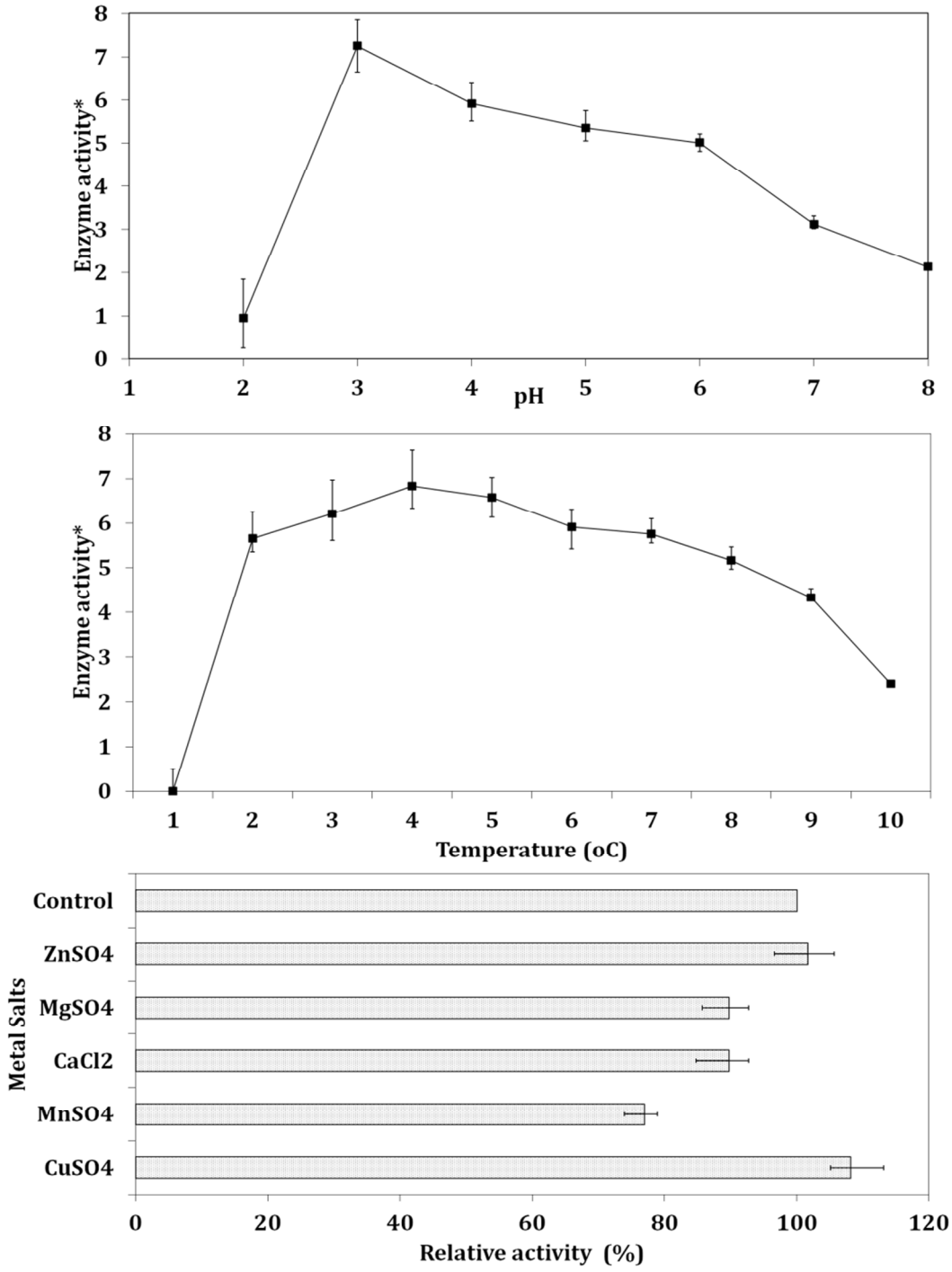


Fig. 5

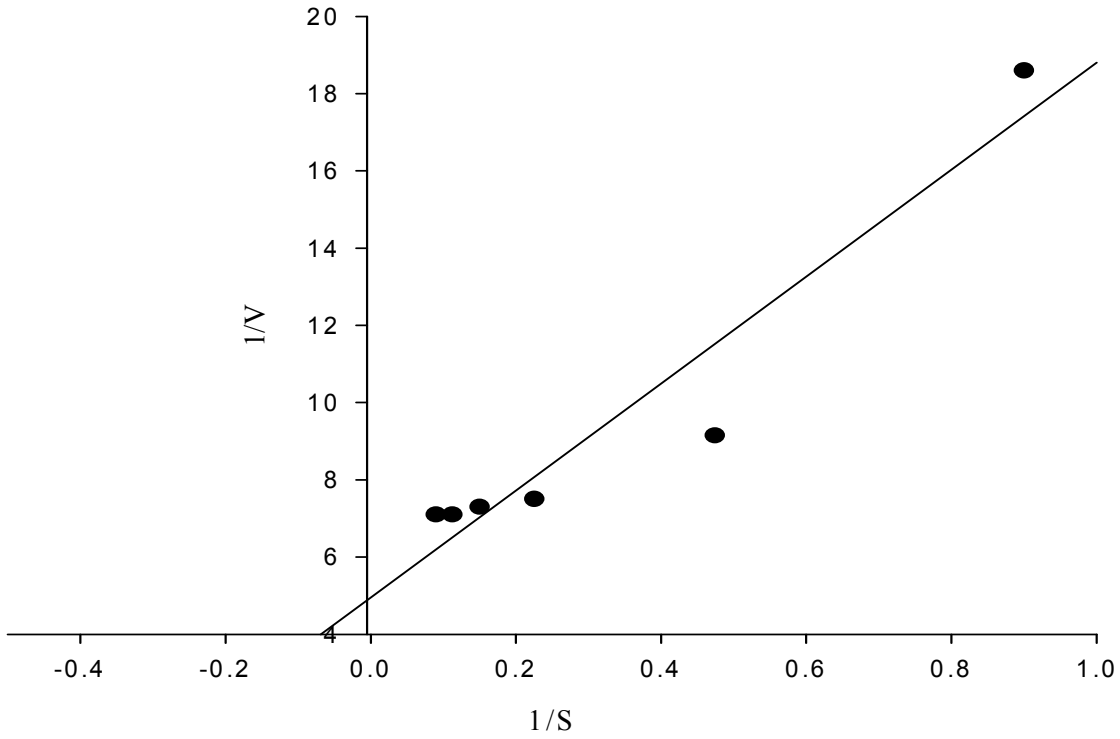


Fig. 6

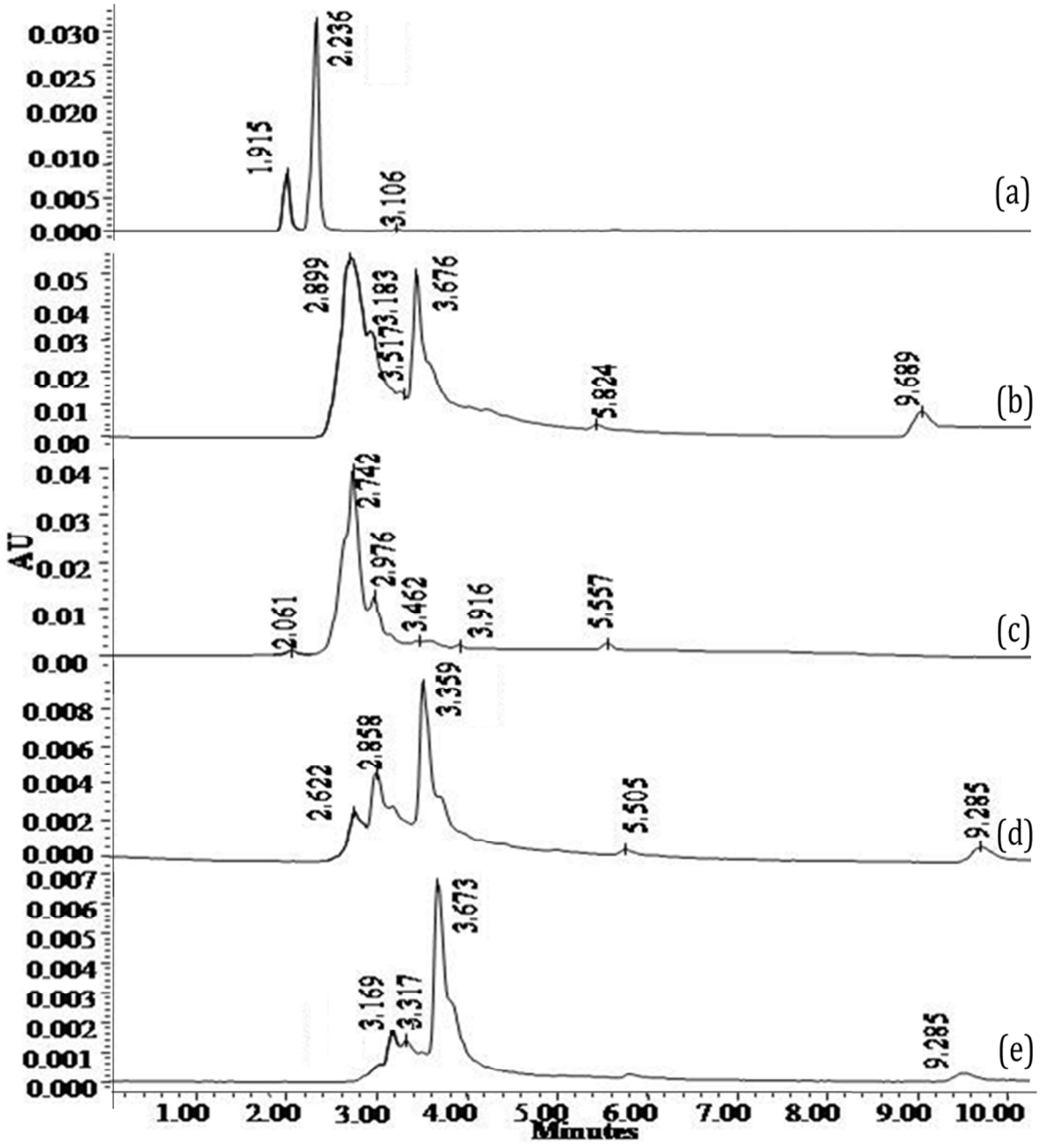


Fig. 7

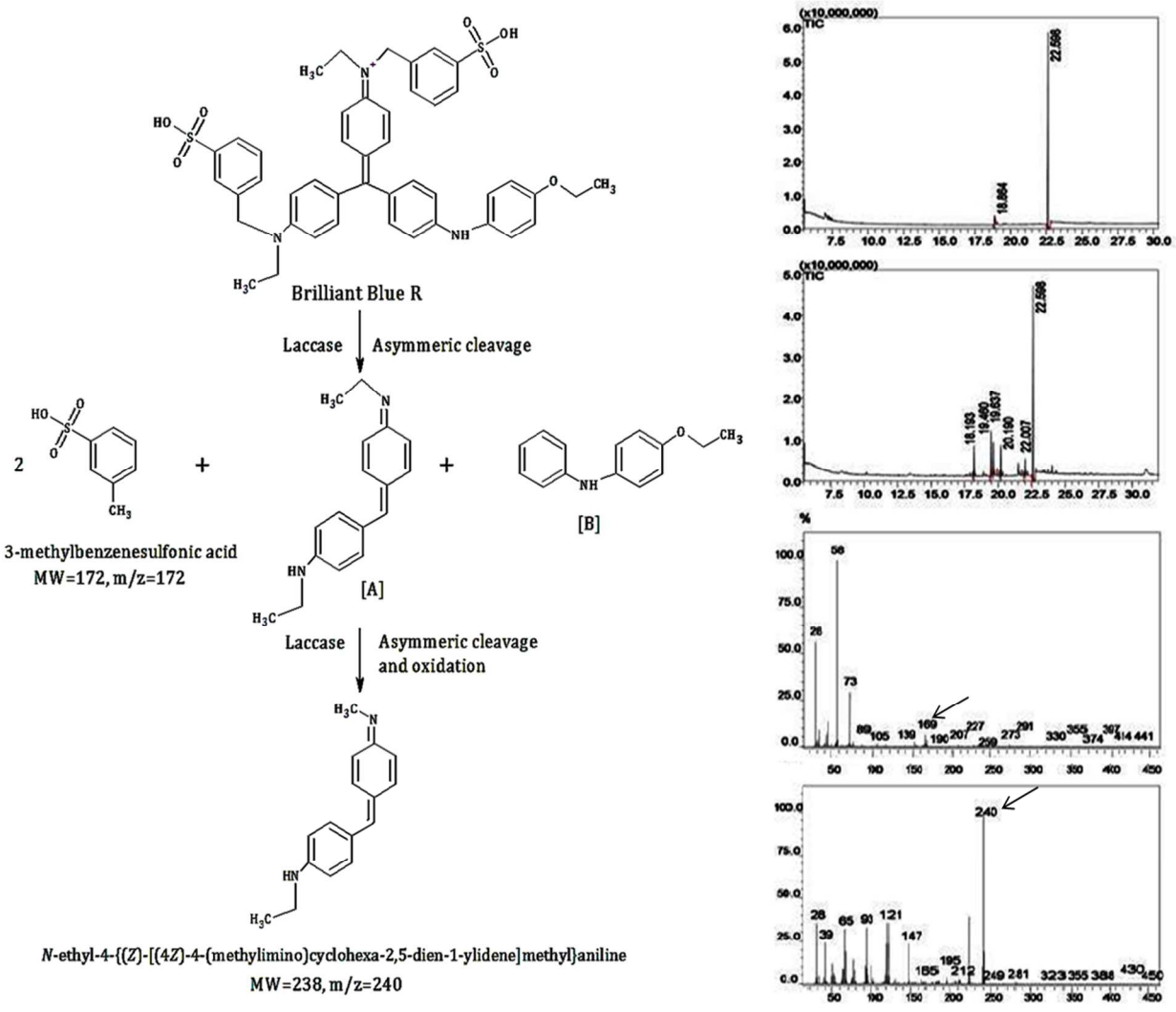
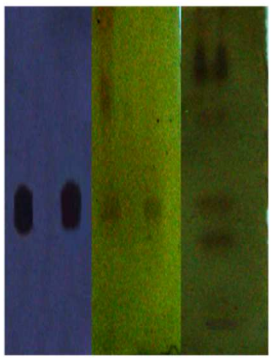
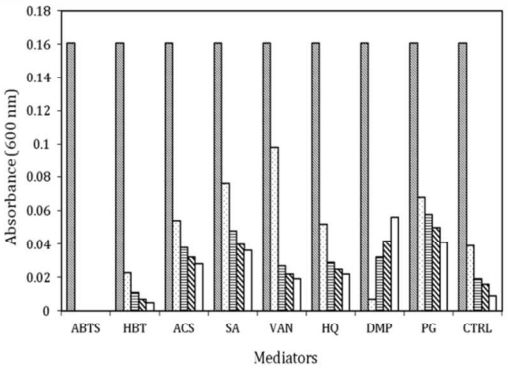


Fig. 8

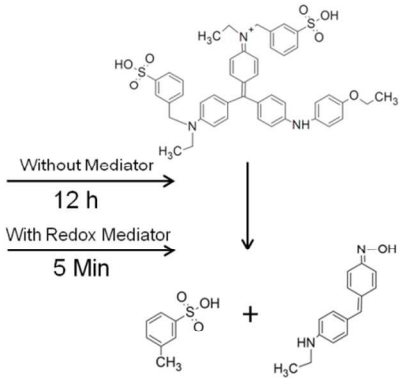
Table of content entry



Purified Laccase



Effect of different mediators on decolorization



Redox mediator significantly enhance the textile dye degradation potential of plant laccase