



Random Mutagenesis Methods for Hyper-Production of Laccase and its Effect on Azo Dye Decolourization

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This work focuses on the improvement of the wild strain for the hyper production of laccase through random mutagenesis. Maximum laccase production (46956 U/L) was observed in mutant *T. hirsuta*, when rice bran (5 g) at 75 % moisture (w/v) was used with 1 mL inoculum at pH 6 and 25 °C in the presence of 1 % (w/v) glucose as a carbon source, 0.1 % (w/v) sodium nitrate as a nitrogen source, 2 % of (v/v) glycerol as an inducer in submerged fermentation under static conditions. Laccase purification was performed by precipitating the enzyme with 90 % ammonium sulphate followed by ion exchange chromatography. The purified laccase had an apparent molecular mass of 49.9 kDa with monomeric structure estimated by SDS-PAGE. The three azo dyes could be efficiently decolourized by the purified laccase in the presence of a HBT as redox mediators after 3 h of incubation at pH 5. Laccase mediated degradation of ponceau was monitored by UV-visible, IR-spectroscopy, HPLC and GCMS. Furthermore, the decolourization products of ponceau were found to be aromatic amines and less phytotoxic nature than the untreated dye.

Keywords: Laccase, Random mutagenesis, Azo dyes, Decolourization, Redox mediators.

INTRODUCTION

Over the past few decades, much attention has been paid on the discharge of textile effluents containing synthetic dyes [1,2]. Wastewaters discharged from the textile industries pose a serious threat to the environment, as a large amount of chemically different synthetic dyes are used. Azo dyes are the largest diverse group of dyes which are extensively used in the textile, paper and printing industries [3-5]. Azo dyes are recalcitrant in nature; this can lead to acute effects on exposed organisms due to toxicity of dyes, phytoplankton forms abnormal colourations and reduction in photosynthesis because of the absorbance of light that enters the water. Recently it was reported that discharge of azo dyes in river and lakes alters the pH, increases the BOD and COD level, which in turn leads to more difficulty for the waste water treatment [6]. However, the biodegradation metabolite consists of aromatic amines and thus the dye stuff toxicity (*i.e.* mortality, genotoxicity, mutagenicity and carcinogenicity) was studied on both aquatic organisms (fish, algae, bacteria, *etc.*) and plants. Chronic effects of dye-stuffs, especially of azo dyes were seldom directly mutagenic or carcinogenic [7,8]. A number of conventional physico-chemical methods have been employed to remove azo dyes but these methods are often very effective but they are expensive and involve the formation of concentrated sludge

that creates a secondary disposal problem [9,10]. Recently, a lot of research on the treatment of textile dye stuff has been developed through biotechnological approach to eradicate this pollution source in an eco-efficient manner [11]. Current technologies for the degradation of azo dyes by microbial cultures and the enzymes derived from them have been recently reviewed [12]. In recent years, white-rot fungus has attracted increasing attention, as their lignolytic enzymes have the ability to degrade recalcitrant compounds and synthetic dyes [13]. Laccase is a multi-copper oxidase and are widely distributed in fungi, higher plants and bacteria. Laccase plays important role in the global carbon cycle and play a critical role in degrading a wide range of xenoaromatic compound present in the textile dyes [14]. Laccase is an extracellular glycoprotein enzyme produced by white rot fungi and are able to oxidize wide range of organic substrates, such as phenols, aromatic amines, heterocyclic compounds but unable to oxidize the non-phenolic compounds which have a higher redox potential [15]. Recently, it has been proved that this enzyme was able to oxidize non-phenolic structures effectively only in the presence of mediators. The purpose of this study was to evaluate the potential of wild and mutant strain for the production laccase and also to determine the efficacy of purified enzyme for the decolourization of azo dyes with exogenous redox mediator.

EXPERIMENTAL

All microbiology reagents and other chemicals were of highest purity and analytical grade. Ethidium bromide (EtBr), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and the azo dyes such as naphthol blue black, ponceau S and trypan blue were purchased from Sigma Aldrich, India. These azo dyes were selected based on the higher stability to the environmental factors. Minimal broth, peptone, yeast extract and agar powder were obtained from Hi-media laboratory, India.

Microorganism: *Phanerochaete chrysosporium* MTCC 787, *Trametes hirsuta* MTCC 1171, *Bacillus subtilis* MTCC 736 and *Pseudomonas aeruginosa* MTCC 2297 were procured in Microbial Type Culture Collection form Chandigarh, India. The young cultures of white rot fungus *T. hirsuta* MTCC 1171 and *P. chrysosporium* MTCC 787 was inoculated into 50 mL 2 % malt extract broth in 250 mL Erlenmeyer flasks at 28 °C, with continuous agitation at 150 rpm for 7 days. The mycelium was collected, washed twice with a salt solution (NaCl, 0.8 % w/v) homogenized for 1 min with sterile distilled water (1:5 v/v, mycelium:water). The selected bacterial cultures were aseptically transferred to 250 mL Erlenmeyer flasks containing 50 mL nutrient broth and incubated at 37 °C for 24 h. The bacterial and fungal suspensions were used for further analysis.

Screening of microorganism for laccase production: The ability of selected bacterial and fungal strains to produce intracellular and extracellular laccase respectively was visualized using plate assay method. The plate assay method allowed rapid determination of the presence of laccase in the intracellular and extracellular fluid. 15 mL of sterile agarose (0.5 %) medium containing 0.5 mM of ABTS per mL in sodium acetate buffer (pH 4.5, 0.1 M) was placed on a sterile glass plate (5 cm × 5 cm) and made well of (3 mm diameter) impregnated with 20 µL fluid. The development of an intense bluish-green colour around the wells inoculated with 20 µL fluid was considered as a positive test for laccase activity.

Random mutagenesis and mutant selection: Two steps of random mutagenesis approach were performed for the development of highly mutated and stable fungal and bacterial strains. In the first step, 4 mL of selected fungal mycelium suspension and 0.1 mL of young cells suspension of selected bacteria (10^6 cells) were separately transferred into sterile petri dishes of 80 mm diameter having a flat bottom. Then the cell suspensions were exposure to UV-A mutagen with 320 nm at 25 cm distance, receiving the radiation for regular intervals of 5, 10, 15, 20, 25 and 30 min. Immediately after the UV radiation, the plates were kept at dark room to avoid photoreactivation. The mutated fungal and bacterial cell suspensions was separately diluted in phosphate buffer and plated on PDA and nutrient agar respectively. After the period of incubation, the mutated strains possessing higher rate of laccase activity were screened out. During the second step, all the best mutant cultures obtained from first mutation were again subjected to ethidium bromide mutagens (100 µg/mL) at specified time intervals 30, 60, 90 and 120 min and double mutated strains were obtained. Among all mutated strains, the best potential strains were screened out for the hyper production of laccase and further work was continued on that mutant.

Optimization of parameters: The effect of various carbon source (glucose, lactose, maltose and sucrose), nitrogen source (sodium nitrate, peptone, beef extract and yeast extract), temperature (25, 35, 45 and 55 °C) and, pH (4-9) and inducers (glycerol, Tween 80, copper sulphate, glycerol + Tween 80) were investigated for the production of laccase using all the four mutant strain under static conditions.

Laccase production

Fungal laccase production: Laccase production was carried out in submerged fermentation using lignocellulosic substrates like wheat bran and rice bran were purchased from the local market, Kumbakonam, Tamilnadu, India. The production medium used for laccase production had the following composition (g/L): glucose-20.0; KH_2PO_4 -2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.01; $(\text{NH}_4)_2\text{SO}_4$ -0.3; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -0.01; KCl-1 and L-Glutamin-1. Submerged fermentation was performed separately for each wild and mutant strain in 250 mL Erlenmeyer flasks containing 100 mL production medium and 5 g lignocellulosic substrate. The contents were autoclaved at 121 °C for 15 min. 5 mL mycelium suspension (10^8 spores cells/mL) of fungal strain were transferred aseptically to the content and adjusted to 72 % moisture (w/w) with pre-optimized medium of pH 6.0 and incubated at 25 °C in a rotatory shaker (120 rpm) for 14 days.

Similarly, the bacterial laccase production was carried out separately for wild and mutant strain in 1000 mL Erlenmeyer flasks containing 500 mL nutrient broth amended with the log phase of bacterial cultures approximately (2.5×10^6 CFU/mL) and incubated on a rotary shaker (90 rev min^{-1}) with pre-optimized medium for 24 h.

Laccase purification: After the period of incubation the bacterial intracellular laccase enzyme was purified according to Diamantidis *et al.* [16]. For the fungal extracellular laccase production, 500 mL of supernatant of fungal culture were obtained by centrifugation at 6,000 g for 15 min. All purification steps were carried out at 4 °C. The extract further precipitated with 60-80 % ammonium sulphate and precipitated proteins were collected by centrifugation at 8,000 rpm for 0.5 h at 4 °C. The resulting pellets were dialyzed against 100 mM sodium phosphate buffer, pH 7.0. The enzyme collected was applied to an ion exchange chromatography (DEAE cellulose) (Bio-Rad) equilibrated with 100 mM sodium phosphate buffer, pH 7.0, at a flow rate of 0.4 mL/min. The column was washed with 200 mM NaCl in 100 mM sodium phosphate buffer, pH 7.0. The retained proteins were eluted with 600 mM NaCl in 100 mM sodium phosphate buffer, pH 7.0. Fractions with laccase activity were pooled, concentrated and then stored at 4 °C.

Laccase activity: Laccase activity was determined by the oxidation of ABTS method. The nonphenolic dye ABTS is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green colour can be correlated to enzyme activity and read at wavelength of 420 nm using spectrophotometer. The assay mixture contained 1.0 mL ABTS (3 mM), 0.8 mL sodium acetate buffer (0.1 M) of pH 4.5 and 200 µL of culture supernatant in a total volume of 2 mL. The reaction was held at 32 °C for 10 min; the 1 mL of 80 %

trichloroacetic acid (TCA) was added to stop the reaction. Oxidation of ABTS was monitored by determining the increase in A420 ($\epsilon_{420} = 3.6 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$). Absorbance was read out at wavelength of 420 nm using spectrophotometer against an abiotic control without laccase as a blank solution. One unit was defined as the amount of the laccase that oxidized 1 μmol of ABTS substrate per min [17].

Molecular mass determination through SDS-PAGE:

To verify the purity and subunit molecular mass, the enzyme was run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as specified by Laemmli [18]. The purified enzyme sample were dissolved in a minimum amount of distilled water and subjected to SDS-PAGE on 10 % polyacrylamide gels using standard molecular weight protein markers. Protein was visualized by staining the gel with silver staining.

Decolourization assay: A dye decolourization experiment was performed using selected stable azo dyes such as naphthol blue black, ponceau S and trypan blue. In order to achieve higher decolourization potentiality of purified laccase from the mutant *T. hirsuta*, the decolourization test was conducted in the presence and absence of different mediators such as veratryl alcohol (VA), 1-hydroxybenzotriazole (HBT), acetosyringone (AS) and 4-hydroxybenzoic acid (HA). The reaction was carried out step wise for each azo dye in a separate flask containing 2.5 mL of azo dye (50 mg/L), 500 μL of purified laccase enzyme (825 U/L) dissolved in 20 mM sodium acetate buffer and 2 mL of mediator (0.1 mM) at pH 5. Enzymatic decolourization reaction was performed with an addition of an enzyme and incubated at 30 °C with mild shaking. Enzyme dose was optimized such that dye was degraded by 25 % after a cuvet was placed in the spectrophotometer. Dye decolourization was determined by measuring residual absorption at the appropriate wavelength for each azo dye. A UV-Vis spectrophotometer was used for the absorbance measurement. A control test containing same amount of dyes without an enzyme was also performed simultaneously. The percentage decolourization was calculated according to Saratale *et al.* [19,20]:

$$\text{Decolourization (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \quad (1)$$

The average decolourization rate ($\mu\text{g h}^{-1}$) was calculated based on the method of Jadhav *et al.* [21]:

$$\text{Average decolourization rate} = \frac{C \times \%D \times 10000}{100 \times t} \quad (2)$$

where, C is the initial concentration of dye (mg L^{-1}) and % D is the dye decolourization (%) after time t (h).

FT-IR spectrum analysis: FT-IR analysis was performed in order to find out the changes in surface functional group of azo dye degraded metabolites, before and after decolourization the absorption. FT-IR spectra the samples are recorded using FT-IR spectrum 2000 Perkin Elmer spectrophotometer, in the mid IR region of 4000-400 cm^{-1} . The control and sample were prepared using spectroscopy pure KBr (1:20), the pellets were fixed in the sample holder and analyzed.

HPLC analysis: The enzymatic degraded product was examined by HPLC. HPLC analysis was carried out in an

isocratic system (Shimadzu with HPLC system with LC-10AT vp Pump) equipped with dual absorbance detector using C18 column with a 4.6 mm inside diameter and 25 cm height. HPLC grade acetonitrile:water:methanol (80:10:10) as mobile phase at the flow rate of 1.2 mL/min for 7 min at of 520 nm. In all cases selected azo dyes were used as control.

GC-MS analysis: GC-MS analysis was carried out for the identification of different metabolites produced during enzymatical degradation of azo dyes. GC-MS analysis (The Agilent 5975C Series GC/MSD) was carried out by using HP-5ms Column. Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1} . The injector temperature was maintained at 280 °C with oven conditions as: 40 °C kept constant for 1 min increased up to 325 °C with 10 °C min^{-1} raised up to 325 °C. The metabolites were identified by their fragmentation pattern and using NIST library.

Phytotoxicity study: Phytotoxicity assay was performed to measures the toxicity effect of azo dye and it's produced metabolites after decolourization by laccase enzyme. It was carried out using seeds of *Brassica nigra* and *Vigna radiata*. The uniformly selected seeds were surface-sterilized for 15 min in a solution of 1.2 % sodium hypochlorite to avoid fungal contaminant and then washed with sterile distilled water. The 500 ppm solution of azo dye and ethyl acetate extracted degradation metabolites of dye applied for toxicity testing. Ten healthy seeds of each crop were separately sowed into the plastic pot containing 15 g of washed and oven dried sand. The assay carried out at room temperature by daily watering 10 mL of dye solution (500 ppm) and its degradation metabolites (500 ppm). Control set was carried out at the same time by watering the seeds with distilled water. Germination (%) and the length of plumle (shoot) and radicle (root) was recorded after 9 days. Germination % was calculated as follows:

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sowed}} \times 100$$

RESULTS AND DISCUSSION

The plate assay method was used to screen the laccase producing bacteria and white rot fungi using agarose medium amended with ABTS as an indicator compound. Among the selected bacteria and fungi, *B. subtilis*, *P. aeruginosa* and *T. hirsuta* shows laccase positive (Fig. 1). From the primary screening of plate assay method it was clearly elucidated that the *T. hirsuta* shows prominent hollow zone around the well while there was no zone formation in *P. chrysosporium* which indicates negative for laccase production. The laccase positive bacteria and fungi were selected for further studies as the oxidative polymerization of guaiacol to form reddish brown zones in the medium. Sivakumar *et al.* [22] reported that *Ganoderma* sp was able to oxidize the phenolic and non-phenolic compounds. ABTS and guaiacol are considered unique laccase substrates in the absence of hydrogen peroxidase. The laccase positive wild bacterial and fungal cultures was grown on a basal medium in the presence of glucose, sodium nitrate, copper sulphate at 30 °C for 21 days. After the period of incubation the laccase activity was significantly higher in *T. hirsuta* when compared to bacterial culture such as *B. subtilis*

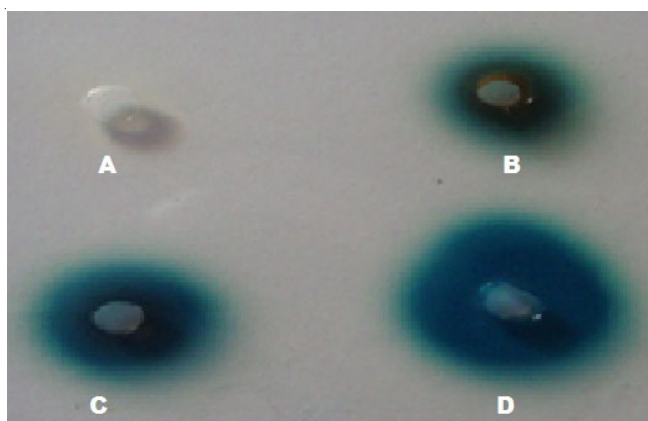


Fig. 1. Plate assay for demonstration of laccase activity in intracellular and extracellular fluid of selected bacteria and fungi respectively (A) 20 μ L of extracellular fluid from 21 days old culture of *P. chrysosporium* (B) 20 μ L of intracellular fluid from 24 h culture of *B. subtilis* (C) 20 μ L of intracellular fluid from 24 h culture of *P. aeruginosa* (D) 20 μ L of extracellular fluid from 21 days old culture of *T. hirsuta*

and *P. aeruginosa* (Fig. 2c). The mycelium suspension of wild strain *T. hirsuta* was exposed to UV radiation (1.2×10^2 J/m²/s) for 0 to 50 min with 6 cm distance from the source. After 35 min exposure of irradiation, culture was amended with 0.2 % of ethidium bromide in a basal medium. Nearly 85 % kill rate was observed and the maximum amount of laccase production

was observed from the stable mutant of *T. hirsuta* (Fig. 2a and 2b).

It was evident that the pH significantly influenced the extra cellular laccase production in mutant *T. hirsuta*. The fungus was able to release a maximum laccase activity of 42068 U/L at pH 6 after 18 days of incubation. The increase in pH over 6 and decrease the pH below 5.5 drastically reduce the laccase activity (Fig. 3a). The pH of the culture medium is critical to the growth, lignocellulolytic enzymes production and xenobiotics degradation. The optimum pH of laccase production was reported in many white rot fungi, falls between 4.5-6.0 [23]. The optimum pH of purified laccase produced by *Shyzyphyllum commune* IBL-06 was 6. The purified laccase remained quite stable within the pH range of 5-8 within 1 h incubation [24]. In the present study, pH 6.0 was found to be suitable for the maximum growth of laccase production by mutant *T. hirsuta* under submerged fermentation using rice bran as a substrate.

Four different carbon sources such as, maltose, sucrose, glucose and lactose were tested at 1 % for laccase production in mutant *T. hirsuta*. Among the carbon sources, glucose supported a maximum laccase activity of 40924 U/L. Our result reported that (1 %) of glucose concentration increases two fold by the mutant *T. hirsuta* under submerged fermentation (Fig. 3b). Kapdan and Kargi [25] reported that cultivation of *Coriolus versicolour* at 10 g/L glucose resulted in a better fungal growth. Revankar and Lele [26] investigated the effect

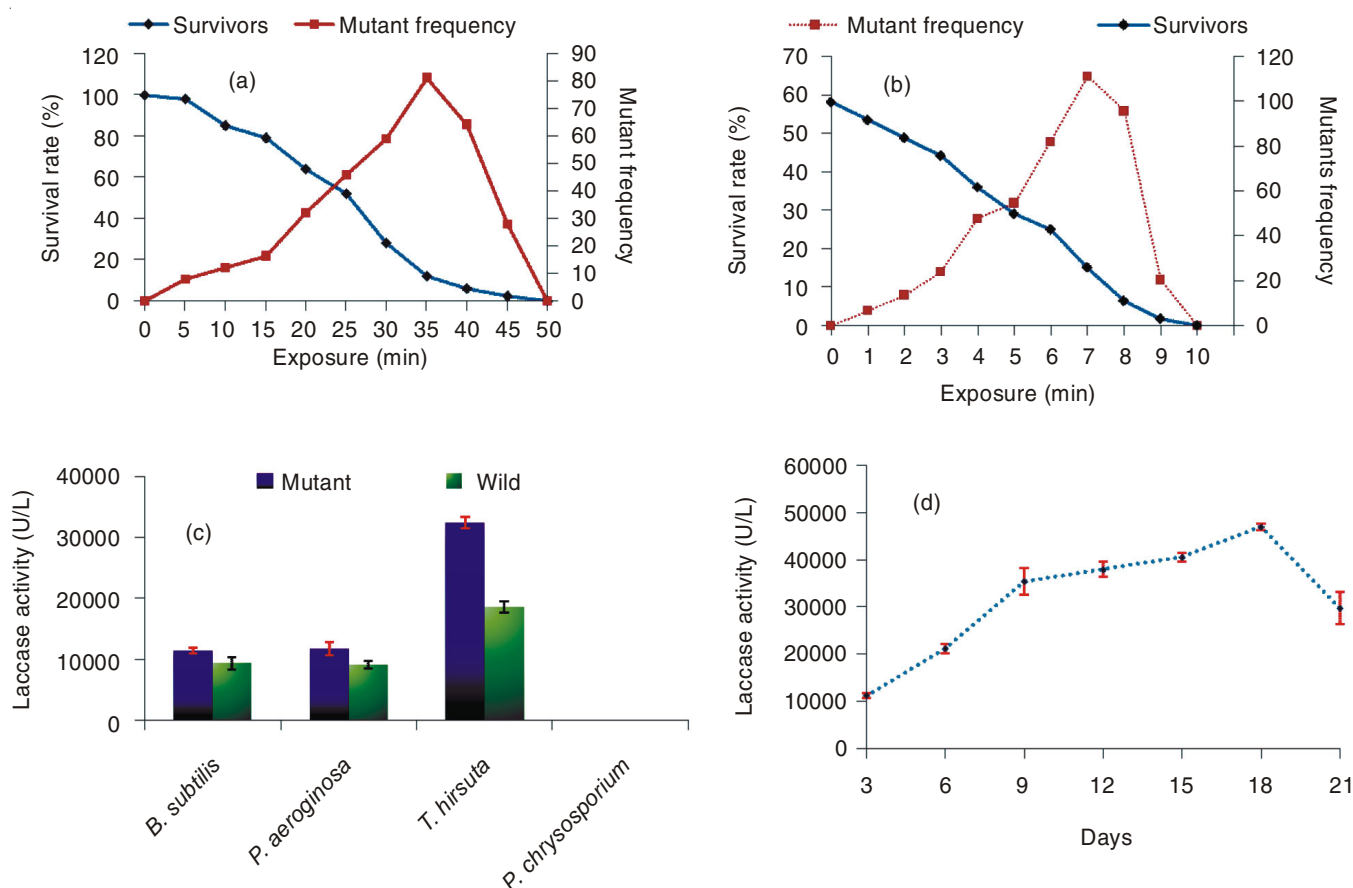


Fig. 2. Effect of random mutagenesis on laccase activity. (a) Effect of UV radiation doses on survival rate and mutant frequency of *T. hirsuta* (b) Effect of ethidium bromide doses on survival rate and mutant frequency of *T. hirsuta* (c) Laccase activity from wild and mutant strains before optimization (d) Hyper-production of laccase under optimized condition by mutant *T. hirsuta*

of different carbon sources (glucose, fructose, sucrose, lactose, starch and glycerol) on laccase production by *T. versicolour* MTCC138 and observed that a 3-fold increase of laccase production resulted when glucose was used instead of fructose and starch.

The fungus was able to release a maximum laccase activity of 41704 U/L at temperature 25 °C (Fig. 3c). The optimal temperature range for fungal laccase activity was ranging from 30 to 60 °C [27]. Laccase had an optimal temperature range of 30 to 65 °C which is similar to that of values obtained for laccases from *Sclerotium rolfsii* SRL [28]. From this, our result reported that the optimal temperature is 25 °C, which is closely related to the values of 30 °C.

Among the four different nitrogen sources tested sodium nitrate supported the maximum laccase production of 41080 U/L (Fig. 3d). The most widely used nitrogen sources for fungal lignolytic enzyme production are ammonium salts such as tartrate or chloride [29], whereas ammonium nitrate favoured high laccase production in white rot fungi namely *Lentinula edodes* [30]. *Trametes versicolour* MTCC 138 showed highest laccase activities by using a complex nitrogen source (yeast extract) and also obtained low laccase activities when using inorganic nitrogen sources [26].

Four different inducers like copper sulphate, Tween 80, glycerol and glycerol + Tween 80 were tested for extracellular laccase production in *T. hirsuta*. Among them, copper sulphate supported the maximum laccase production of 42016 U/L (Fig. 3e). Niladevi and Prema [31] obtained maximum laccase activity when copper sulphate was used at a concentration of 1 mM. Copper sulphate (7.9 U/mL) also proved to be a promising inducer for laccase production by *Streptomyces psammoticus* as similar to that in most of the fungi. Our results reported that CuSO₄ at 0.2 % concentration gives higher laccase activity.

Hyper production of laccase was investigated using mutant *T. hirsuta* under optimized condition. The maximum amount 46956 U/L of laccase was observed in the mutant *T. hirsuta* at 18 days of incubation under static condition (Fig. 2d). The optimum conditions were: pH-5, temperature-25 °C, moisture content-72 % (w/w), glucose-1 %, sodium nitrate-0.2 % and copper sulphate-0.2 %. To our best of knowledge the values of laccase activity obtained in the present study are higher than those found in the current literature. The highest laccase productivity with rice bran in liquid medium was 22 U/g substrate at 15 days cultivation [32]. Stredansky and Conti [33] reported that *Pleurotus florida* produced high amount of laccase (4.60 U/mL) in the malt extract broth after 12th day

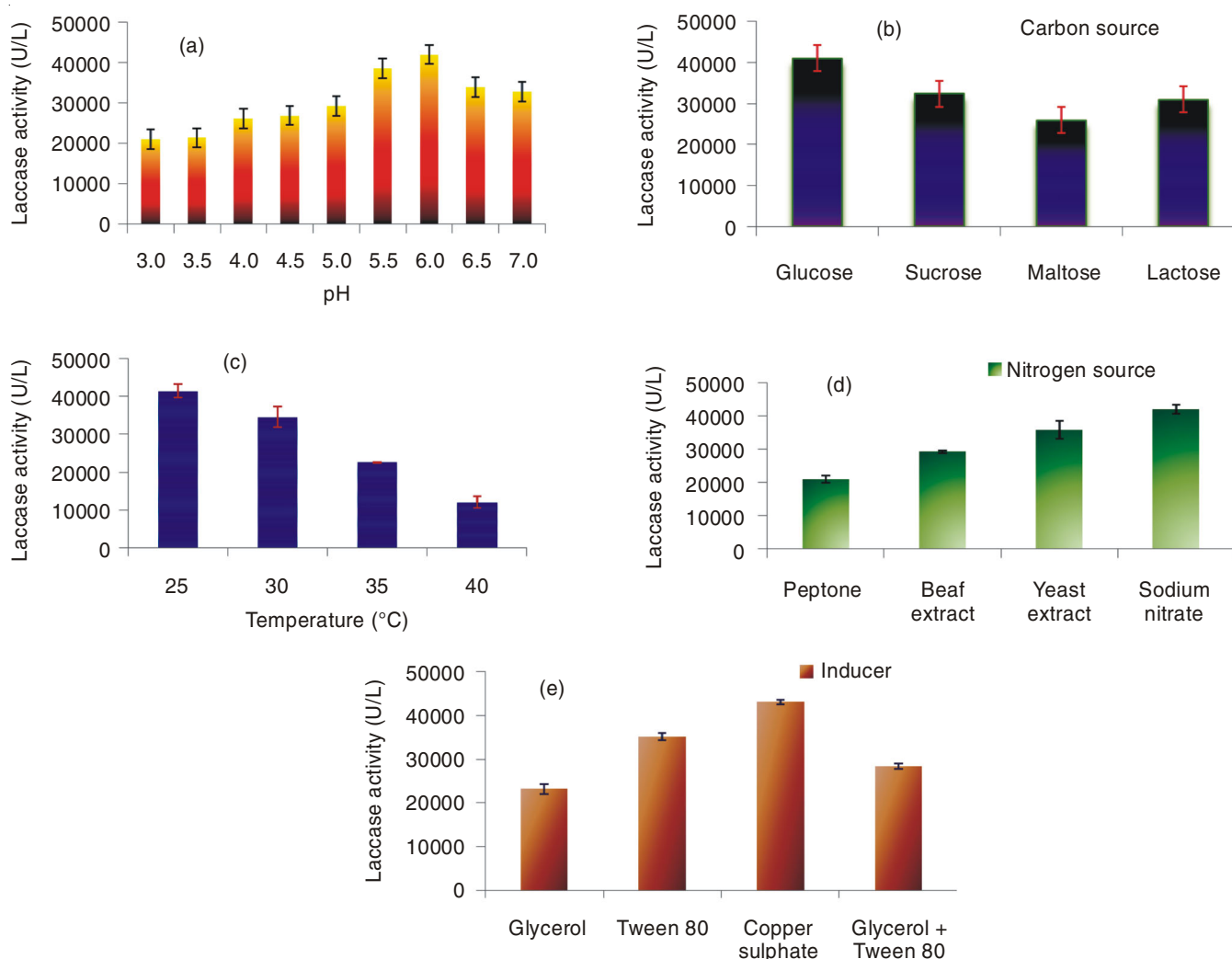


Fig. 3. Effect of (a) pH, (b) carbon sources (c) temperature (d) nitrogen sources (e) inducer on submerged fermentation using rice bran by mutant *T. hirsuta*

under stationary conditions. Laccase production using banana skin as a substrate produced 63 U/L on the third day and, then, it sharply increased up to a maximum activity of nearly 1600 U/L at the 18th day of cultivation [34]. Leatham and Stahmann [35] reported that *Botryosphaeria rhodina* MAMB-05 grown on essential oil-extracted orange bagasse in solid state fermentation without added nutrients produced laccase about 46 U/mL. Laccase activity in the medium reached a maximum on the 19th day. Our result reported that the mutant *Trametes hirsuta* was very stable in laccase production during four sub culturing and showed consistent high laccase activity in the basal medium along with the substrate rice bran over the period tested. The mutant strain showed 58 % improvement in the activity when compared to wild variety. The improvements of enzyme production of mutant strain depend on the mutagenic method used in the experiment.

The purified laccase from a mutant *T. hirsuta* appeared as a monomeric protein with single band during SDS analysis (Fig. 4). The molecular mass of purified denatured laccase was estimated to be 49 ± 1 kDa by SDS-PAGE analysis.

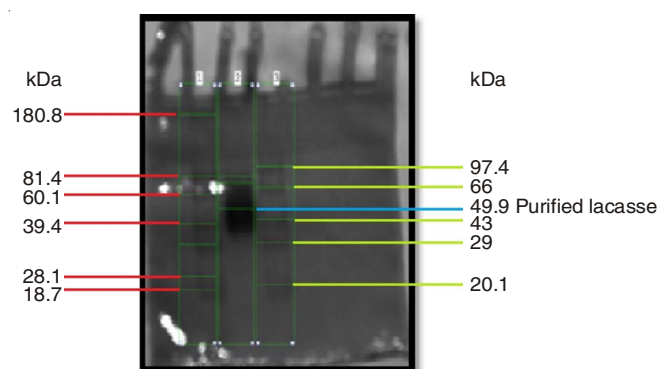


Fig. 4. SDS-PAGE of the purified laccase from mutant *T. hirsuta* Lane 1: high standard markers; Lane 2: purified laccase enzyme; Lane 3: medium range marker

Results of effective degradation of stable azo dyes such as ponceau S, trypan blue and naphthol blue black using laccase in absence or presence of mediators are illustrated in Fig. 5. Among the four redox mediators tested the HBT significantly enhances the degradation potential of laccase on ponceau S and trypan blue, whereas the veratryl alcohol shows very effective degradation only on naphthol blue black. However, the remaining mediators had little impact on degradation of selected dyes. Up to 89 and 66 % of degradation was observed with addition of HBT on ponceau S & trypan blue, respectively, whereas 75 % degradation was observed in naphthol blue

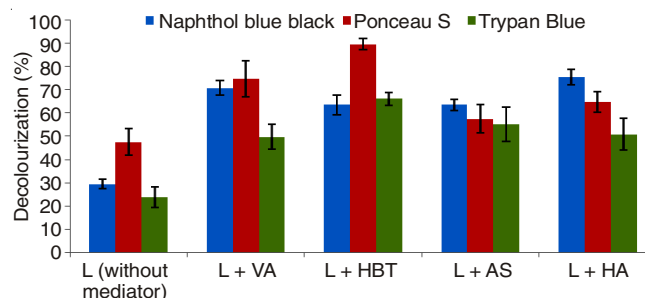


Fig. 5. Decolourization of azo dyes by laccase (L) in absence and presence of redox mediators { veratryl alcohol (VA), 1-hydroxybenzotriazole (HBT), acetosyringone (AS) and 4-hydroxybenzoic acid (HA) } after 3 h incubation at 30 °C. Data is represented as mean \pm standard error, $n = 3$

black within 3 h of incubation time at pH 5. Our results revealed that the mediator increases the catalytic activity of laccase towards the degradation of azo dyes. Moreover, the redox mediators are laccase substrates and act as electrons shuttle between the enzyme active site and the complex compounds, which enable the oxidation of target compounds [36]. Recently, the laccase-mediated systems are one of the most powerful systems for enzymatical removal of textile pollutant, especially the synthetic azo dyes [37,38]. It was observed that the laccase from the mutant *T. hirsuta* represent an ecofriendly, inexpensive and promising tool for the removal of various azo dyes from textile dye effluents.

The decolourization potential of a laccase can be tested by using different types of selected stable azo dyes. The average decolourization rate of various azo dyes by laccase from the mutant *T. hirsuta* was significantly higher than the wild strain with the help of redox mediators (Table-1). Effective degradation of azo dyes using laccase mediator systems depends upon the concentrations of the mediator, laccase activity, pH of the reaction carried out, redox reversibility of the radical-substrate reaction and nature of the azo dyes used [13].

FTIR spectra obtained from control dye ponceau S showed specific peaks at $1120.46\text{--}692.17\text{ cm}^{-1}$ for C-H deformation, 1225.85 cm^{-1} for C-N vibrations, 1404.81 cm^{-1} for NO_2 stretching of aromatic nitro compound, 1635.33 cm^{-1} for N=N stretching in azo group, 2083.95 cm^{-1} for C \equiv N stretching in saturated nitriles and 3434.88 cm^{-1} for O-H stretches (Fig. 6a). After the enzymatical dye degradation using laccase produced by mutant *T. hirsuta*, a significant reduction in IR peaks was observed in the 1635.33 cm^{-1} region of metabolites confirmed the cleavage of azo group of the dye. The peak at 3369.9 cm^{-1} with supporting peak at 2946.8 and near 2834.9 indicated N-H stretching of amines. A C=C stretch of aromatic compound forms peaks

TABLE-1
COMPARATIVE AVERAGE DECOLOURIZATION RATE OF AZO DYES BY LACCASE WILD (W) AND MUTANT (M) *T. hirsuta* IN THE ABSENCE AND PRESENCE OF REDOX MEDIATORS { VERATRYL ALCOHOL (VA), 1-HYDROXYBENZOTRIAZOLE (HBT), ACETOSYRINGONE (AS) AND 4-HYDROXYBENZOIC ACID (HA) } AFTER 3 h INCUBATION AT 30 °C

Name of azo dyes	λ_{max}	Average decolourization rate ($\mu\text{g h}^{-1}$)								Without redox mediator	
		VA		HBT		AS		HA			
		W	M	W	M	W	M	W	M	W	M
Naphthol blue black	618	6830	12166	5997	10500	6830	10495	6997	12328	4831	5997
Ponceau S	352	8996	12328	8996	14827	8663	9329	7996	11162	7996	8996
Trypan blue	607	3831	8330	5997	11162	6664	9163	3831	8829	3831	4998

at 1452.9, 1415 and 1113.5 cm^{-1} shows S=O stretching of sulphonamides and sulphonic acids, respectively. Moreover, a peak at 1026 cm^{-1} for C-H deformation suggests cleavage of dye molecule (Fig. 6b). Disappearance of major peaks and formation of new peaks in the IR spectrum of mutant *T. hirsuta* metabolites suggest the biotransformation of dye into distinct metabolites. The HPLC analysis of ponceau S before the laccase mediator treatment showed a single peak at retention time 2.2 (Fig. 7a) whereas, the detection of two or more peaks in HPLC elution profile after the laccase mediator degradation of ponceau S, clearly indicated the degradation of the parent dyes (Fig. 7b).

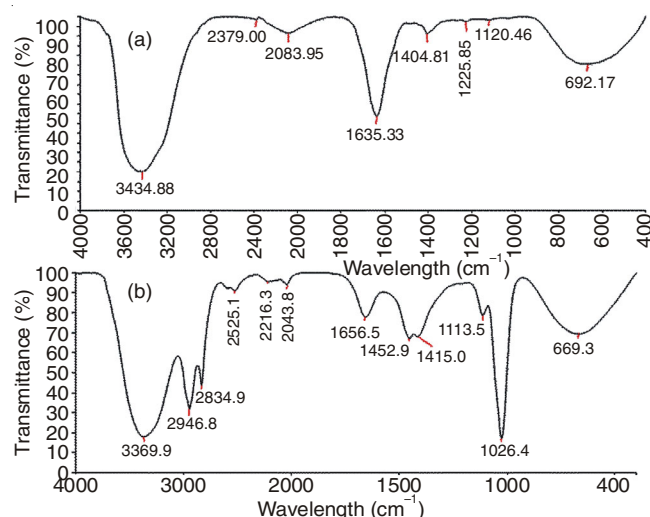


Fig. 6. FTIR spectrum (a) control dye ponceau S (b) degraded metabolites of ponceau S by mutant *T. hirsuta*

GC-MS analysis was carried out in order to verify degradation product formed during the enzymatical decolourization. Furthermore, the GC-MS analyses of the metabolites raised from the enzymatical degradation of dye ponceau S by mutant *T. hirsuta* demonstrated the asymmetric cleavage of dye with the yields three metabolites such as 12-methyl-E,E-2,13-octadecadien-1-ol-13.7 {55/69 m/z }, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-14.5 {154 m/z } and N-hexadecanoic acid-15.8 {256 m/z }. Furthermore, in most of the research it was clearly elucidated that veratryl alcohol oxidase was responsible for the asymmetric cleavage of azo dyes [39].

Over the last few decades, in India, the untreated dyeing effluent causes severe environmental problems and health hazards. Further, they are being discharged into the water bodies and cause severe contamination of surface and ground water. Thus, it was of concern to assess the phytotoxicity of

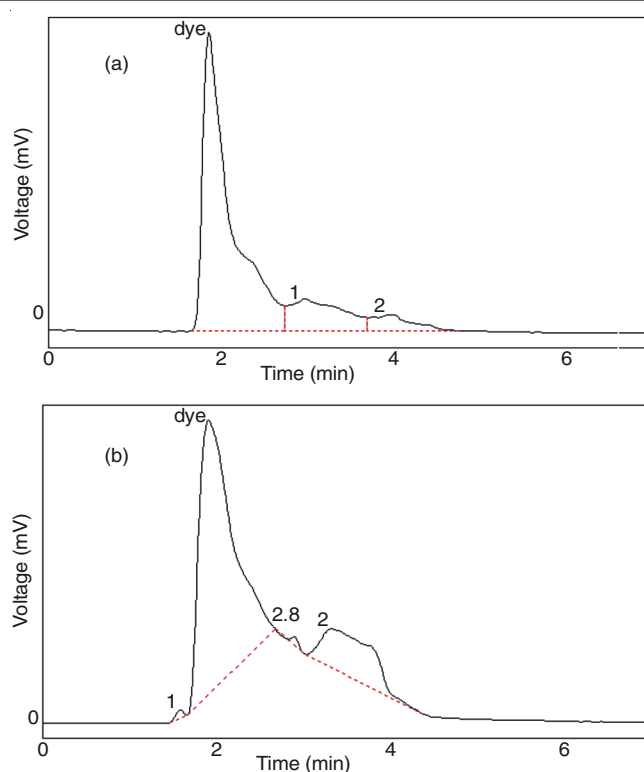


Fig. 7. HPLC chromatogram (a) before the enzymatical treatment of ponceau S with concentration of 50 ppm (b) Laccase mediated degraded metabolites of ponceau S with concentration of 50 ppm

the dye before and after degradation. Table-2 represents the phytotoxicity analysis of the ponceau S and its metabolites obtained after decolourization. 100 % seed germination was observed in water for both the plant *B. nigra* and *V. radiata*. Whereas, seed germination was inhibited up to 90 and 95 %, when seeds were treated with 500 ppm concentration of ponceau S in *B. nigra* and *V. radiata*, respectively, this indicates the toxic nature of the dyes to plants. Furthermore, higher inhibitory effect was observed on plumule and radical length when treated with a ponceau S (500 ppm). In contrast, plumule length and radical length in *P. mungo* and *S. vulgare* were found equivalent to control set (distilled water) with 90 % germination when treated with 500 ppm concentration of the degradation products. The outcomes of the phytotoxicity studies indicate that the control dye compound was toxic and the metabolites had a less toxic effect on the crop plant seeds.

Conclusion

In this study, we have developed a simple and cost-effective technique for the hyper production of laccase from the mutant *T. hirsuta*. Our result also suggests that the efficiency

TABLE-2
PHYTOTOXICITY STUDIES OF PONCEAU DYE AND ITS DEGRADED METABOLITES WITH *B. nigra* AND *V. radiata*

Parameters	<i>Brassica nigra</i>			<i>Vagina radiata</i>		
	Distilled water	Ponceau dye	Metabolite	Distilled water	Ponceau dye	Metabolite
Germination (%)	100	20	90	100	30	95
Plumule (cm)	23.90 ± 0.98	6.50 ± 0.62	20.50 ± 0.99	1.78 ± 0.055	0.55 ± 0.058	1.41 ± 0.06
Radical (cm)	1.28 ± 0.057	0.59 ± 0.064	1.65 ± 0.058	24.70 ± 0.87	4.70 ± 0.87	18.3 ± 0.73

Values are mean of three experiments standard error mean (SEM) (±), Seeds germinated in Ponceau dye and degraded metabolites are significantly different from the seeds germinated in distilled water at P < 0.001 by one-way analysis of variance (ANNOVA) with Tukey-Kramer comparison test.

of laccase mediators system for the decolourization of selected azo dyes depend on the stability and chemical nature of dyes. Furthermore, our study also demonstrates that the laccase mediators system effectively transforms the toxic nature of the dyes into non-toxic, thereby enhancing its feasibility in practical applications.

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