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Full Length Research Paper

Decolourization and degradation of azo Dye, Synozol Red HF6BN, by *Pleurotus ostreatus*

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The present paper focuses on the use of fungus, *Pleurotus ostreatus*, to decolorize and degrade azo dye, Synazol Red HF6BN. Decolorization study showed that *P. ostreatus* was able to decolorize 96% Synazol Red HF6BN in 24 days. It was also found that 94% Synazol Red HF6BN containing dye effluent was decolorized by *P. ostreatus* after 30 days of incubation at room temperature. The enzyme exhibited highest activity at 70°C and at pH 6.0. The enzyme activity was enhanced in the presence of metal cations. High performance liquid chromatography analysis confirmed that this fungal strain is capable of degrading Synazol Red HF6BN dye into metabolites. The observation of no zones of inhibition on agar plates and growth of *Vigna radiata* in the presence of dye extracted sample indicated that the fungal degraded dye metabolites are nontoxic to beneficial micro-flora and plant growth. Therefore, *P. ostreatus* has promising potential in colour removal from textile wastewater containing azo dyes.

Key words: Azo dyes, decolorization, *Pleurotus ostreatus*, bioremediation.

INTRODUCTION

A great number of dyes and other chemicals are used in textile industry. There are more than 100,000 commercially available dyes with over 10,000 different dyes and pigments used in industries, representing an annual consumption of around 7×10^5 tonnes worldwide (Akhtar et al., 2005). Among all the dyestuff, the azo dyes are considered the most important and a major group of dyes mostly used in industry, representing 70% of total dyes produced per year (Kumar et al., 2007; Dos Santos et al., 2007; Jadhav et al., 2007). They are extensively used in textile dyeing due to their favorable characteristics such as superior fastness to the applied fabric, high stability to light bright color, resistance to microbial attack, water-fastness and simple application techniques. However, nearly 50% of reactive dyes may be lost in the effluent after the dyeing of cellulose fibers, and are highly recalcitrant to conventional wastewater treatment processes (Aksu and Cagatay, 2006).

Effluents from the textile industries containing dye are highly colored and are therefore visually identifiable (Kilic et al., 2007). The discharge of these industrial effluents into aquatic ecosystems and their efficient removal from textile industry is still a major environmental challenge not only for aesthetic reasons, but also for the alteration of the solubility of gases in water, their bio-recalcitrant nature, and their effects on the ecosystem due to the toxic intermediates produced (mutagenic and/or carcinogenic) (Dos Santos et al., 2007; Pavan et al., 2008). Although electrochemical destruction (Mohana et al., 2007), photocatalysis and adsorption (Hasnat et al., 2007) are effective tertiary treatments, these methods are not economically viable. Different techniques including almost all the known physical and chemical and biological techniques were described for decolorization and the final conclusion was that each process alone might not be able to meet the requirements (Peralta-Zamora et al., 2003).

In recent years, several microorganisms have been investigated for decolorization of reactive dyes, and its effectiveness depends on the adaptability and the activity of selected microorganisms (Kodam et al., 2005; Aksu et al., 2007; Jadhav et al., 2007; Dave and Dave, 2009). The role of fungi in the treatment of wastewater has been extensively researched and due to an increased cell-to-surface ratio, fungi have a greater physical and enzymatic contact with the environment. Basidiomycetes are considered to be efficient laccase producers, (Martínez et al., 2005; Mendonc et al., 2008). In particular, the

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white rot fungi (WRF) (Revankar and Lele, 2006) produce large repertoire of extracellular lignin-modifying enzymes (Arora and Sharma, 2009) which are able to degrade and detoxify (Kumar et al., 2007) a wide range of xenobiotic compounds under aerobic conditions (Mohorcic et al., 2006). Laccase, EC 1.10.3.2, p-diphenol: oxygen oxidoreductase, is part of a larger group of enzymes termed the multicopper oxidases (MOC), (Komori et al., 2009) belonging to the group of blue-copper proteins (Minussi et al., 2007). It is an important class of enzyme found in many organisms, including plants such as the varnish tree *Rhus vernicifera*, (Morozova et al., 2007) fungi, bacteria and humans (Augustine et al., 2008). This enzyme is generally extracellular and catalyzes the oxidation of several phenolic compounds, aromatic amines, thiols and some inorganic compounds using molecular oxygen as electron acceptor (Arora and Sharma, 2009; Pezzella et al., 2009) and have a great potential in various biotechnological processes. Fungal laccases have been confirmed for their ability to degrade several azo dyes (Tauber et al., 2005; Husain, 2006). The decolorization efficiency ranged from 70 to 90% when investigating nine dyes with different structure degraded by white rot fungi *Pleurotus ostreatus* (Zhao, 2004).

The aim of the present work was to exploit the biodecolourization of Synazol Red HF-6BN by *P. ostreatus* with the following objectives: (1) to assess the ability of the fungal cultures to decolorize the actual dye industry waste, (2) confirmation of degradation of the dye and (3) to assess the toxicity of the degraded products.

MATERIALS AND METHODS

Microorganisms and growth conditions

The fungus, *P. ostreatus*, was a kind gift from Mycology and Plant Pathology Department, Punjab University, Lahore, Pakistan. The solid medium used for fungal growth contained per liter: 10 g of malt extract, 4 g of yeast extract, 4 g of glucose and 20 g of agar (pH 5.5). For laccase production and induction studies, 3 ml of homogenized mycelium were used for inoculation of 1000-ml Erlenmeyer flask containing 300 ml of culture medium. This salt basal medium contained (per liter) glucose, 10 g; peptone, 5 g; yeast extract, 1 g; ammonium tartrate, 2 g; KH_2 PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; trace elements solution, 1 ml. The pH was adjusted to 5.5.

Decolorization study

Dye concentration of the decolourized broth was quantified by comparing its absorbance with the absorbance of known concentrations of Synazol Red HF6BN and this was used to calculate the dye removal rate (mg L^{-1}) and expressed in percentage of decolorization (Chen et al., 1999).

$$\text{Decolorization (\%)} = \frac{I - F}{I} \times 100$$

Where, I is the initial absorbance and F is the absorbance of decolorized medium.

Decolorization of dye from a manufacturing industry's effluent

To check the efficacy of fungus to decolorize the industrial effluent, a laboratory-scale experiment was set up. Two plastic containers were taken. In the first container, 8 L of dye effluent was taken along with 1.5 L of *P. ostreatus*. In the second container, only 8 L of dye effluent (temperature, 33°C; pH, 7.6; dissolved oxygen, 0.154 ± 0.04 g L^{-1}; Synazol Red HF6BN 2.160 ± 0.03 µg mL^{-1}) was taken and 20 mg L^{-1} of Synazol Red HF6BN stress was maintained in each container. The experiment was carried out at room temperature (28 ± 2°C). After 10, 20 and 30 days of incubation, samples were taken, centrifuged and supernatants used to estimate the amount of Synazol in dye effluents by ultraviolet UV) visible spectroscopic analysis (Hitachi U-2800, Tokyo, Japan) by measuring the optical density at 463 (Khalaf, 2008). The percent decolourization was calculated by taking untreated dye solution as control (100%).

High-performance liquid chromatography (HPLC)

The biodegraded products were monitored by HPLC. The HPLC analysis was carried out (Waters model no. 2690) on a C18 column (symmetry, 250 × 4.6 mm) with methanol: acetonitrile (1:1) as mobile phase with at flow rate of 1.0 ml/min and UV detector at 540 nm (Dawkar et al., 2010).

Enzyme assay

Laccase activity was determined by using azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma, USA) as substrate at 420 nm. The measurements were made with 100 mM sodium acetate buffer (pH 5.0) at 30°C for 30 min (Hatvani and Mecs, 2001). One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µmol of ABTS per min.

Effect of temperature, pH and metal ions on the enzyme activity

The optimum temperature of the laccase was determined by incubating the reaction mixture for 30 min at different temperatures ranging from 30 to 90°C. The pH profile of the enzyme was evaluated by incubating the reaction mixture for 30 min in the presence of appropriate buffers: 50 mM sodium acetate (pH 4.5 to 6.0), 50 mM sodium phosphate (pH 6.0 to 8.0), and 50 mM Tris-HCl (pH 8.0 to 10.0). The activity of each sample was quantified by the assay method as earlier described. The metal ion effect on enzyme activity was examined by chloride salts of various metals. Each metal was added in the reaction mixture at a final concentration of 0.1 mM and laccase activity was determined at 30°C and pH 5.0. No metal ions were added in the control assay.

Microbial and phytotoxicity assay

The decolorized dye at the concentration of 100 mg L^{-1} was tested for its toxic effect (Chen, 2002) on the agriculturally important soil bacterial flora according to Mali et al. (2000). *Bacillus cereus* and *Azotobacter* sp. were inoculated on minimal salt medium. Two wells of 2 mm diameter were made on the minimal salt medium containing plates. Both were filled with 1.0 mg/L of decolorized centrifuged broth. The plates were incubated at 30°C for 48 h. Zone of inhibition surrounding the well represented the index of toxicity.

Phytotoxicity test was also performed in order to assess the toxicity of the treated dye sample by fungus at concentration of 100 mg/L according to Dawkar et al. (2010). For this purpose soil was

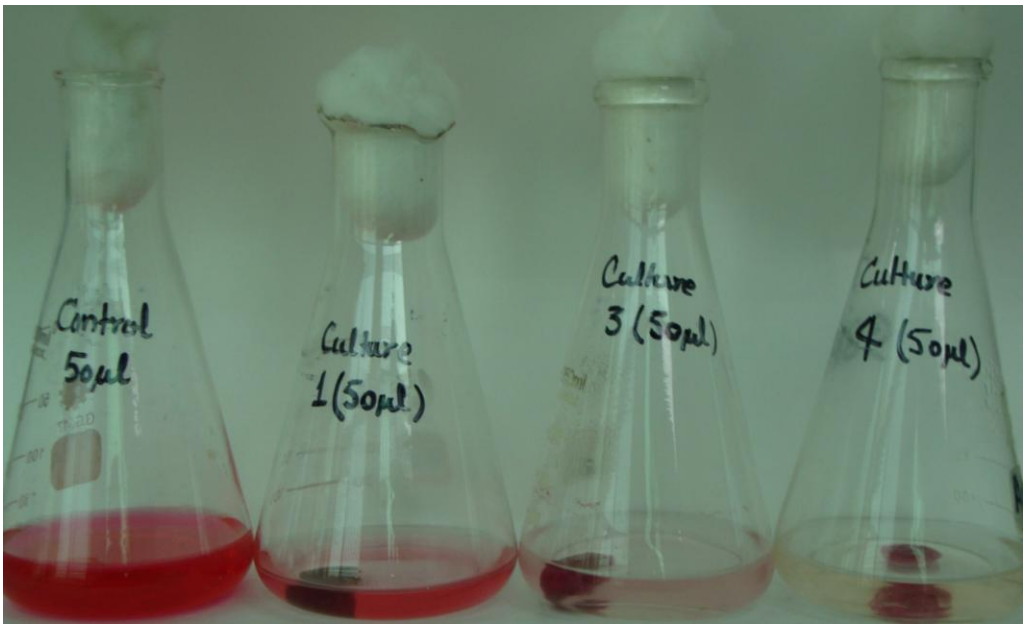

Figure 1. Gradual decolorization of Synazol Red HF6BN by *P. ostreatus* from the cultural flasks at room temperature.

Table 1. Decolorization (%) of Synazol Red HF6BN by *P. ostreatus* incubated at 30°C for different time period.

Fungal isolate	Time period (day)			
	6	12	18	24
P. ostreatus	40	66	82	96

sterilized by autoclaving and almost equal quantity of sterilized soil was taken in pots. Seeds of mung beans (*Vigna radiata*) were taken and sterilized with $HgCl_2$ (1%). After washing thrice with $HgCl_2$, they were rinsed thoroughly with distilled water and inoculated (four in each pot). Pots were watered regularly with 15 ml of supernatant of seven days old cultures of *P. ostreatus*. For control pots, tap water was used instead of fungal supernatant. The growth of *V. radiata* watered with fungal decolorized water was compared with the growth of *V. radiata* watered with simple tap water after seven days incubation of 12 (dark) : 12 (light) time period.

Statistical analysis

Observations were made and all the experiments were repeated two or more times. The results reported are average values.

RESULTS AND DISCUSSION

Decolorizing ability of the fungus

Synazol Red HF6BN processing capability of the *P. ostreatus* was checked by adding dye at 20 mg L^{-1} in the culture medium. *P. ostreatus* could decolorize 96% of dye from the medium after 24 days of incubation. The fungus was also capable of decolorizing dye by 40, 66 and 82% from the medium after six, 12 and 18 days, respectively (Figure 1; Table 1). Bio-decolorization of dyeing wastewaters by microbial enzymes is a promising, eco-friendly and cost competitive alternative. Usman et al. (2011) described that *Corynebacterium* sp. could decolorize 60% (Reactive Black5) and 76% (Reactive Yellow15) from the medium containing 100 mg L^{-1} after two days. Degradation of azo dyes by filamentous fungi, such as whit rot fungi have already been reported (Martins et al., 2001). Compared to other fungal oxidative enzymes, laccases can act oxidatively, non-specifically at the aromatic rings and has the potential to degrade a wide range of compounds. Laccases have gained much attention over the last number of years in many industrial and environmental fields due to their wide substrate specificity (Sadhasivam et al., 2009).

Decolorization of Synazol Red from industrial effluent

In order to assess the ability of *P. ostreatus* fungus to remove Synazol Red HF6BN from dye contaminated Industrial effluents, a lab-scale experiment was performed. *P. ostreatus* was capable to decolorize 43, 68, and 94% dye from the industrial effluent after 10, 20 and

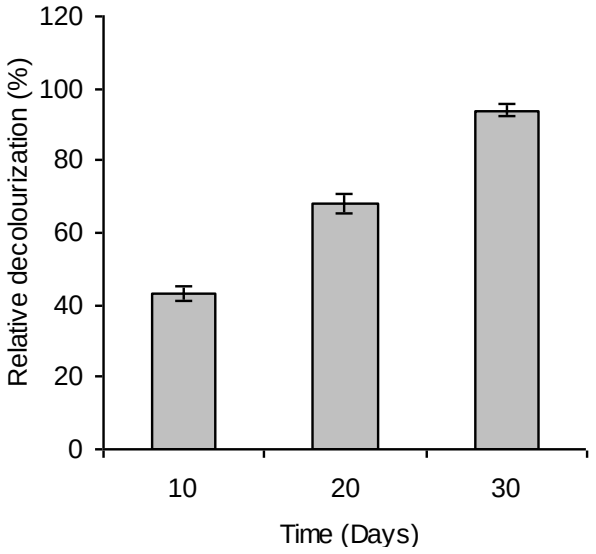

Figure 2. Decolorization of Synazol Red HF6BN by *P. ostreatus* from the industrial effluent after 10, 20 and 30 days of incubation at room temperature.

30 days of incubation at room temperature (Figure 2). Ali et al. (2008) reported that the decolorization of acid red 151, orange II, sulfur black and Drimarine blue K_2RL was 68.64, 43.23, 21.74, and 39.45%, respectively by *Aspergillus niger* in liquid medium under static condition. Jin et al. (2007) reported 89.9% optimum decolorization rate of reactive black RC, reactive yellow HF2-GL, reactive blue BGFN and reactive black B-150 at pH 3.0 after 48 h of incubation. Similarly, removal of Congo red from an aqueous solution by fungus *A. niger* was reported by Fu and Viraraghavan (2002).

Effect of temperature on enzyme activity

Extracellular laccase produced by *P. ostreatus* was characterized with regard to pH optimum and thermostatbility. The activity of crude laccase isolated from culture filtrate of *P. ostreatus* was determined at various pH values and temperatures. *P. ostreatus* laccase activity was maximum at 70°C (348%) whereas at 30°C (218%), 40°C (254%), 50°C (290%) and 90°C (311%) at pH 5 (Figure 3a). The temperature optimum of the laccase was 70°C with ABTS as a substrate in buffer of pH 5. The optimum temperature of laccase I from *Pleurotus eryngii* was 65°C and that of laccase II from the same organism was 55°C (Munoz et al., 1997). As in the present study, laccase from *P. ostreatus* showed maximum activity at 70°C. So it belongs to laccase 1 category of the enzyme.

Zouari-Mechichi et al. (2006) reported that *Trametes trogii* laccase in crude form showed optimum activity at pH 7 at room temperature for 24 h but retained more than 50% of its activity at pH 5. The laccase in the crude extract was also stable for 24 h at 50°C. In one of the studies from the same laboratory, the laccase from *A. niger* showed the maximum activity at 40°C (232%) whereas in *Nigrospora* sp. the maximum enzyme activity was determined at 50°C (152%) (unpublished data).

Effect of pH on enzyme activity

Experiments were performed to elucidate whether pH interfere laccase activity or not, and it was assessed that *P. ostreatus* laccase activity was maximum at pH 5 (350%). In contrast, pH 6 (256%), pH 7 (186%), pH 8 (100%), and pH 9 (58%) showed decrease in enzymatic activity (Figure 3b). Jung et al. (2002) reported that laccase of *Trichophyton rubrum* was more stable at pH 6, although pH optima depend on the substrate used (Fukushima and Kirk, 1995). The activity of many laccases decrease beyond optimum pH (Jung et al., 2002), but this laccase showed a high relative activity over a broad pH range from 5 to 9. This could be a very useful characteristic for various industrial applications.

Effect of metal ions on enzyme activity

Laccase activity of *P. ostreatus* was enhanced by 8%, 6%, 6%, 4%, and 4% in Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} and there was no change in enzymatic activity was found in Co^{2+} and Ni^{2+} (Fig. 4). It is clear from the laccase activity that the activity wasnot dependent on the divalent metal ions. The maximum relative increase in laccase activity was 28% (Co^{2+}) and 23% (Mg^{2+}) in *A. niger* and *Nigrospora* sp., respectively. No change in laccase activity was estimated in the presence of Fe^{2+} and Zn^{2+} in *A. niger*, while 5% activity decrease was determined in the presence of Mn^{2+} in *Nigrospora* sp. (unpublished data).

HPLC analysis

The HPLC analysis of the dye sample collected at 0 h incubation showed one detectable peaks at 2.41 min (Figure 5a). An extracted sample (seven days) showed four detectable peaks at retention time 2.40, 9.40, 11.88 and 12.12 min. The presence of extra peaks in the fungal treated dye sample indicates clearly that the dye has been converted into its metabolites as compared to the original dye molecule (Figure 5b).

Toxicity assay

No zone of inhibition was observed in the treated dye, indicating that the biodegraded or decolourized products were non-toxic to the tested beneficial bacterial flora of the soil (Figure 6). In the present investigation, phytotoxicity study showed good germination rate of *V. radiata* in the fungal decolorized water and in tap water, indicating that metabolites of the dye produced in the presence of *P. ostreatus* are found to be nontoxic to the growth of the plants (Figure 7).

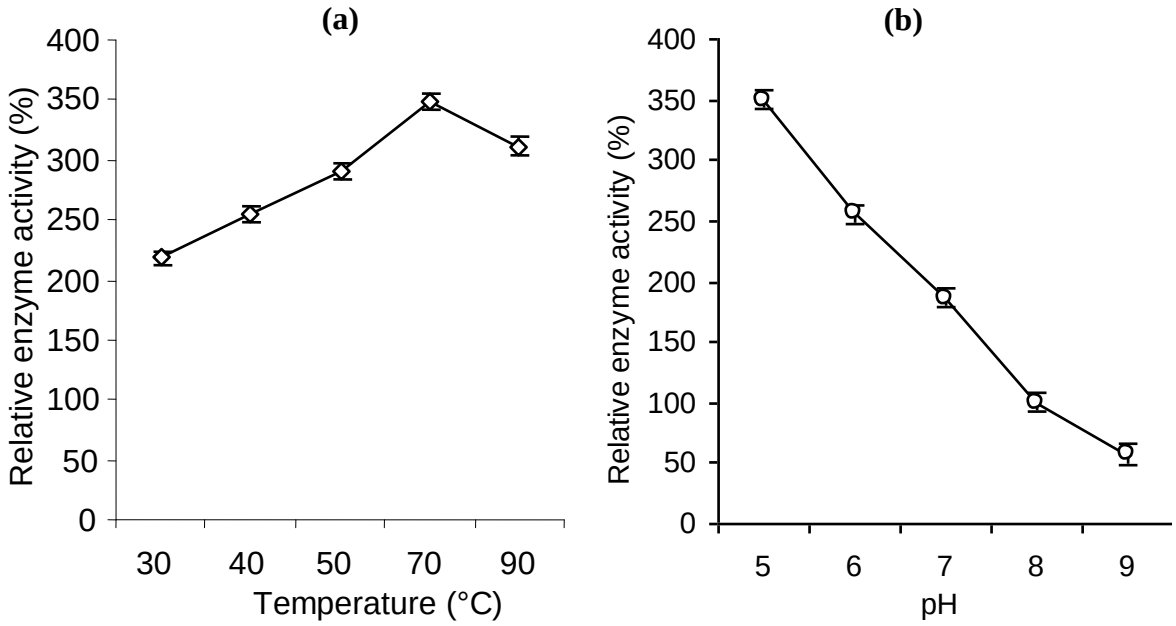

Figure 3. Effect of temperature and pH on the activity of laccase. (a) Effect of temperature on laccase activity. The enzyme activity was measured at various temperatures at pH 5.0 without addition of any metal ions in the reaction mixture. (b) Effect of pH on laccase activity. Activity was examined as described in the experimental section.

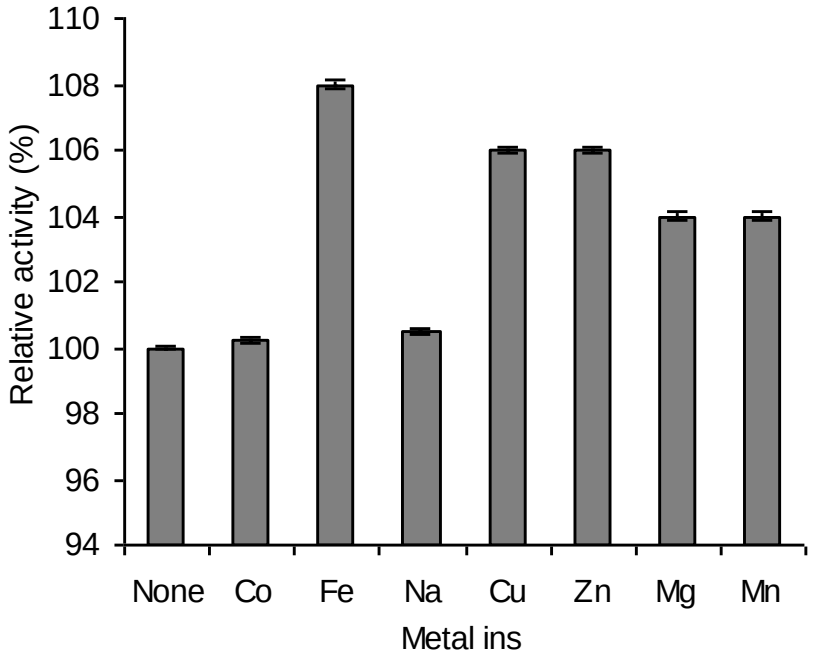

Figure 4. Effect of metal ions on the enzyme activity of laccase. The chloride salt of each metal cation was used at a final concentration of 0.1 mM. The activity was examined at 30°C and pH 5.0 as described in the experimental section.

Brilliant green, fast green, methylene blue, and Congo red removal and their toxicity after biological treatment have been reported by Mali et al. (2000). Despite this fact, untreated dyeing effluents may cause serious environmental and health hazards. These effluents are being disposed off in water bodies that could be used for

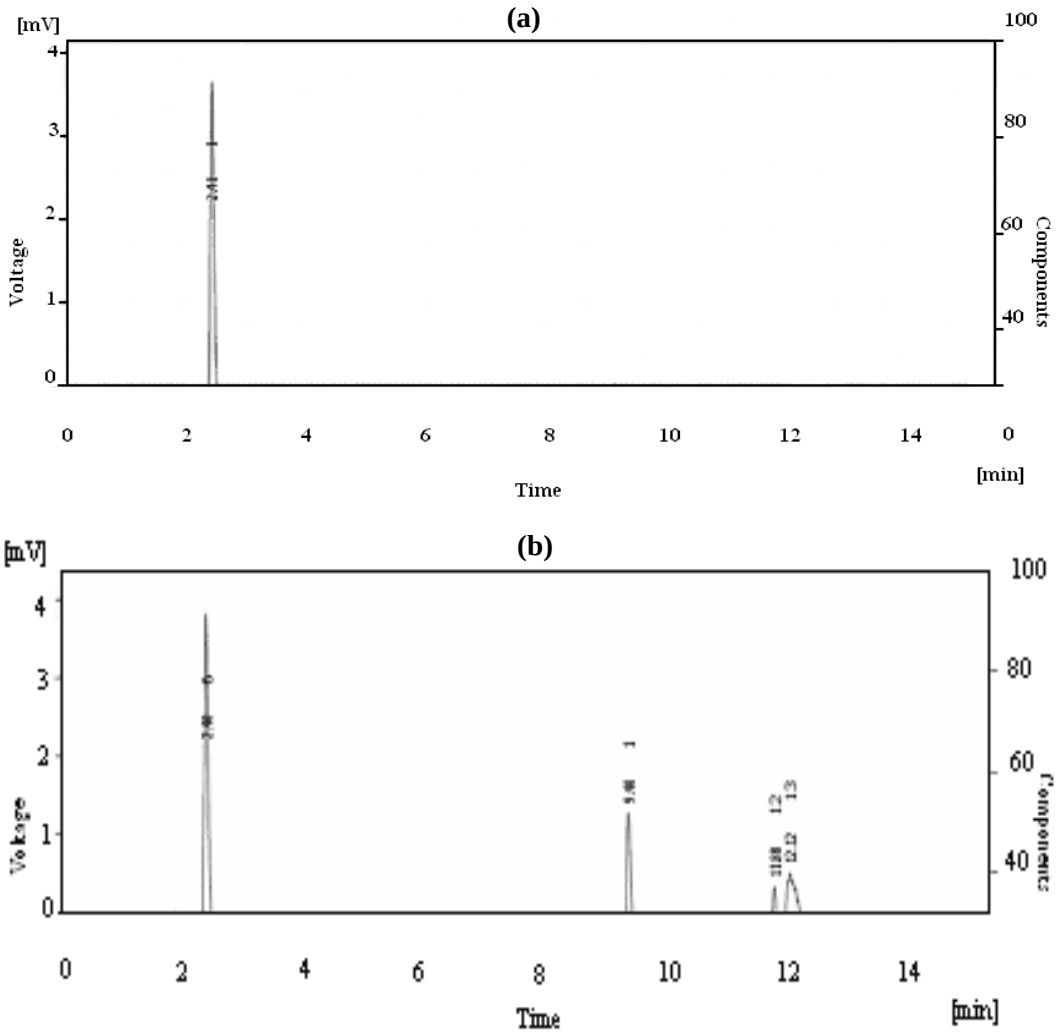

Figure 5. HPLC analysis of reactive Synazol Red HF6BN with a mobile phase of profile of methanol : acetonitrile (1:1) at 1.0 ml min^{-1} and detection at 540. The column was C-18 (250 × 4.6 mm). A represents dye chromatogram, while B represents *P. ostreatus* degraded dye products extracted after seven days of incubation at 30°C.

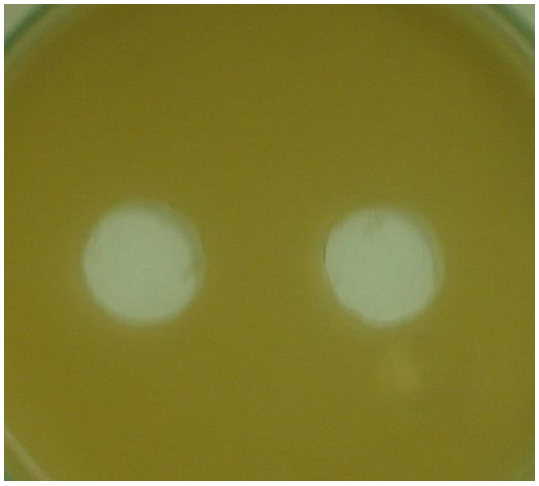

Figure 6. No zone of inhibition was observed around the fungal-decolorized broth after incubating *B. cereus* at 30°C for 48 h.

the agriculture purpose. In the present study, phyto-toxicity study showed good germination rate of *V. radiata* in the metabolites extracted after decolorization and in tap, indicating that metabolites of the dye produced are found to be non-toxic to the growth of the plants (Figure 7). Dawkar et al. (2010) reported that metabolites were non-toxic with respect to *Sorghum bicolor* and *Triticum aestivum*. Similarly, Kalyani et al. (2008) reported that *Sorghum vulgare* and *Phaseolus mungo* showed good germination rate as well as significant growth in the plumule and radical of both plants in the Red BLI metabolites extracted after decolorization as compared to dye sample.

In summary, the *P. ostreatus* was able to decolorize 96% Synazol Red HF6BN in 24 days. The fungus was also capable of decolorizing 94% dye effluent containing Synazol Red HF6BN after 30 days of incubation at 28 ± 2°C. The crude enzyme exhibited highest activity at 70°C also observed in the presence of metal cations. HPLC

Figure 7. The growth of *Vigna radiata* in dye treated wastewater indicates that *P. ostreatus* treated wastewater is safe for plant growth.

clearly indicated that *P. ostreatus* was capable of degrading Synazol Red HF6BN into metabolites. The no inhibition zones on agar plates and *V. radiata* growth in the presence of dye extracted sample indicate that the dye metabolites are non-toxic to beneficial micro-flora and plant growth. Therefore, *P. ostreatus* can be employed for colour removal from textile wastewater containing azo dyes.

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