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Biodegradation of triphenylmethane dye cotton blue by *Penicillium ochrochloron* MTCC 517

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Abstract

Triphenylmethane dyes belong to the most important group of synthetic colorants and are used extensively in the textile industries for dying cotton, wool, silk, nylon, etc. They are generally considered as the xenobiotic compounds, which are very recalcitrant to biodegradation. *Penicillium ochrochloron* decolorizes cotton blue (50 mg l^{-1}) within 2.5 h under static condition at pH 6.5 and temperature 25 °C. TLC, FTIR and HPLC analysis confirms biodegradation of cotton blue. FTIR spectroscopy and GC–MS analysis indicated sulphonamide and triphenylmethane as the final products of cotton blue degradation. The pH, temperature and maturity of biomass affected the rate of decolorization. Presence of lignin peroxidase, tyrosinase and aminopyrine N-demethylase activities in the cell homogenate as well as increase in the extracellular activity of lignin peroxidase suggests the role of these enzymes in the decolorization process. The phytotoxicity and microbial toxicity studies of extracted metabolites suggest the less toxic nature of them.

Keywords: *Penicillium ochrochloron*; Cotton blue; Biodegradation; Lignin peroxidase; Aminopyrine N-demethylase

1. Introduction

Large numbers of chemically different dyes are used for various industrial applications and significant proportion appears in the form of wastewater and is spilled into the environment. Improper chemical disposal of dyes leads to the reduction in sunlight penetration that causes decrease in photosynthetic activity and dissolved oxygen. The physical and chemical treatments available have limited use and are having high operational cost [1]. Synthetic dyes used are recalcitrant to remove by conventional wastewater treatments such as adsorption, photo-oxidation, coagulation, flocculation, photodegradation and chemical degradation. The biological degradation is of great value as it has inexpensive, ecofriendly and has less sludge producing properties. Currently an extensive research is focused to find optimal microbial biomass, which is as cheap as possible for removal of contaminating dyes from large volumes of polluted water [2]. For bioremediation of synthetic colorants several microorganisms including bacteria and fungi can be employed, as they are easy to manipulate, having fast growth rate and high adaptive capacity.

The ability of fungi to transform a wide variety of hazardous chemicals was aroused interest in using them in bioremediation [1]. The white rot fungi are unique among eukaryotes for having evolved nonspecific methods for the degradation of lignin; curiously they do not use lignin as a carbon source for their growth [3]. The abilities of three lignin-degrading fungi, *Phanerochaete chrysosporium*, *Phanerochaete sordida* and *Tramates hirsuta* to degrade pentachlorophenyl (PCP) and creosol in soil have been comparatively studied [4]. *P. chrysosporium* has been shown to degrade a number of toxic xenobiotics and several miscellaneous compounds like sulfonated azo dyes due to presence of enzymes, viz. laccases, polyphenol oxidases, lignin peroxidases, reductases and methyl transferases [5]. Decolorization of recalcitrant dye amaranth by *Ganoderma* sp. has been reported earlier [6]. Three different species of *Penicillium* namely *P. commune*, *P. freii* and *P. allii* were shown to remove 96, 64 and 65%, respectively, of direct violet dye after 2 h [7].

Penicillium ochrochloron is used for testing mold resistance. It is also used for the production of (1-3)β-D-glucanases and β-glucosidase [8]. The fungus is well known for the

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bioleaching of metals specially copper [9]. These properties of *P. ochrochloron* encouraged us to use this fungus for textile dye biodegradation. The present study was focused on the degradation of cotton blue, a triphenyl methane dye. Cotton blue is extensively used in textile industries for dyeing cotton especially and no report is available that suggests the decolorization of cotton blue using microbial treatment. *P. ochrochloron* effectively decolorizes cotton blue within 2.5 h (50 mg l^{-1}) under static anoxic condition at pH 6.5 and 25 °C. Further microbial and phytotoxicity studies of the extracted metabolites after dye degradation supports its ecofriendly nature.

2. Materials and methods

2.1. Dyes and chemicals

Czapek dox agar and czapek dox broth (composition (g l^{-1}): sucrose 30, sodium nitrate 3, dipotassium phosphate 1, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01) were obtained from Hi-media, India. *n*-Propanol, cotton blue, NADH, dichlorophenol indophenol (DCIP), catechol and ethyl acetate were purchased from SRL Chemicals, India.

2.2. Organisms and culture conditions

P. ochrochloron MTCC 517 strain used in the present study was obtained from Institute of Microbial Technology, Chandigarh, India and was routinely maintained on czapek dox agar. The mycelium used for decolorization was grown in czapek dox broth at 29 °C at pH 6.5.

2.3. Decolorization experiments

P. ochrochloron grown at 29 °C for 96 h was routinely used for decolorization purpose under static conditions at 25 °C unless otherwise stated. The flasks containing 100 ml sterile czapek dox broth were inoculated with 0.5 ± 0.1 g (wet weight) of fungus grown on czapek dox agar, for 72 h in a petriplate.

Cotton blue 50 mg l^{-1} was separately added to the broth culture containing 96 h grown *P. ochrochloron* in 250 ml Erlenmeyer flask. Abiotic controls without microorganisms were always included. The aliquots (4 ml) were withdrawn, centrifuged to separate fungal biomass and absorbance of the supernatant was measured at respective wavelengths. The percent decolorization was calculated as:

$$\%\text{Decolorization} = \frac{\text{initial absorbance} - \text{observed absorbance}}{\text{initial absorbance}} \times 100.$$

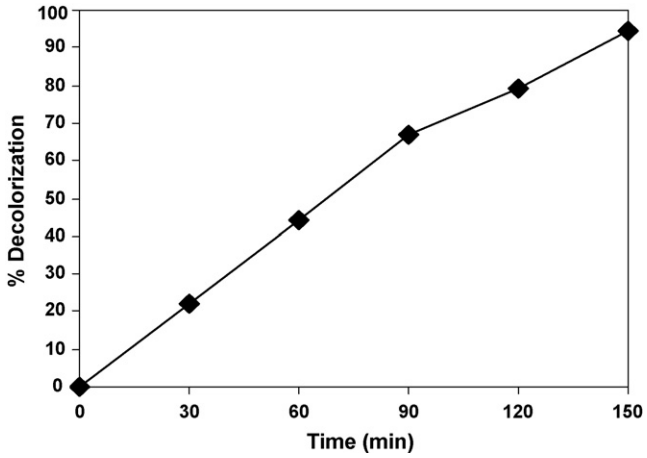

Fig. 1. Percent decolorization of cotton blue.

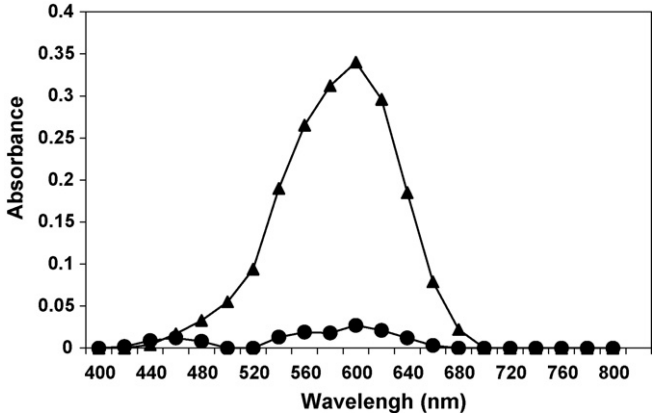

Fig. 2. UV–vis spectral analysis of cotton blue (control, ▲) and after decolorization (2.5 h, ●).

2.3.1. Decolorization at different pH and temperatures

The decolorization studies of cotton blue (50 mg l^{-1}) were carried out at pH 3, 5, 7, 9 and 11 by adjusting pH of the medium using 0.1N HCl and 0.1N NaOH. The fungus was grown at respective pH for 96 h at 29 °C temperature under static condition and used for decolorization studies.

Similarly the decolorization of cotton blue (50 mg l^{-1}) was carried out at different temperatures 5, 25, 30, 40 and 50 °C by using 96 h grown fungus at 29 °C. Flasks were incubated at

Table 1
Effect of pH and temperature on the decolorization of cotton blue

Parameters	pH					Temperature (°C)				
	3	5	7	9	11	5	25	30	40	50
%Decolorization	80	83	93	88	80	80	93	93	80	80
Time for decolorization (h)	6.5	5.5	2.5	4.0	5.0	8.0	2.5	4.5	6.0	6.5

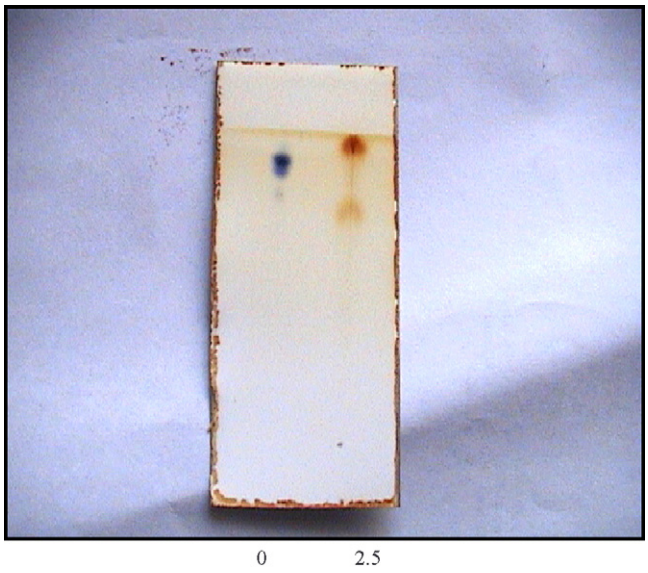

Fig. 3. TLC analysis of control (0 h) and its degraded metabolites (2.5 h).

respective temperatures for 30 min before addition of the dye and decolorization was observed.

2.3.2. Decolorization by using fungal biomass of different maturity

Fungal biomass of different maturity ranging from 12 to 120 h grown at 29 °C was used to study its effect on decolorization of cotton blue 50 mg l^{-1}.

Table 2
Effect of biomass maturity on the rate of decolorization of cotton blue

Maturity of fungal biomass (h)	Time required for decolorization (h)	%Decolorization
60	6.5	74
72	4.5	83
84	2.5	90
96	2.5	93
108	3.5	91

2.4. Preparation of enzyme samples and enzyme assays

Five-gram mycelium before and after decolorization was cut in to small pieces and blended using mixer for 2 min for getting fine mycelial fragments. Chilled properly and again homogenized for three times in hand homogenizer for 30 s, filtered and the filtrate was used for enzyme assay. Supernatant medium, before and after decolorization, was used as it is for the study of extracellular enzyme activities. Laccase, lignin peroxidase, tyrosinase activities were assayed in cell free extract as well as in the supernatant medium before and after decolorization, while NADH–DCIP reductase, aminopyrine N-demethylase (AND) and Malachite green (MG) reductase activities were assayed in cell free extract only. Laccase activity was determined in a reaction mixture of 2 ml containing 10% ABTS in 0.1 M acetate buffer (pH 4.9) and increase in absorbance at 420 nm was measured [10]. Tyrosinase activity was determined in a reaction mixture of 2 ml, containing

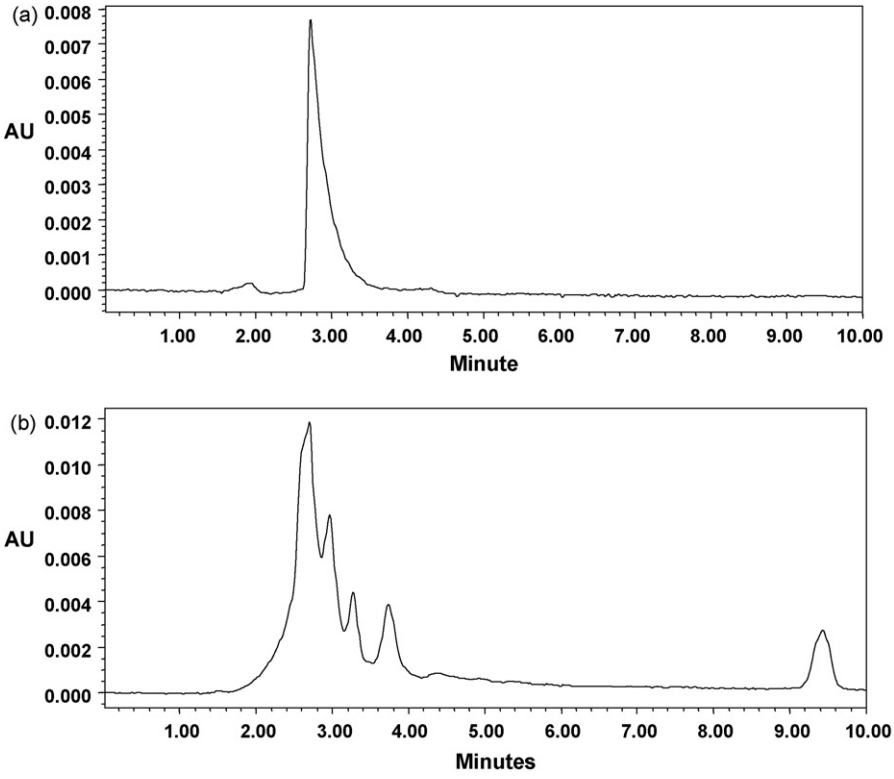

Fig. 4. (a and b) HPLC chromatogram of cotton blue and its extracted metabolites after 2.5 h.

0.01% catechol in 0.1 M phosphate buffer (pH 7.4) at 495 nm [11]. Lignin peroxidase activity was observed 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 [12]. All enzyme assays were run in triplicate and average rates calculated and one unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg $protein^{-1}$.

NADH–DCIP reductase activity was determined using a procedure reported earlier [13]. The assay mixture contained 50 μM DCIP, 50 μM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution in a total volume of 5.0 ml. The NADH–DCIP reduction was calculated using the molar extinction coefficient of 92 mM cm^{-1}. The AND activity was carried as per procedure reported earlier [2].

2.5. Phytotoxicity and microbial toxicity studies

The phytotoxicity studies were carried out using 700 ppm concentration of the cotton blue and its extracted metabolites using seeds of *Triticum aestivum* and *Ervum lens Linn*, with water as a control. The microbial toxicity was carried out using *Azotobacter vinelandii* on nutrient agar plate having composition 1% peptone, 0.5% NaCl, 0.3% yeast extract and 2.5% agar.

2.6. Analytical procedures

The metabolites produced after biodegradation of cotton blue at 0 h (control) and 2.5 h were extracted with equal volumes of ethyl acetate. The extract was dried in petriplate and the crystals as it is were used for FTIR analysis. For HPLC, TLC and UV–vis spectral analysis the crystals were dissolved in small amounts of HPLC grade methanol. HPLC was carried out on waters model equipped with dual λ UV–vis detector and C_{18} column, the mobile phase used was methanol with flow rate 0.5 ml min^{-1} for 10 min. UV–vis spectral analysis was carried out using Hitachi UV–vis spectrophotometer and changes in the absorption spectrum of control and products were recorded. FTIR analysis was carried out using Perkin-Elmer spectrophotometer and changes in %transmission at different wavelengths were observed. TLC analysis was carried out on silica gel using mobile phase methanol, ethyl acetate, *n*-propanol, water and acetic acid in proportion 1:2:3:1:0.2 (v/v). The sam-

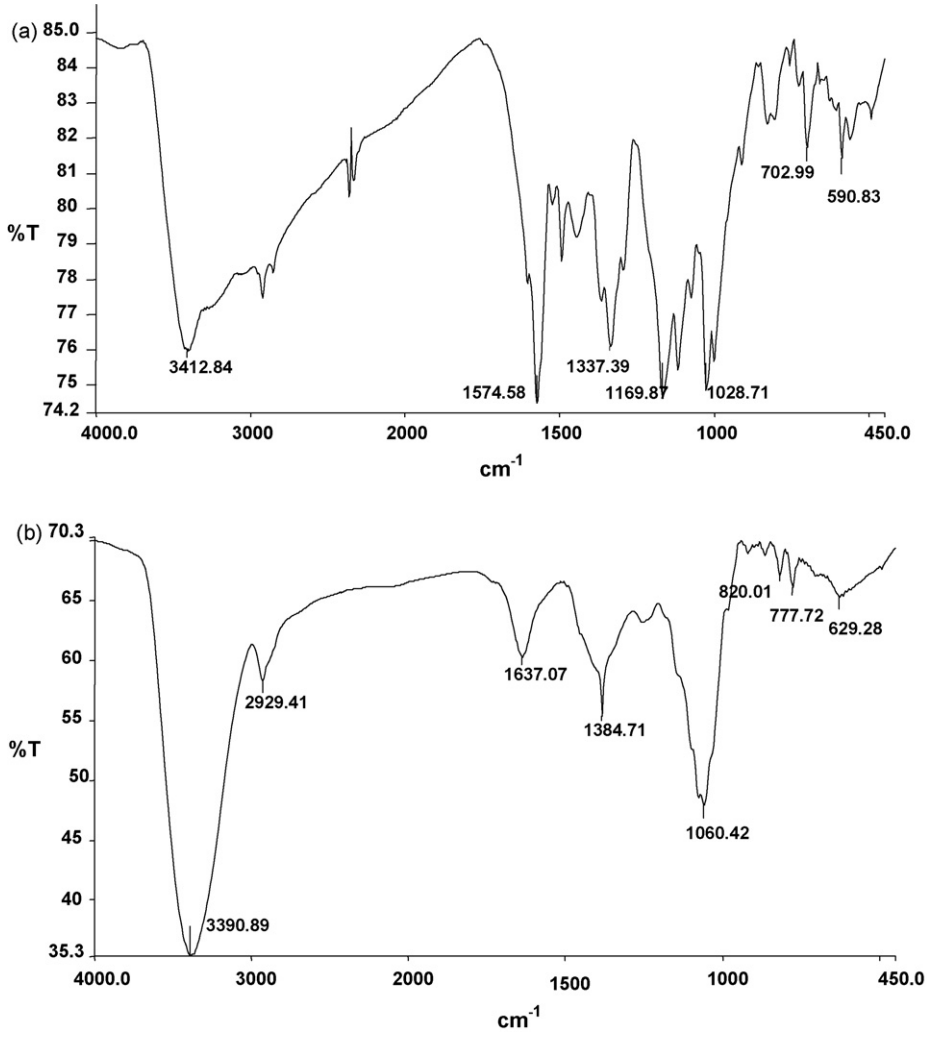

Fig. 5. (a and b) FTIR spectra of cotton blue and its degradation metabolites (2.5 h).

Table 3
Enzyme activities during decolorization

Enzymes	Cell biomass		Supernatant	
	Before decolorization	After decolorization	Before decolorization	After decolorization
Lignin peroxidase[a]	0.11 ± 0.60	0.025 ± 0.005*	0.001 ± 0.0003	0.009 ± 0.003*
Laccase[a]	0.045 ± 0.01	0.022 ± 0.0006	NA	NA
Tyrosinase[a]	0.23 ± 0.10	0.012 ± 0.003	NA	NA
NADH–DCIP reductase[b]	483 ± 7.12	308 ± 1.39	–	–
MG reductase[c]	12.49 ± 0.27	5.03 ± 0.21	–	–
Aminopyrine N-demethylase[d]	0.22 ± 0.0009	0.20 ± 0.0004	–	–

Values are mean of three experiments ± S.E.M., $^{*}P < 0.05$; NA: no activity.
[a] Units $\text{min}^{-1}\ \text{ml}^{-1}$.
[b] μg DCIP reduced $\text{min}^{-1}\ \text{ml}^{-1}$.
[c] μg MG reduced $\text{min}^{-1}\ \text{ml}^{-1}$.
[d] *n* mol formaldehyde liberated min^{-1} mg protein^{-1}.

ples of 0 and 2.5 h were analyzed and developed using iodine chamber.

GC–MS analysis was performed using a QP2010 gas chromatography coupled with mass spectrometer (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature-programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 80 °C for 2 min, then increased linearly at 10 °C min^{-1} to 280 °C, and held for 7 min. The temperature of the injection port was 280 °C and the GC/MS interface was maintained at 290 °C. The Helium carrier gas flow rate was 1.0 ml min^{-1}. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC–MS.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Turkey–Kramer multiple comparisons test was used. Readings were considered significant when P was ≤ 0.05.

3. Results and discussion

Several microorganisms can be used for the removal of dyes from the textile industrial effluent. Among them fungi represents the promising group of microbes for biodegradation of dyes. Previously it have been reported that fungi can be used for the biodegradation of dyes. The yeast *Saccharomyces cerevisiae* was shown to decolorize triphenyl methane dyes [2], Phenolic azo dyes have been shown to be oxidized by the enzyme laccase produced by *Pyricularia oryzae* [14]. *P. chrysosporium* has been shown to affect the bioleaching of organic dyes [15]. While the decolorization of azo-triphenyl methane dyes by lignin peroxidase produced by *P. chrysosporium* have been reported [16]. The role of lignin peroxidase and manganese peroxidase from *P. chrysosporium* in the decolorization of olive mill wastewater have been demonstrated [17].

3.1. Decolorization of cotton blue

Cotton blue (50 mg l^{-1}) was decolorized up to 93% by using *P. ochrochloron* MTCC 517 as in Fig. 1 within 2.5 h at pH 6.5 and at temperature 25 °C this is the fastest biodegradation of cotton blue reported by us than the earlier one [2] which shows only the adsorption of cotton blue on *S. cerevisiae* cells. As we got the decolorization at static condition and not at the shaking condition so we continued our work at static condition. The presence of oxygen would normally inhibit the activity of decolorization, resulting in a low efficiency of color removal [18].

The various physicochemical parameters such as pH, temperature were found to affect the rate of decolorization as well as percent decolorization data shown in Table 1. The pH optimum for decolorization was 6.5 and temperature optimum was 25 °C.

Table 4
Phytotoxicity comparison of cotton blue and its extracted metabolites.

Parameters studied	*Triticum aestivum*			*Ervum lens Linn*		
	Water	Cotton blue (700 ppm)	Extracted metabolites (700 ppm)	Water	Cotton blue (700 ppm)	Extracted metabolites (700 ppm)
Germination (%)	100	70	90	100	80	100
Shoot (cm)	12.37 ± 0.71	5.43 ± 0.71***	8.38 ± 0.98	6.98 ± 0.66	1.62 ± 0.44***	4.56 ± 0.39$$
Root (cm)	4.18 ± 0.39	0.93 ± 0.24***	1.58 ± 0.29	3.68 ± 0.16	0.87 ± 0.17***	2.35 ± 0.18$$$

Phytotoxicity study of cotton blue and its extracted metabolites formed after biodegradation, values are mean of germinated seeds of three experiments, S.E.M. (±), significantly different from the control (seeds germinated in water) at $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no significant difference. Significantly different from cotton blue treated group $^{\$}P < 0.05$, $^{\$\$\$}P < 0.001$, by one-way ANOVA with Turkey–Kramer comparison test.

Fungal biomass of different maturity when used for decolorization, it was observed that up to 48 h there is no sufficient growth of the fungus and it did not show the decolorization of cotton blue, might be due to insufficient amount of enzymes required for the degradation process. Maximum decolorization (93%) of cotton blue (50 mg l^{-1}) was observed by using the 96 h grown biomass. However mature fungal biomass decolorized the same dye concentration (50 mg l^{-1}) much slowly and with decreased percentage removal. The data for the effect of biomass maturity is summarized in Table 2.

3.2. Analysis of extracted metabolites

UV–visible spectral analysis of cotton blue and its degradation metabolites showed decrease in the absorbance at 600 nm (Fig. 2). TLC analysis of the extracted metabolites after decolorization showed presence of three more spots with Rf values 0.93, 0.83 and 0.75 compared to control (0.86) shown in Fig. 3. HPLC analysis showed peak at retention time 2.731 for control as shown in Fig. 4(a) and five different peaks at retention time 2.695, 2.962, 3.270, 3.738 and 9.431 for extracted metabolites as shown in Fig. 4(b).

The FTIR spectra of control cotton blue displays a peak at 3412 cm^{-1} for N–H stretching, peak at 1574 cm^{-1} for C–H ring stretching of benzene. The peak at 1337 cm^{-1} for asymmetric S–O stretching vibrations, peak at 1169 cm^{-1} for aromatic C–N stretching vibrations, peak at 1028 cm^{-1} indicate C–C bending. Peak at 702 cm^{-1} indicates out of plane C–H bending and C–N stretching. The absorption bands below 900 cm^{-1} indicate aromatic nature of the compound. Hence FTIR spectra confirm the structure of cotton blue in Fig. 5(a). The FTIR spectra of 2.5 h extracted metabolite show peak at 3390 cm^{-1} for N–H stretching of amine, peak at 2929 cm^{-1} for C–H stretching of alkyl benzene, peak at 1637 cm^{-1} for N–H bending, C–C stretching and C–H bending and peak at 1384 cm^{-1} for aromatic S–O stretching vibrations. The peak at 629 cm^{-1} for C–S stretching and peak at 820 cm^{-1} indicate out of plane C–H bending and 777 cm^{-1} for N–H and C–H bending vibrations. All these peaks indicate formation of aromatic amines. The peak at 1060 cm^{-1} for S–O stretching indicates formation of sulphoxides or sulphonamides in Fig. 5(b).

Triphenylmethane dyes cleaved by enzyme action, symmetrically or asymmetrically depending on the structure of substrate and active site of an enzyme. We have proposed the degradation pathway of cotton blue by *P. ochrochloron*, where there is asymmetric cleavage of cotton blue catalyzed by lignin peroxidase as depicted in Fig. 6. The GC–MS analysis of extracted metabolites showed the formation of sulphonamides (MW 172, mass peak at 172 *m/z*, relative abundance 10% with retention time 20.8 as a major peak) and triphenylmethane (MW 343, mass peak at 343 *m/z*, relative abundance 10% with retention time 24.578) (Fig. 7). Hence the present study confirms the biodegradation of cotton blue by *P. ochrochloron*.

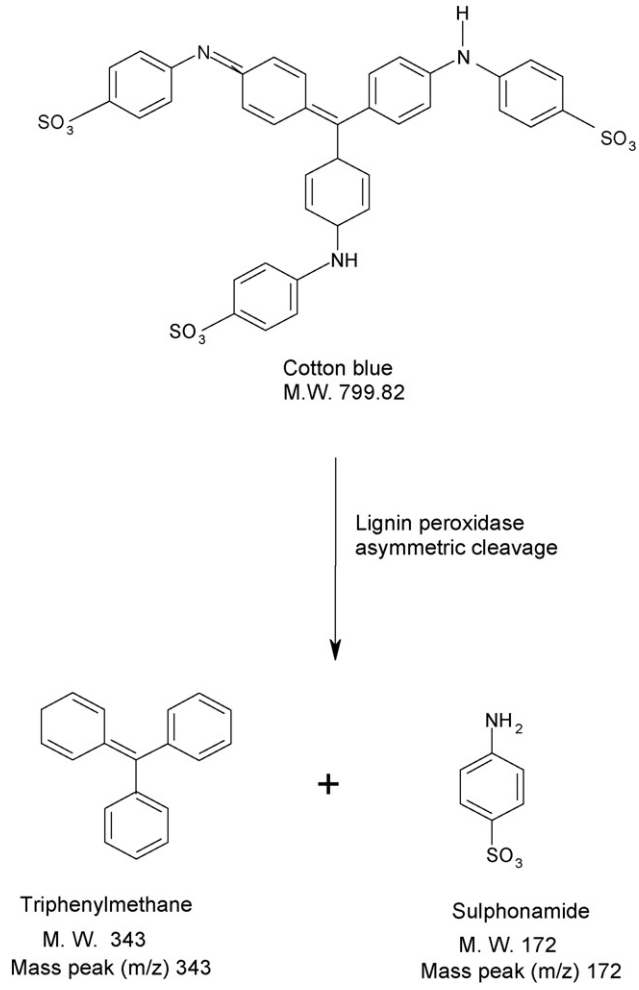

Fig. 6. Proposed pathway for the degradation of cotton blue.

3.3. Enzymatic analysis

The degradation of dyes is mediated by several lignin modifying enzymes, laccase, manganese peroxidase, lignin peroxidase and to some extent by aminopyrine N-demethylase [19]. The relative contributions of these enzymes in decolorization of dyes may be different for each microorganism. Laccase, a sole phenol oxidase produced by *Pycnoporus sanguinues* was found to mediate the degradation of azo and triphenylmethane dyes [20]. Biodegradation of methyl violet is found to be due to demethylation [21]; cytochrome P450 system mediates both the reduction and the N-demethylation reactions [22].

Data shown in Table 3 represents the enzymatic activities present in the control cells (fungal biomass), the cells after decolorization and the supernatant, before and after decolorization. Lignin peroxidase, laccase, tyrosinase, aminopyrine N-demethylase, NADH–DCIP reductase, MG reductase were found to be present in the control cells and cells obtained after decolorization suggests the prominent role of these enzymes during decolorization process. While activity of lignin peroxidase in supernatant before and after decolorization denotes the role of extracellular lignin peroxidase in the decolorization of cotton blue.

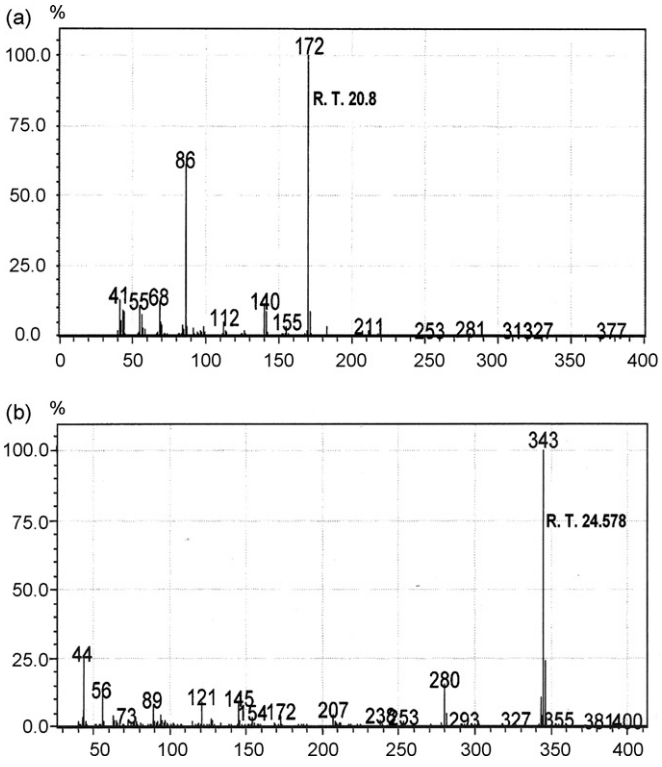

Fig. 7. (a and b) MS spectra of degradation metabolites.

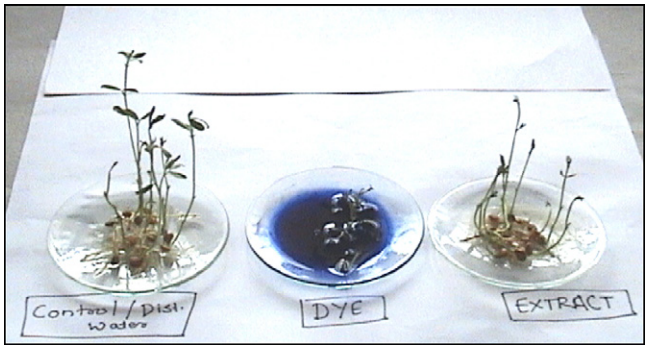

Fig. 8. Phytotoxicity study on *Ervum lens Linn*.

3.4. Toxicity studies

Disposal of the untreated dyeing effluent, without any treatment, in water bodies cause serious environmental and health hazards. Besides the use of dye contaminated water is harmful to agriculture. Thus it was of prime interest to assess the phytotoxicity of the dye and its extracted metabolites after degradation. The relative sensitivities towards the cotton blue and its degradation products in relation to *T. aestivum* and *E. lens Linn* seeds are presented in Table 4. Phytotoxicity study shows well germination rate as well as significant growth in shoot and root for both the plants, grown in metabolites extracted after decolorization, as compared to the dye (700 ppm). Fig. 8 displays the comparative growth of root and shoot in *E. lens Linn* seeds. Hence phytotoxicity studies revealed that biodegradation of the dye by a fungal culture, resulted in its detoxification. Thus treated effluent can be used for ferti-irrigation.

Microbial toxicity on *Azotobacter vinelandii*, a nitrogen fixing bacterium, showed growth inhibitory zone (1.2 cm) surrounding the well containing dye, while product did not show inhibitory zone also confirms the nontoxic nature of the extracted metabolite.

4. Conclusion

- From the above study we confirm the degradation of cotton blue in to sulphonamide and triphenylmethane and the less toxic nature of these metabolites to plants as well as microbes. This is the first report showing the decolorization of cotton blue, which is very recalcitrant for biodegradation.
- The effectiveness of bioremediation of cotton blue by *P. ochrochloron* may be affected by various physical and chemical parameters. The ability of fungus to degrade cotton blue is due to presence of the enzymes involved in dye decolorization process and there is also appearance of extracellular activity of lignin peroxidase.

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