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Bioremediation of the heavy metal complex dye Isolan Dark Blue 2SGL-01 by white rot fungus *Irpex lacteus*

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ABSTRACT

The present study was conducted to evaluate the decolorization and degradation of the chromium metal complex dye Isolan Dark Blue 2SGL-01 by *Irpex lacteus*, a white rot lignolytic fungus. *I. lacteus* effectively decolorized the sulphonated reactive dye at a high concentration of 250 mg/l over a wide range of pH values of 5–9 and temperatures between 20 and 35 °C. Complete (100%) decolorization occurred within 96 h, and *I. lacteus* demonstrated resistance to the metallic dye. UV–vis spectroscopy, HPLC, GC–MS, and FT-IR analyses of the extracted metabolites confirmed that the decolorization process occurred due to degradation of the dye and not merely by adsorption. GC–MS analysis indicated the formation of 1(2H)-naphthalenone, 3,4-dihydro- and 2-naphthalenol as the main metabolite. ICP analysis demonstrated the removal of 13.49% chromium, and phytotoxicity studies using germinated seeds of *Vigna radiata* and *Brassica juncea* demonstrated the nontoxic nature of the metabolites formed during the degradation of Isolan Dark Blue 2SGL-01 dye.

1. Introduction

Dyes are water soluble substances used to furnish color to a variety of materials like textiles, paper, leather, wood and food. The discharge of azo dyes and associated chemicals may induce mutagenesis leading to toxicity in aquatic plants and animals [1–3]. Various effluent treatments including pH neutralization, coagulation, and biological treatments are commonly performed, but such treatments are unable to completely remove dyes from the environment, due to the recalcitrant nature of the dyes [4].

Physical and chemical methods are not often used in the treatment of dye wastewater and in the textile industries because they are expensive and associated with waste management issues [5,6]. The well-studied chemical technique is advanced oxidation, which has the potential to decolorize and destroy dye chromophores. The advanced oxidation process also liberates organochlorinated compounds and other byproducts that cannot be further oxidized, and that may have increased toxicities compared to the original parent dye compounds [7].

The treatment of dye wastewater by physical or chemical methods leads to the generation of secondary pollution by liberating hazardous byproducts [8]. Microbial treatment of dyes has gained popularity due to the ability of microbes to develop resistance by acclimatization to new environments. Biological treatment also helps in the transformation of hazardous chemicals to less toxic compounds [9]. Biological methods are less expensive, highly efficient, and eco-friendly. Dyes containing mainly azo dyes at levels of around 70% are difficult to degrade because they have complicated structures and are artificially synthesized [10,11].

Microbial decolorization of dyes has been carried out using *Klebsiella pneumoniae* [12], *Brevibacillus* spp. [13], *Pseudomonas desmolyticum* [14], *Galactomyces geotrichum*, *Bacillus* spp. [15], *Exiguobacterium* spp. [9], *Streptococcus krainskii* [16], *Staphylococcus arlettae* [17], *Pycnoporus sanguineus* [18], *Saccharomyces cerevisiae* [19], *Trametes hirsuta*, *Pleurotus florida* [20], *Aspergillus fumigatus* XC6 [21], *Phellinus gilvus*, *Pleurotus sajor-caju*, *Pleurotus ostreatus* [22], *Trametes pubescens* [23], and *Phanerochaete chrysosporium* [24]. Fungi were found to be more tolerant than bacteria and more efficient for decolorization as well as degradation of toxic chemicals. Lignolytic fungi produce enzymes including laccase, lignin peroxidase, and manganese peroxidase, which degrade lignin. Lignin degradation can be utilized for dye degradation because dyes also contain mainly aromatic compounds.

White rot fungi are also advantageous because their enzyme productions are enhanced in the absence of nutrients, nitrogen, and

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carbon. In addition, the enzymes produced are extracellular and lack substrate specificity, which allows the fungi to act and degrade a wide range of xenobiotics [25]. The present study was undertaken to decolorize and degrade the metallic dye Isolan Dark Blue 2S-GL01 using *Irpex lacteus*, a white rot lignolytic fungus.

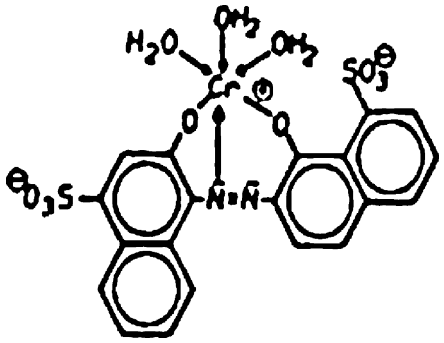
2. Materials and methods

2.1. Culture and conditions

I. lacteus (KACC 43353) was obtained from the Korean Agricultural Culture Collection. Two percentage malt extract agar Petri plates were used to maintain the pure culture at 4 °C, and subculture was performed once per month. For decolorization studies the liquid starter cultures were prepared in 2% malt extract by inoculating 1 cm × 1 cm of fungal mycelia cultured on Petri plates containing the solid agar media. The culture was incubated at 25 °C for 5 days and fresh mycelia were used for dye biodecolorization and biodegradation analyses.

2.2. Dyes and chemicals

The Isolan Dark Blue 2S-GL01, was purchased from Dystar (Korea). Isolan Dark Blue 2S-GL01 is a 1:2 metal complex, monosulphonated dye used for dying wool and polyamide fibres. It has a solubility of 30 g/l at 25–90 °C. The dye contains chromium (III) metal at a concentration of 2.9% and the dye was used without any purification. All chemicals used in the present study were of analytical grade. The chemical structure of the Isolan Dark Blue 2S-GL01 is depicted below.



2.3. Biodecolorization studies

Decolorization studies were conducted at different pH values, temperatures, and concentrations. The malt extract medium was prepared and pH of medium was adjusted to 5, 6, 7, 8, or 9 with 0.1 N HCl or 0.1 N NaOH. Decolorization studies were performed at four different temperatures, 20 °C, 25 °C, 30 °C, and 35 °C. Dye concentrations of 50, 100, 150, 200, and 250 mg/l were tested for decolorization. 250 ml conical flasks containing 100 ml of dye solution at a concentration of 100 mg/l were inoculated with 0.1 g of *I. lacteus* seed culture and maintained at 25 °C for pH and concentration analyses and at different temperatures for temperature dependent decolorization studies. The cultures were shaken at 150 rpm. The concentrations were maintained at 100 mg/l for decolorization experiments at different pH values and temperatures. Spectrum analysis of dye was carried between 200 and 700 nm and the maximum absorbance was found to be 578 nm. So the decolorization analyses were carried out by measuring absorbance at every 24 h at 578 nm. Three milliliters of the suspension was taken and centrifuged at 10,000 rpm for 10 min and clear supernatant was utilized for analysis. The control was maintained without inoculation of fungal culture. All experiments were carried out in triplicate.

$$\%\ \text{of decolorization} = \frac{A_0 - A_t}{A_0} \times 100$$

A_0 is the absorbance measured at 0 h and A_t is the absorbance measured at t h (t = 24, 48, 72, 96).

2.4. UV–vis spectrum analysis

After complete decolorization, supernatants of control and experimental cultures were subjected to UV–vis spectrum analyses in the wavelength range of 200–700 nm to check for complete decolorization and as well as for the formation of new peaks. The peaks of experimental samples were compared with the peaks of control.

2.5. HPLC analysis of biodegradation

Biodegradation analyses were performed once complete decolorization was observed. Dye at an initial concentration of 250 mg/l was used for analyzing dye degradation. Six hundred milliliters of decolorized dye containing the fungus was centrifuged at 10,000 × *g* and filtered through Whatman filter paper #1 and the clear solution was used for extraction. The extraction was performed using equal volumes of ethylacetate. The extracted metabolites were dried over anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator. The metabolites were then dissolved in 2 ml of 100% methanol, which was subsequently used for analyses. Compounds from positive control samples containing only the dye and negative control fungal culture filtrate were prepared following same procedure. The samples were analyzed by HPLC (Waters model no. 2690) using a reverse phase column with dimensions of 5 μm and 4.6 mm × 150 mm at a temperature of 35 °C. The mobile phase, acetonitrile, with a gradient of 10–90% was used at a flow rate of 1.0 ml/min. Chromatographic runs were carried out for 35 min and the peaks were detected by the photo-diode detector functioning at 254 nm.

2.6. Metabolite identification by GC–MS

The metabolites extracted from negative control, positive control and experimental sample using ethylacetate were dissolved in methanol and used for GC–MS analyses. The following conditions were maintained during analysis: an injector temperature of 300 °C and a constant oven temperature of 100 °C for 2 min, which was then increased to 250 °C at a rate of 10 °C/min followed by an increase to 280 °C at 30 °C/min. The mobile phase applied for the elution of the metabolites was helium gas at a flow rate of 1.1 ml/min. The run time was for 20 min and the compound analysis was performed using mass spectra obtained from the NIST library.

2.7. FT-IR analysis of the functional groups of the metabolites

The metabolites obtained after ethyl acetate extraction were freeze dried. The obtained powder was mixed with pure KBr at a ratio of 5:95 and then samples were fixed in the sample holder. FT-IR analysis was performed using a FT-IR spectrophotometer (Perkin Elmer model PE1600) within the mid IR region of frequency 400–4000 cm^{-1} at a scan speed of 16 cm/s.

2.8. ICP analysis for chromium estimation

The control dye sample was prepared by dissolving Isolan Dark Blue 2S-GL01 dye at a concentration of 250 mg/l. The same concentration of the dye was used for decolorization analyses with the

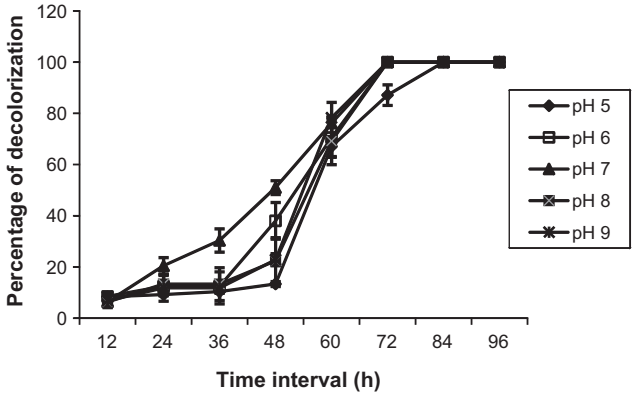


Fig. 1. Decolorization of Isolan Dark Blue 2SGL-01 at different pH values at 25 °C and a concentration of 100 mg/l.

I. lacteus fungal culture and allowed to stand for 24 h after decolorization. Five milliliters of the control and decolorized dye samples were taken and centrifuged at 10,000 rpm. The supernatant was filtered through 0.22 μm Steritop Millipore filters. The chromium ion concentration in sample was estimated using inductive coupled plasma analyses (Leemans Labs Inc., USA). Chromium standard samples were used for standardization and the chromium contents in control and experimental sample were obtained.

2.9. Phytotoxicity studies

The crude Isolan Dark Blue 2SGL-01 dye dissolved in water and the decolorized dye were directly subjected to phytotoxicity evaluations using seeds of *Brassica juncea* and *Vigna radiata*. The normal tap water was supplied for seeds which were maintained as positive control. For the phytotoxicity analysis control dye concentration was 250 mg/l, same concentration was used for dye degradation and after degradation they were subjected to phytotoxicity studies. Seeds of *B. juncea* and *V. radiata* were placed over tissue paper in Petri plates and twice every day they were poured with tap water for positive control and with crude dye solution, degraded dye solution for the experimental. The germination percentage, shoot lengths, root lengths and fresh weight of the seeds were measured after seven days of experiment to estimate the phytotoxicity.

3. Results and discussion

3.1. Effects of pH on rate of decolorization

Decolorization experiments were carried out at different pH values in order to test the ability of *I. lacteus* to decolorize dye over a wide range of pH conditions including acidic, neutral, and basic. The experiment was performed using a dye concentration of 100 mg/l and carried at 25 °C temperature. The percentages of decolorization at pH values of 5, 6, 7, 8, and 9 are shown in Fig. 1. The rate of decolorization was high when the pH was basic and neutral. Under acidic conditions, the decolorization rate was slow but complete decolorization was achieved at all pH values tested. Complete decolorization occurred on the third day for all tested pH values except for pH 5, for which decolorization occurred after three and a half day. Dye industry effluents have an average pH of 7.5–8.0 [26] and decolorization was found to be faster in this pH range, which supports the potential use of *I. lacteus* for effective removal of dye compounds from industrial effluents. The optimum pH for decolorization of dyes by bacteria *Exiguobacterium* and yeast *Trichosporon beigelii* [9,27] was 7, which coincides with the results of our study, in which decolorization was maximized at a pH of 7. In addition, the percentages of decolorization at pH values of 7 and 8 did not differ significantly. The fungus was able to grow at various pH tested and growth of fungus was noticed to be independent of the tested pH.

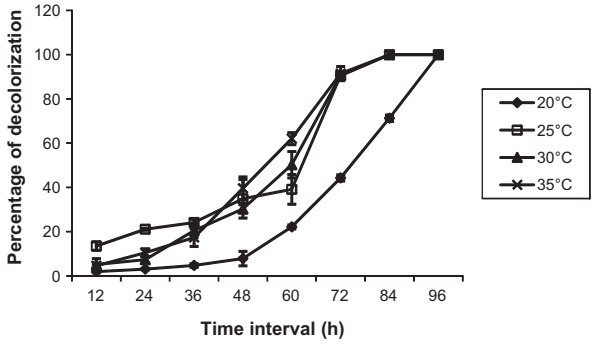


Fig. 2. Effect of temperature on the decolorization of Isolan Dark Blue 2SGL-01 at pH 6.5 and a concentration of 100 mg/l.

3.2. Effects of temperature on the rate of decolorization

The effect of temperature on decolorization was evaluated by conducting experiments at different temperatures of 20 °C, 25 °C, 30 °C, and 35 °C with a constant pH of 6.5 and concentration of 100 mg/l. The maximum rate of decolorization was observed at a temperature of 35 °C, followed by 30 °C and 25 °C. The results are presented in Fig. 2. At a temperature of 20 °C, decolorization was very slow compared to the other temperatures but a sudden rise in the decolorization rate was noticed after the second day. The decolorization reached more than 90% on the third day but was only about 45% at 20 °C, and complete decolorization was observed on the fourth day. The decreased rate of decolorization at 20 °C is attributed to reduction in growth of the mycelia. The growth of *I. lacteus* was affected by temperature, as optimum growth of fungus was around 25–30 °C, at low temperature growth of fungus was reduced which in turn decreases the rate of decolorization.

3.3. Effects of concentration on the rate of decolorization

Isolan Dark Blue 2SGL-01 dye concentrations of 50, 100, 150, 200, and 250 mg/l were tested for decolorization by *I. lacteus*, and the results are presented in Fig. 3. The rate of decolorization decreased as initial concentration of dye increased. Complete decolorization for concentrations of 50, 100, and 150 mg/l was observed at the end of third day, whereas for concentrations of 200 and 250 mg/l, complete decolorization occurred on the fourth day. The decolorization of dye at higher concentrations 200 mg/l

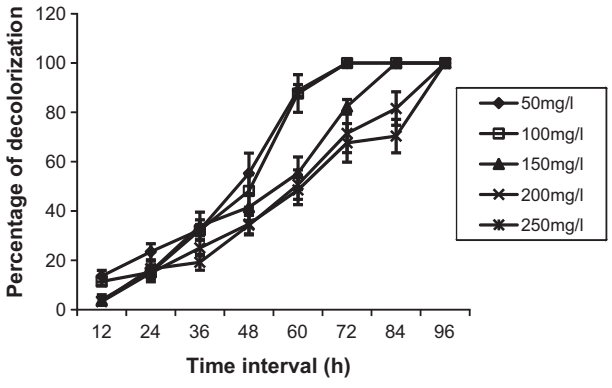


Fig. 3. Decolorization of Isolan Dark Blue 2SGL-01 at various concentrations at 25 °C and pH 6.5.

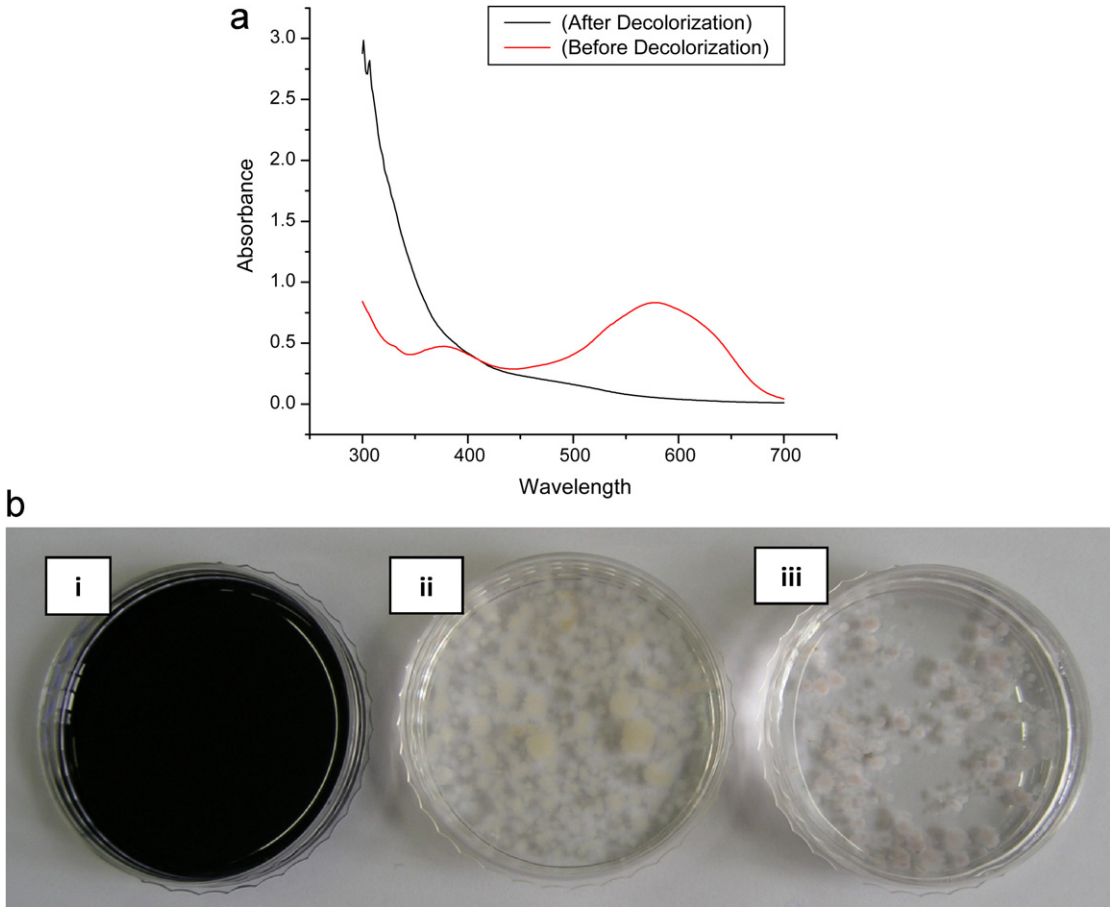


Fig. 4. (a). UV-Visible spectrum analysis of the original dye and after decolorization of Isolan Dark Blue 2SGL-01. (b). (i) Isolan Dark Blue 2SGL-01 dye solution (ii) the fungal mycelia grown in 2% malt extract broth (iii) the fungal mycelia obtained after complete decolorization of the dye.

and 250 mg/l was observed after a short time of four days. Dye concentrations of 70 and 100 mg/l were previously found to be toxic to the actinomycete *S. krainskii* [16]. In a different study, 85% biodecolorization of Orange II dye by *P. chrysosporium* at a concentration of 100 mg/l occurred by the fifth day [24]. In the present study, *I. lacteus* was able to decolorize Isolan Dark Blue 2SGL-01 dye even at high concentrations and in less time compared with the decolorization of Reactive Black 5 by *I. lacteus* [28] and to other microorganisms with the other dyes.

3.4. Detection of decolorization and degradation by UV–visible spectroscopic analysis

The UV–visible spectroscopic absorbance was obtained at 578 nm for complete decolorization, where an absorbance of zero corresponds to 100% decolorization. Twenty-four hours following complete decolorization, spectral analysis in the wavelength range of 200–700 nm revealed complete absences of the peaks at 578 nm and 378 nm, as represented in Fig. 4a. Fig. 4a indicates the complete degradation of the Isolan Dark Blue 2SGL-01 dye. The degradation of dye is characterized by decrease in major peak or by formation of new peaks [29]. The microbial removal of dyes can occur by either of two ways by adsorption of dyes on surface of bacteria or fungi or by degradation of dyes by microorganisms. According to Chen et al. [29], adsorption of the dyes will lead to decrease of peak in the treated sample approximately in proportion to untreated dye. If the dye has been removed by biodegradation, peak formed at major visible light absorbance of the dye will completely disappear or new peak will be formed. They also have proposed that if adsorption of the dye would have occurred the cell mats will be deeply colored. But the *I. lacteus* mycelia were not colored deep blue as given in Fig. 4b after the removal of dye which supports dye degradation.

The graph shown in Fig. 4a reveals the absence of two major peaks, indicating that decolorization associated with degradation of dye parent compound had been occurred. The reduction of molecule by breaking azo bond by electron transfer was first mechanism in the degradation of dyes. The redox mediators of dye degradation may be quinolic groups in case of carbon surface and extracellular enzymes or coenzymes when degradation was carried out by microorganisms. This reduction leads to loss of dye color [30]. To confirm degradation, further HPLC analysis and GC–MS analyses were carried out.

3.5. Biodegradation analysis by HPLC

The metabolites extracted from control samples containing only dye and not treated with fungus, metabolites extracted from fungal culture filtrate and experimental samples were subjected to HPLC analysis to confirm the degradation of dye compounds. HPLC chromatogram depicted new peaks, indicating the formation of new metabolites, while disappearance of other peaks demonstrates removal of some compounds, as seen in Fig. 5. The peaks obtained from all the three samples were compared and peaks obtained at retention times for negative control samples were neglected in positive control sample and experimental sample, to eliminate peaks obtained for fungal extracellular metabolites. The peak obtained at a retention time of 19.9 min in the positive control sample was drastically reduced, and peaks were

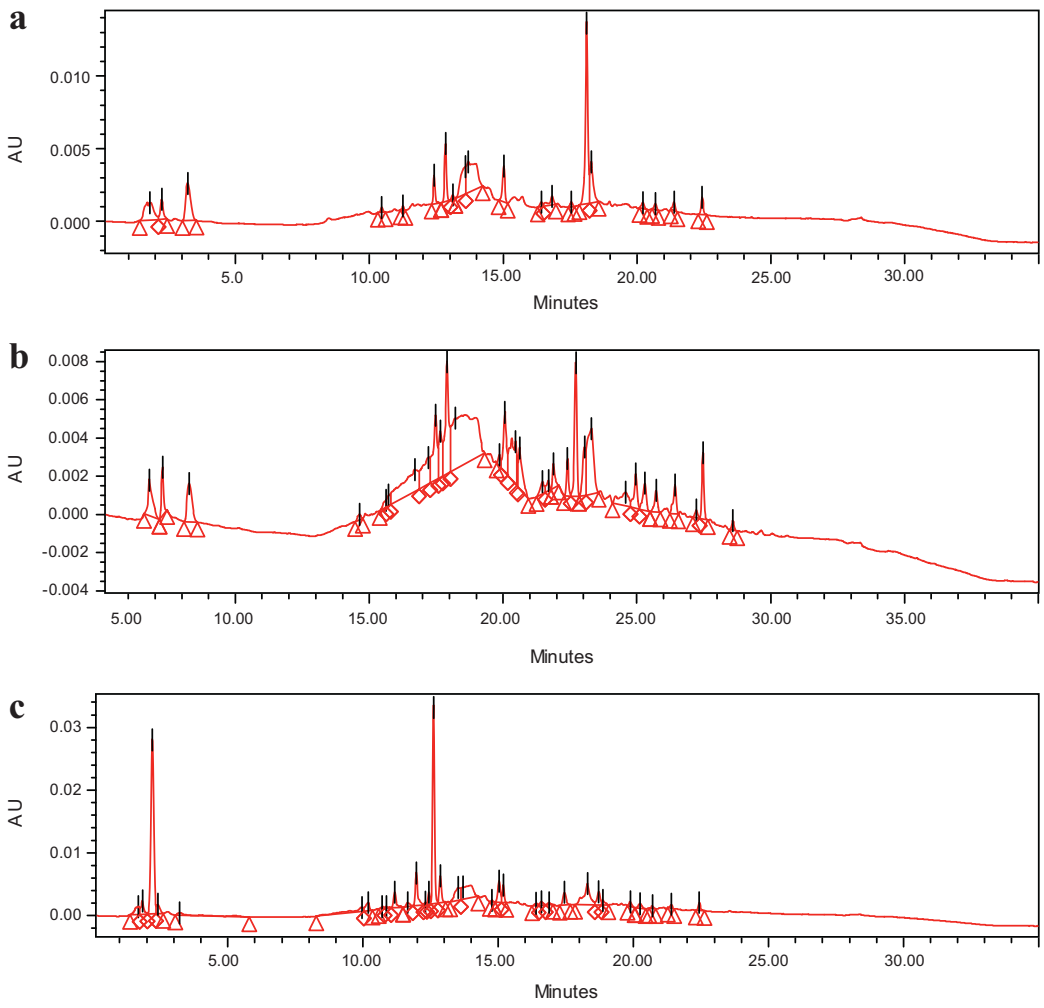


Fig. 5. High performance liquid chromatographic elution profile of the metabolites extracted (a) from fungal culture grown without dye. (b) before decolorization of Isolan Dark Blue 2SGL-01 (c) after 24 h of decolorization (4 days of dye treatment with fungi) of Isolan Dark Blue 2SGL-01.

not found at retention times of 19.5 min, 22.2 min, and 23.5 min in the experimental sample. Many new peaks were formed at retention times of 10.8, 15.1, 18.7 and 18.8 min indicating the formation of new compounds. It is clear from HPLC analysis that the Isolan Dark Blue 2SGL01 dye was successfully degraded.

3.6. GC–MS analysis for the identification of degraded metabolites

GC–MS was carried out for the identification of metabolites formed by degradation of the parent dye compound. The chromatogram of the GC–MS analysis is presented in Fig. 6. The compounds that are identified in fungal cultural filtrate (negative control) were eliminated from compounds identified in untreated dye and fungal treated dye sample and then dye degraded metabolites were detected. The main metabolites obtained after degradation were identified to be 1(2H)-naphthalenone, 3,4-dihydro- and 2-naphthalenol using NIST library. The first step in degradation of azo dye is commonly by breakage of azo bond which reduces color of dye. The azo bond cleavage has occurred by repeated electron transfer reactions, oxidative activation of dyes leading to formation of carbonium ion followed by nucleophilic attack on cationic species. The lignin peroxidases and manganese peroxidases are oxidized by H_2O_2 which are then reduced back by the azo dyes. Manganese peroxidase is responsible for the oxidation of many phenolic compounds [31]. The *I. lacteus* is also known to be source of several enzymes like manganese peroxidase, lignin peroxidase, especially laccase involved in degradation of wood. The degradation of azo disperse orange dye by white rot fungus *Pleurotus ostreatus* proposed the role of lignin peroxidase in the breaking of azo bonds [32]. The degradation of Isolan Dark Blue dye could be contributed by one of the following enzymes.

3.7. Functional group identification of metabolites by FT-IR spectroscopy

The FT-IR spectra obtained from extracted compounds of Isolan Dark Blue 2SGL-01 dye and extracted metabolites after complete decolorization revealed changes in peaks, as shown in Fig. 7. The broader peaks obtained at 3394.78 and 3420.36 cm^{-1} from dye and degraded metabolites, respectively; represent hydrogen O–H stretch of the phenolic groups and alcohols, which also indicates presence of aromatic compounds in the dye and in degraded dye metabolites. The formation of new peaks in regions of 2953.62 and 2869.81 cm^{-1} for metabolites after degradation represent H–C–H asymmetric and symmetric stretches of the alkanes. Both control and experimental samples formed peaks at 2933.31 and 2931.79 cm^{-1}, respectively, and the additional peak in control sample at 2881.35 cm^{-1} corresponds to H–C–H asymmetric and symmetric stretches of alkanes. However, the positions of peaks at different wave numbers, as compared with the control dye compounds, indicate that there may be changes in nature of the

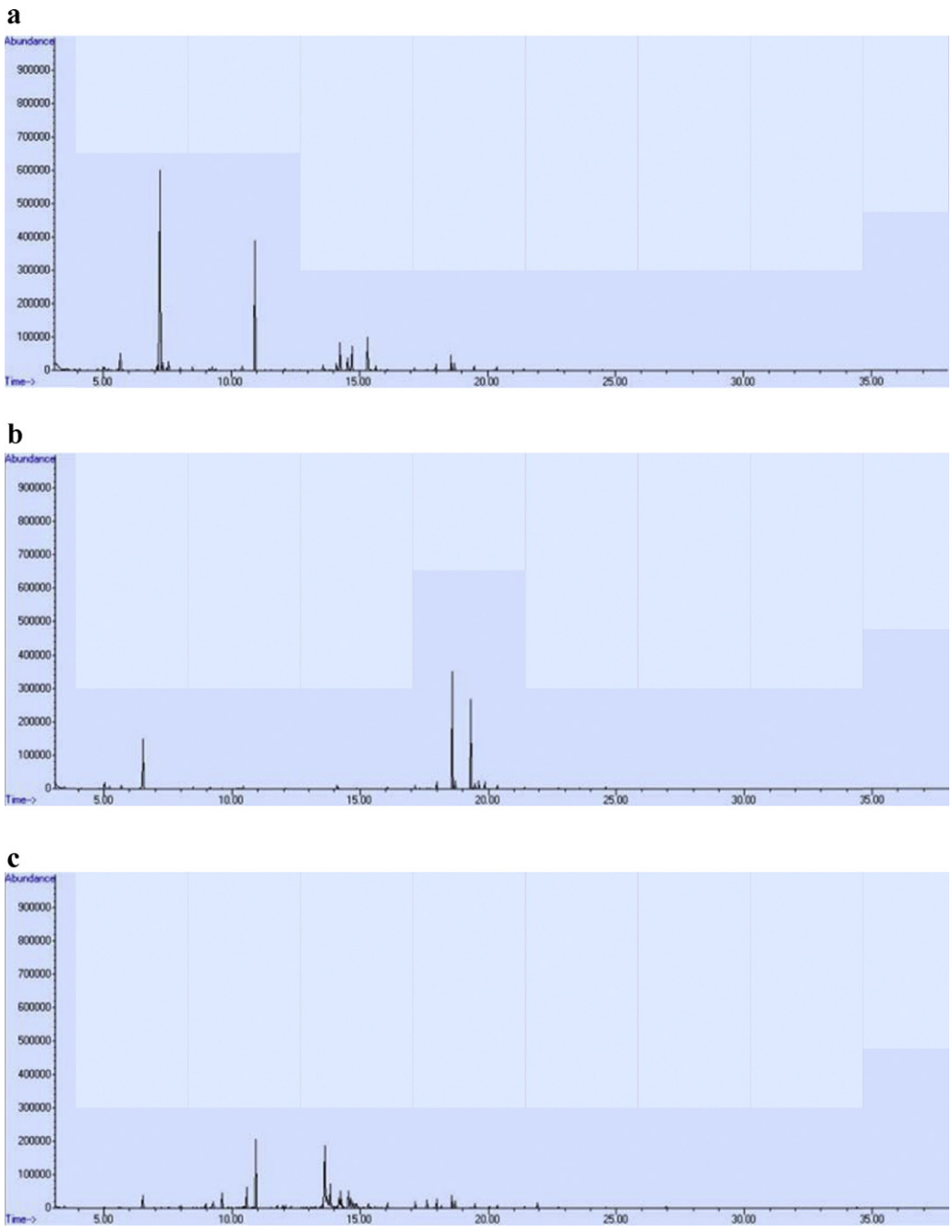


Fig. 6. Gas chromatographic analysis of the metabolites (a) from fungal culture grown without dye (b) before decolorization of Isolan Dark Blue 2SGL-01 (c) after 24 h of decolorization (4 days of dye treatment with fungi) of Isolan Dark Blue 2SGL-01.

metabolites. The formation of peaks at 2564.62 and 2100.22 cm^{-1} represent C≡C stretch and hydrogen bonded O–H stretch of the carboxylic acids, The peaks at 1666.76 and 1697.09 cm^{-1} represent C=O stretches of carboxylic acids. The new peaks at 1382.94 and 1222.33 cm^{-1} represent the O–H bend of phenolic groups and C=O stretch of the ethers, respectively. The presence of C–H bend of alkenes is represented by peaks at 808.58 and 782.13 cm^{-1} in control dye sample as well as by the peaks at 764.30 and 718.37 cm^{-1} in experimental sample. The formation of a new peak at 653.99 cm^{-1} in metabolites after degradation shows the presence of C–H bends of alkyne. The peaks obtained between 675 and 870 also indicates C–H bend of phenyl ring substitution bands, peaks obtained for metabolites extracted before and after the treatment of dye with fungi varies which might be due to the substitution at different carbon number.

3.8. ICP analysis for chromium estimation

Isolan Dark Blue 2SGL-01 dye is a metallic dye consisting of chromium (III) at a concentration of 2.9%. The concentration of chromium after complete decolorization estimated by ICP analysis was 6.190 ppm and in control, it was estimated to be 7.155 ppm. The percentage of chromium removed from dye was 13.49% at a dye concentration of 250 ppm. When the dye is degraded, chromium is released as free chromium ions and 13.49% of chromium is removed from the medium due to fungal bioadsorption. This suggests that

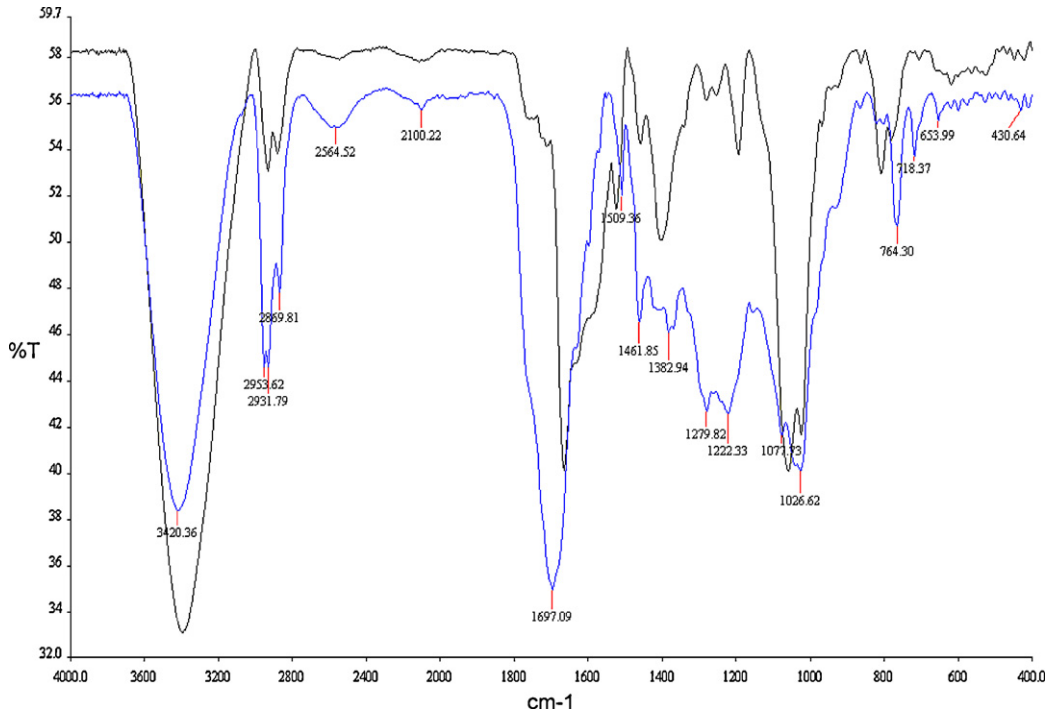


Fig. 7. FT-IR spectra of the metabolites obtained before and after degradation of the Isolan Dark Blue 2SGL-01 dye.

Table 1
Phytotoxicity test results of the extracted metabolites from the control dye and degraded dye.

Parameters	*Vigna radiata*			*Brassica juncea*		
	Water	Isolan Dark Blue 2SGL-01	Treated dye	Water	Isolan Dark Blue 2SGL-01	Treated dye
Germination (%)	100	100	100	100	100	100
Radicle (cm)	1.69 ± 0.39	0.72 ± 0.12	1.11 ± 0.21	1.77 ± 0.17	0.45 ± 0.13	1.13 ± 0.07
Plumule (cm)	0	0	0	1.53 ± 0.23	0.59 ± 0.06	0.98 ± 0.09
Fresh weight (g)	1.85 ± 0.17	1.76 ± 0.27	1.20 ± 0.22	0.21 ± 0.03	0.10 ± 0.01	0.09 ± 0.02

dye degradation is accompanied by the adsorption of chromium, which is a heavy metal.

3.9. Phytotoxicity

Dyes are often toxic. Therefore, they must be removed or degraded before effluents are liberated into water sources. New metabolites are formed during the degradation of original dye compounds that are usually nontoxic, but sometimes primary and secondary amines and other aromatic compounds are liberated, and these are more toxic than the dye compounds themselves. Therefore, to evaluate the toxicity of newly formed metabolites, phytotoxicity studies were carried out and the results obtained are presented in Table 1. The germinations of *V. radiata* and *B. juncea* seeds showed no differences, as all samples exhibited 100% germination. A marked difference was observed in radicle lengths and plumule lengths. The radicle length of seeds grown in water was found to be highest, whereas seeds grown with dye solution at a concentration of 250 ppm had lower radicle length. The seeds germinated using treated dye sample regained radicle length comparatively as of seeds grown with plain water. Similar results were noticed with the plumule length also, decrease in plumule length was observed with seeds grown with the dye solution. The fresh weights of germinated seeds grown with control and treated dye were lower than the fresh weights of germinated seeds grown in plain water. These results suggest that the metabolites formed after degradation of the dye were nontoxic to the plants compared to parent dye compound. The untreated dye reduced the normal growth of plant whereas it has been regained little when dye was treated with *I. lacteus*.

4. Conclusion

This study evaluated the bioremediation of the heavy metal complex dye Isolan Dark Blue 2SGL-01 by the white rot fungus *I. lacteus*. Our results demonstrate the efficacy of the fungus to decolorize and degrade the dye over very broad ranges of pH, temperature, and concentration. The removal of chromium at lower proportions, demonstrates the bioremediation ability of fungus and the nontoxic nature of resulting degradation products in plant germination studies suggests the potential use of *I. lacteus* in bioremediation of textile dyes, especially metallic dyes.

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