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Microbiolgy

Optimization of Culture Condition and Influence of Mediators on Degradation of Reactive Magenta-HB Dye Using *Ganoderma cupreum* AG-1

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Abstract

Reactive dyes are important chemical pollutants from the textile industries. Utilization of microbes including white rot fungi for decolorization of synthetic dyes is one promising strategy of an environmental friendly and cost-competitive alternative to physico-chemical decomposition processes for the treatment of industrial effluents. The present study investigates the biodegradation and decolorization of reactive dyes using white rot fungi *Ganoderma cupreum* AG-1. Physico-chemical parameters including carbon and nitrogen sources, initial glucose and yeast extract concentration, pH, temperature, initial dye concentration, along with the effect of various metal ions and mediators were studied for the decolorization of Reactive Magenta-HB dye. The fungal isolate efficiently decolorized Reactive Magenta HB within 48 h at 28°C, at pH 5.0 and under optimized conditions 94.20 ±1% dye decolorization was obtained within 48 h. The effect of mediators on laccase, manganese peroxidase (MnP) and manganese independent peroxidase (MnIP) were also studied. The decolorization of Reactive Magenta-HB dye was monitored by UV–Visible spectroscopy while dye degradation was studied by HPTLC and FTIR spectroscopy.

Highlights

- Spectrum of various textile dyes was studied to determine for maximum percent decolorization efficiency of *Ganoderma cupreum* AG-1, which showed maximum percent decolorization of Reactive Magenta-HB dye.
- One single factorial design was adopted for the media optimization study of the decolorization of Reactive Magenta-HB dye.
- Supplementation of metal ion (Ca^{2+}) and mediator (veratryl alcohol) have shown positive influence upon decolorization efficiency.
- Various ligninolytic enzyme activities were determined which showed an increase in activities with the supplementation of mediators like tyrosine and veratryl alcohol and 1.26 fold rise was observed in laccase activity in presence of Veratryl Alcohol.
- Degradation analysis was done using FTIR and HPTLC techniques, change in the spectra of decolorized sample and different R_f value of different bands suggests structural transformations occurred in the dye structure due to biological treatment.

Keywords: Reactive Magenta-HB dye, decolorization, laccase, peroxidases, mediators, veratryl Alcohol, *Ganoderma cupreum* AG-1.

Introduction

Synthetic dyes are extensively used in several industries including textile, paper, printing, cosmetics and pharmaceuticals (Marmion, 1991). There are many structural varieties, such as, acidic, basic, disperse, azo, diazo, anthraquinone based and metal complex dyes. It is estimated that 10–15% of the dyes are lost in the effluent during the dyeing process (Zollinger, 1987). Therefore, many dyes are difficult to decolorize due to their complex structure and synthetic origin. Especially, brightly colored, water-soluble reactive and acid dyes are the most problematic; as they tend to pass through conventional treatment systems unaffectedly (Willmott *et al.,* 1998). Color can be removed from effluent by various chemical and physical methods including adsorption, coagulation-flocculation, ion-exchange, oxidation and electrochemical methods (Lin *et al.,* 1994; Lin *et al.,* 1996). The physico-chemical treatments are found to be effective but their application is limited due to excess usage of chemicals, sludge production, subsequent disposal problems, high installation as well as operating costs (Vandevivere *et al.,* 1998; Sarioglu *et al.,* 2007). Alternatively, dye decolorization using microbes has received great attention in recent years due to its efficient application (Abadulla *et al.,* 2000; Claus *et al.,* 2002; Zille *et al.,* 2003; Couto *et al.,* 2005). Decolorization of dye waste water is a challenging process of the textile industry and the great potential of microbial decolorizing can be adopted as an effective tool. In the recent past there has been an intensive research on bioremediation of dyes and the use of white rot fungi/ ligninolytic fungi is turning into a promising alternative to replace or supplement present treatment processes (Dos Santos *et al.,* 2004; Ashger *et al.,* 2006). The abilities of white rot fungi in mineralization of xenobiotics to CO_2 and water through the highly oxidative and nonspecific ligninolytic system are well documented, which are also responsible for decolorization and degradation of a wide range of dyes (Boer *et al.,* 2004; Patel *et al.,* 2009). The extracellular enzyme system of white rot fungi include laccase (E.C. 1.10.3.2), manganese peroxidase (MnP, E.C. 1.11.1.13), and lignin peroxidase (LiP, E.C. 1.11.1.14) are well reported to decolorize various dyes of different chemical structures (Levin *et al.,* 2004). The biodegradation of four ring polycyclic hydrocarbon (PAH), pyrene was also studied in submerged culture of white rot fungi by Patel et al. (2010). The physiology of ligninolytic enzyme production by white rot fungi for ligninolysis or recalcitrant pollutant degradation has been studied extensively.

Redox mediators (RM) are molecules that reversibly oxidize and reduce by conferring their capacity to serve as an electron carrier in multiple redox reactions. They are known to accelerate reactions by lowering the activation energy of the total reaction (Stolz *et al.,* 2001). The rate of color removal is highest when the redox potential of the system is at its most negative, and the rate falls as the redox potential of the system rises (Liu *et al.,* 2009). The effect of redox mediators on the decolorization rates has generally been investigated with model compounds as well as in the decolorization of textile wastewaters. The first studies focused on the application of RM on the reductive biotransformation of contaminants were carried out during decolorization of dye in high rate UASB reactors (Cervantes *et al.,* 2002; Van der zee *et al.,* 2001). However, the effectiveness of redox mediators in enhancing the decolorization of textile wastewater is still unclear due to the wide range of redox potentials among dyes (-180 to -430 mV) present in wastewater, high COD and diverse properties of the different dyes (Dos Santos *et al.,* 2004).

In order to exploit the potential of *Ganoderma cupreum* AG-1 various process parameters like carbon and nitrogen sources, initial glucose and yeast extract concentration, pH and temperature were optimized to develop an economic decolorization process. Furthermore, relationship between ligninolytic enzyme production and decolorization of reactive azo dye by fungi *Ganoderma cupreum* AG-1 was assessed. Degradation analysis was studied using UV–Visible, HPTLC and FTIR.

Demand and necessity of synthetic dyes are continuously growing for dye manufacturing and dye utilizing companies. However, the major consumers of dyes are textiles industries. Most of the textile processing units give some type of primary treatment to meet the requirements of common effluent treatment plants. The effluents receive a combination of physico-chemical treatments followed by activated sludge treatment. However, the treatments are not sufficient enough to remove the color completely and consequently show adverse effect on different ecosystems. As a result, dyes find their way into receiving water bodies which has resulted in extensive pollution of surface and ground waters in the surrounding areas.

Thus, we undertook the present study with following objectives:

- Screening of various dyes for decolorization study using fungal isolate.

- Optimization of physico-chemical parameters to obtain maximum decolorization.
- Study of various enzymes involved in dye decolorization and degradation.
- Study the role and effect of mediators on dye decolorization.
- Dye degradation study using various analytical methods.

Materials and Methods

Media, Chemicals and Dyes

Malt extract agar (MEA) medium (g % w/v): Malt extract powder (2.0), Glucose (0.5), Yeast extract (0.05), $MgSO_4.7H_2O$ (0.05), and KH_2PO_4 (0.05), KCl (0.05).

Basal medium (g % w/v): Glucose (0.5), Yeast extract (0.05), $MgSO_4.7H_2O$ (0.05), and KH_2PO_4 (0.05), KCl (0.05).

All chemicals used were of analytical grade. Hydrochloric acid, ethyl acetate and n-propanol were purchased from Hi Media Labs. (Mumbai), India. Ortho-dianisidine was purchased from Sd-fine chemicals (Mumbai), India. 2, 2-Azino-bis (3 ethylbenzthiozoline-6- sulphonic acid) (ABTS) was purchased from Sigma, (St. Louis, MO, USA) and 2, 6-Dimethoxyphenol (DMP) was purchased from Lancaster (Lancs, UK).

All mediators (veratryl alcohol, *p*-coumaric acid, vanillin, vanillic acid, L-tyrosine, syringaldehyde, veratryaldehyde, 1-hydroxy benzotriazole, 4-hydroxy Benzaldehyde and *p*-hydroxy benzyl alcohol) were purchased from Hi Media Labs. (Mumbai), India.

Different dyes which were used in this study were obtained from Meghmani Enterprise Pvt. Ltd., Ahemadabad, (Gujarat), India.

Micro-organisms and culture conditions

The fungal isolate *Ganoderma cupreum* AG-1 was maintained on the plate containing 2% malt extract agar amended with chloramphenicol (0.5 g l^{-1}) and incubated at 28°C. MEA incorporated with 0.1 g l^{-1} ortho-dianisidine was used for the screening of phenol oxidase activity. Potential isolate was sub cultured and maintained at 4 °C on malt extract agar slants.

Spectrum of dyes decolorized using Ganoderma cupreum AG-1

Erlenmeyer flasks (250 ml) containing 100 ml of basal culture medium were inoculated with 8 agar plugs of size 8 mm diameter punched from the leading edge of pre-grown fungal culture on MEA plates and incubated at 28°C for 120 h. On the 5th day of incubation 0.1 g l^{-1} of dye was added from the autoclaved stock and incubated for 48 h at 28°C. Uninoculated basal medium served as abiotic control. Dye decolorization was determined spectrophotometrically by monitoring the absorbance of samples at γ_{max} of the respective dyes using a UV–Visible spectrophotometer (Shimadzu UV 1800, Japan). A sample of 2 ml from inoculated flasks was withdrawn at a regular interval of 48 h and centrifuged at 10,000 rpm at 4°C for 15–20 mins. The clear supernatant obtained was used to determine percent decolorization.

On the basis of the mean amount of decolorization of three replicates, the decolorization expressed in % of the initial dye concentration was calculated as follows:

$$\% \text{ Decolorization} = 100 \times (A_0 - A_t) / A_0$$

Where A_0 is the absorbance value of the initial dye concentration and A_t is the absorbance value of the dye concentration in sample at time t.

Optimization of process parameters for enhanced decolorization of Reactive Magenta-HB dye

A single factorial strategy was adopted for the optimization of the decolorization of Reactive Magenta-HB dye by varying one factor at a time. All the decolorization experiments were performed in triplicates.

Effect of carbon and nitrogen source on decolorization of Reactive Magenta-HB dye.

To enhance the decolorization performance of Reactive Magenta-HB, the decolorization medium was supplemented with different carbon sources like glucose, fructose, mannose, xylose, maltose, lactose and sucrose, individually added at a concentration of 5 g l^{-1}. Similarly, in other set various organic nitrogen sources (yeast extract, beef extract, peptone, urea) and inorganic nitrogen sources (ammonium sulfate, ammonium nitrate) were added at a concentration of 0.5 g l^{-1} to the medium to study the effect of nitrogen sources on decolorization process. To optimize the concentration of glucose (0.25-2.0 g % w/v) and yeast extract (0.02-2.5 g % w/v) were added in the medium and decolorization efficiency was monitored.

Effect of physico-chemical parameters on decolorization of Reactive Magenta-HB dye.

Physicochemical parameters like temperature and pH were investigated by varying the temperature in the range of 20° to 45° C and pH in the range of 3 to 9. The flasks containing 100 ml of basal media were inoculated with 8 agar plugs and incubated at 28°C under shaking conditions for 120 h. On the 5th day of incubation 0.1 g l^{-1} dye was added and incubated for 48 h at 28°C. Percent decolorization was measured by the formula mentioned earlier.

Effect of initial dye concentration on decolorization of Reactive Magenta-HB dye.

The dye decolorization efficiency of the fungus was monitored by varying the concentration (0.1-1.0 g l^{-1}) of Reactive Magenta-HB in the medium. Each flask containing 100 ml basal media were inoculated with 8 agar plugs and incubated at 28°C under shaking conditions for 120 h. On the 5th day of incubation 0.1 g l^{-1} dye was added and incubated for 48 h at 28°C. Percent decolorization was measured using supernatant by the formula mentioned earlier.

Effect of Metal ions on decolorization of Reactive Magenta-HB dye

Metal ions play an important role as cofactors in various enzymatic reactions and therefore, they can influence the decolorization efficiency. To determine the efficacy of metal ions on decolorization various metal ions like Cd^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+} were added at a concentration (1 mM) to the 100 ml basal media. On the 5th day of incubation 0.1 g l^{-1} dye was added and incubated for 48 h at 28°C. Percent decolorization was measured using supernatant by the formula mentioned earlier.

Effect of Redox Mediators on decolorization of Reactive Magenta-HB dye

Laccase-based mediators have shown their significant effect on microbial growth and ligninolytic enzymes which involves in the decolorization of different dyes. The effect of various mediators like veratryl alcohol, *p*-coumaric acid, vanillin, vanillic acid, L-tyrosine, syringaldehyde, veratryaldehyde, 1-hydroxy benzotriazole, 4-hydroxy Benzaldehyde and *p*- hydroxy benzyl alcohol on decolorization of dye was studied. All the mediators were added at a concentration of 1 mM to the basal medium. On the 5th day of incubation 0.1 g l^{-1} dye was added and incubated for 48 h at 28°C. Percent decolorization was measured using supernatant by the formula mentioned earlier.

Laccase, MnP and MnIP activity were assayed spectrophotometrically (Shimadzu UV 1800, Japan) from extracted sample. Laccase activity (E.C. 1.10.3.2) was determined by measuring the oxidation of 2, 2-Azino-Bis-3-ethyl-benzthiozoline- 6-sulphonic acid (ABTS) at 420 nm as described by Niku *et al.* (1990). Manganese peroxidase (MnP, E.C. 1.11.1.13) activity was measured by oxidation of 2, 6- dimethoxy phenol (DMP) at 469 nm and MnP activity was corrected for manganese-independent peroxidase (MnIP) activity by subtracting the MnIP activity obtained at pH 3.25 in absence of $MnSO_4$ at 469 nm. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µM of substrate per min at room temperature.

2.4.6 Repeated batch decolorization study of Reactive Magenta-HB dye

Repeated batch decolorization study was carried out to understand the stability and performance of veratryl alcohol mediated and non-mediated decolorization process. After 1st cycle of decolorization readdition of the same concentration of dye (0.1g l^{-1}) was made till the same extent of decolorization could be achieved and the process was repeated till decolorization occurred. All the assays were performed in triplicates with the uninoculated media as a control.

In vitro decolorization study of degradation of various dyes

The reaction was carried out in sugar tubes at 28°C under static condition. The reaction mixture contained an aliquot of crude filtrate (10 ml), Reactive Magenta–HB dye (0.01 g l^{-1}), Reactive Violet 1 dye (0.01 g l^{-1}) and Reactive Blue 3R (0.01 g l^{-1}). Residual dye content was monitored at the respective absorbance maxima of each dye. A control with distilled water was kept in parallel. Decolorization activity was estimated as the decrease in absorbance at the maximum visible wavelength for each dye. The effect on the crude filtrate decolorization after the addition of Veratryl Alcohol (1 mM) was also evaluated.

Degradation of Reactive Magenta-HB dye using Analytical methods

Decolorization was monitored by UV–Visible spectroscopic analysis (Shimadzu UV 1800, Japan), whereas biodegradation was monitored using HPTLC and FTIR spectroscopy. The HPTLC analysis was carried out using

the precoated silica gel 60 F254 plate (Merck, Germany). A 5 µl of the sample was spotted on HPTLC plates using micro syringe. The solvent system used was n-propanol: ethyl acetate: water (5:1:4 ratio). The dye chromatogram was observed by exposing to the ultraviolet light (254 nm) and in visible light using Camag HPTLC scanner 3. The biodegradation of Reactive Magenta-HB was further characterized by FTIR spectroscopy (Fourier transform infrared spectroscopy, Perkin Elmer Spectra GX). The FTIR analysis was carried out in the mid IR region of 400–4,000 cm^{-1}. The control and degraded dye samples were mixed with spectroscopically pure KBr in the ratio of 5:95 to form a uniform pellets, which was then fixed in sample holder, and the analysis was carried out.

Results and Discussion

Spectrum of dyes decolorized by using Ganoderma cupreum AG-1

The fungi *Ganoderma cupreum* AG-1 was screened for the decolorization of nine different dyes. The efficiency of decolorization for various dyes is illustrated Table 1. The maximum decolorization efficiency was observed for Reactive Magenta-HB (94.20 ± 1%) within 48 h. The variation in decolorization efficiency and the variation in time for decolorization of different dyes may be due to molecular complexity of the dyes, culture conditions and the enzyme system of the fungi (Katia *et al.* 2006; Santos *et al.* 2006). A low rate for decolorization can also be attributed to higher molecular weight and the presence of group like $–NO_2$ and $–SO_3$ groups in the dyes (Hu and Wu 2001; Xiu *et al.*, 2009) reported. Decolorization of azo, triphenylmethane and anthraquinone dyes varied between 30 to 97 % in 7 days by *Trametes sp.* SQ01 with concomitant production of laccase. Ismat *et al.* (2010) reported Novosal direct dye decolorization by *Agaricus biotrocus* A66 which varied in the region of 26–78 % in 120 h. The results with us are in accordance with those obtained by Katia *et al.* (2006) who reported decolorization of 28 reactive dyes using *Trametes villosa and P. sanguineus.*

Table 1: Spectrum of dyes decolorized by using *Ganoderma cupreum* AG-1.

Dyes	λ_{max} (nm)	% Decolorization
Reactive Magenta HB	540	**94.20**
Reactive Blue 3R	577	82.15
Reactive Violet 1	517	82.49
Reactive Violet 5R	560	65.61
Reactive Blue 160	612	91.02
Reactive Red M5B	520	90.40
Reactive Red 141	512	71.82
Reactive Red HE8B	540	71.59
Reactive Red ME6B	545	78.58

Optimization of physico-chemical parameters

Effect of carbon sources on decolorization of Reactive Magenta-HB dye

To determine the role of suitable carbon source on decolorization efficiency the medium was supplemented with different carbon sources, among various carbon sources maximum decolorization obtained was 94.76 ± 1.2% in the flask supplemented with glucose as carbon source followed by mannose and xylose with 92.14 ± 1% and 90.77 ± 0.5% decolorization respectively as shown in Fig. 1 (a). The obtained results indicate glucose is the most common and simple carbon source which can be easily metabolized by the fungal biomass. Fazli *et al.* (2010) using *Ganoderma spp*. reported the maximum color removal of 75.4% after 5 days when glucose was supplemented in the medium. Biodegradation of reactive dyes like Reactive Blue 19 and Reactive Black 5 was carried out by Petchoy *et al.* (2010) in which glucose is used as carbon source. However, Fazli *et al.* (2010) reported glycerol as best carbon source for maximum decolorization (95.3%) of reactive blue dye by *Ganoderma spp* on the 5th day of incubation. Furthermore, to determine an effective concentration of glucose in the flasks were supplemented with variable concentrations of glucose and maximum decolorization (95.67 ± 0.5%) was observed with 0.5% w/v glucose concentration. Fig. 2 (a) depicts the concentration of glucose increases, dye decolorization efficiency initially increases and maximum decolorization (95.67 ± 0.5%) was observed with 0.5 % glucose concentration. With further rise in the concentration of glucose, a decrease in dye decolorization was observed. This may be because higher concentrations of glucose may create a highly acidic condition or catabolite repression leading to a negative effect on dye decolorization. The results obtained are in accordance with other reports wherein carbon-limited conditions trigger ligninolytic enzyme activity in white rot fungi, which is required for pollutant degradation (Kapdan and Kargi, 2002).

Effect of nitrogen sources on decolorization of Reactive Magenta-HB dye

In case of nitrogen source, the maximum decolorization (94.84 ± 1.2%) was observed after 120 h of incubation when the yeast extract was used as shown in Fig. 1 (b). However, peptone and beef extract showed lower efficiency for dye decolorization than yeast extract (81.97 ± 0.3% and 82.65 ± 1%) and significantly less efficient (35 ± 0.6% and 29 ± 0.1%) was observed with urea and ammonium sulfate, respectively. The obtained results suggest organic compounds act as a better nitrogen source in comparison to inorganic nitrogen sources. The results obtained are in agreement with those of Saratale *et al.* (2009) who studied the addition of yeast extract in synthetic media that showed maximum decolorization of Navy Blue HER by *Trichosporon beigelii*. Ponraj *et al.* (2011) also found yeast extract as the most effective nitrogen source for the decolorization of True blue dye by *Aspergillus flavus*. However, Kashif *et al.* (2011) reported ammonium sulfate as the best nitrogen source for maximum decolorization (89.1%) of Solar Golden Yellow R dye by *Pleurotus ostreatus* species. Furthermore, to optimize the yeast extract concentration in the medium different concentration of yeast extract (0.02-2.5% w/v) on dye decolorization were studied. Fig. 2 (b) depicts as the concentration of yeast extract increased, percent decolorization increased and maximum decolorization (97.64 ± 1%) was obtained with a concentration of 2.0% (w/v). The presence of various carbon and nitrogen sources in the medium lead to stimulatory or inhibitory effect on the induction of the enzyme system, involved in the decolorization of Reactive Magenta dye resulting in the variation of percent decolorization.

Effect of pH on decolorization of Reactive Magenta-HB dye

The pH of the culture medium is critical to the growth, metabolic activity, ligninolytic enzyme production and dye degradation by white rot fungi. To investigate the effect of pH on Reactive Magenta dye decolorization by *Ganoderma cupreum* AG-1, the initial pH of the medium was adjusted in the range of 3 to 9. The decolorization efficiency increased with increased in pH from 4.5 to 6 and maximum decolorization (96.48 ± 1.5%) was obtained at pH 5.0, further rise in pH (7 to 9) showed drastic decrease in percent decolorization 50-28 ± 2.1%, respectively as in Fig. 1 (c). The results obtained indicate, acidic condition favors Reactive Magenta-HB dye decolorization. At high pH values, reactive dye solutions are more negatively charged, and dye removal efficiency by white rot fungi is readily decreased (Tak *et al.*, 2004). Various researchers reported optimum ligninolytic enzyme production as well as dye decolorization capabilities in an acidic pH range from 4 to 6. (Mazmanci and Ali, 2010; Shazia and Safia, 2011; Ali and Mohamedy, 2012). Cripps *et al*. (1990) also reported the enzymatic oxidation of azo dyes by crude lignin peroxidase at pH 4.5.

Effect of temperature on decolorization of Reactive Magenta-HB dye

Incubation temperature is an important process parameter that varies from one microorganism to other microorganisms. A change in the incubation temperature may affect the growth and enzyme activities of microorganisms. The effect of varying incubation temperature on decolorization of Reactive Magenta-HB by *Ganoderma cupreum* AG-1 is shown in Figure 1 (d) and the maximum decolorization (94 ± 1.8%) was observed in the shake flask incubated at 28°C for 48 h.

The dye decolorization ability of fungal culture decreased with an increase in incubation temperature up to 45°C. The obtained result suggests the fungal culture exhibited better dye decolorization in the lower temperature ranges (28–37°C). Similar results were also shown by Abedin (2008) for the decolorization of crystal violet and malachite green by *Fusarium solani* in a low temperature range. Haq *et al.* (2008) also reported 30°C as an optimum temperature for the decolorization of Cibacron Red FN-2BL dye by *Schizophyllum commune* IBL 6. The temperatures higher than 30°C, reduces the decolorization ability of fungi indicates that either the fungi is not able to produce the oxidases or peroxidases for decolorization or they get denatured at high temperature (Baldrian and Snajdr, 2006; Niladevi *et al.*, 2007). These results are also in consistent with the findings of various researchers who explained that fungal growth was supported in a limited temperature range with dye removal (Tan *et al.*, 2000; Nyanhango *et al.*, 2002; Masud and Anantharaman, 2006).

Effect of initial dye concentrations on decolorization of Reactive Magenta-HB dye

In the present study different concentrations of the dye were used to investigate the effect of initial dye concentrations on dye removal efficiency under optimum conditions. The dye solutions were varied with the dye concentration increasing from 0.1 to 1 g l^{-1}. The dye was

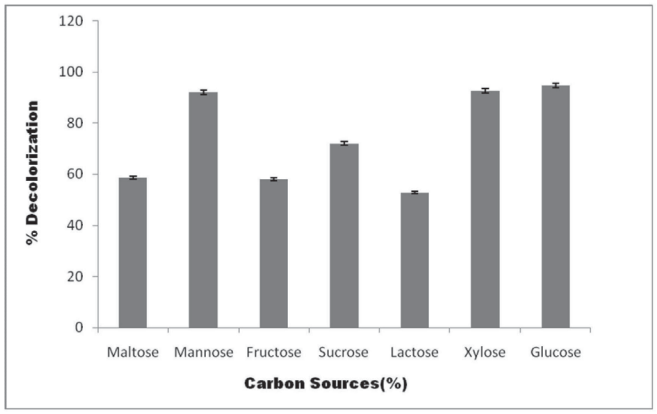

Fig. 1 (a): Effect of carbon sources on decolorization of Reactive Magenta-HB dye.

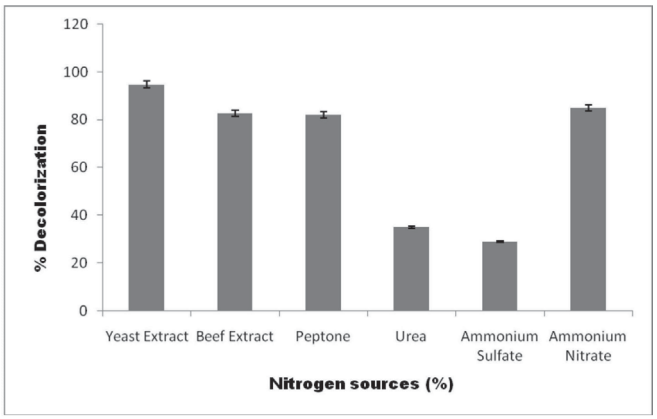

Fig. 1(b): Effect of nitrogen sources on decolorization of Reactive Magenta-HB dye.

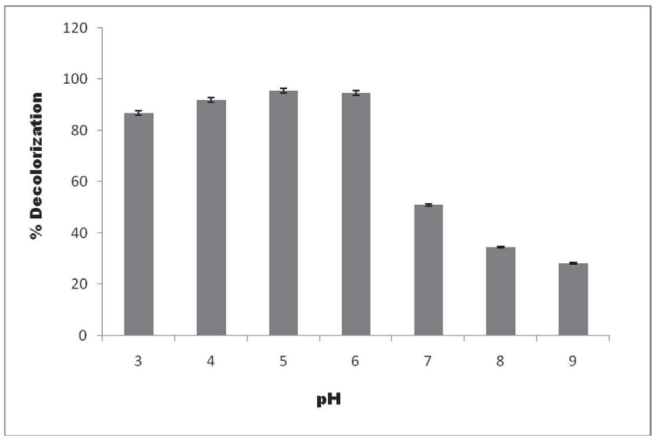

Fig. 1(c): Effect of pH on decolorization of Reactive Magenta-HB dye.

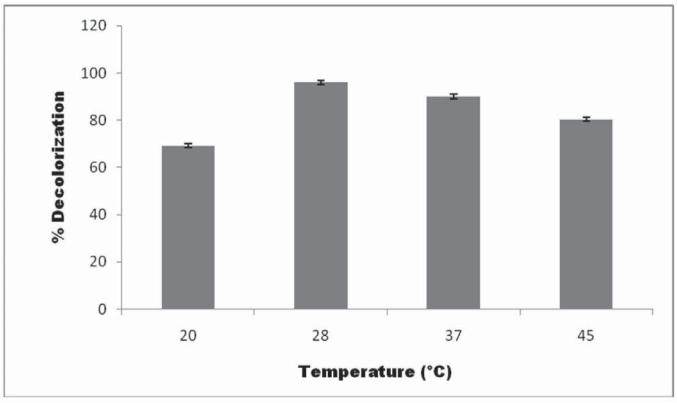

Fig. 1 (d): Effect of temperature on decolorization of Reactive Magenta-HB dye.

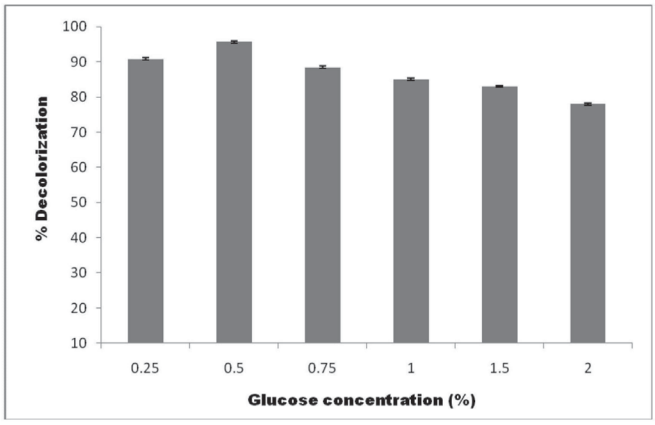

Fig. 2(a): Effect of initial glucose concentration on decolorization of Reactive Magenta-HB dye

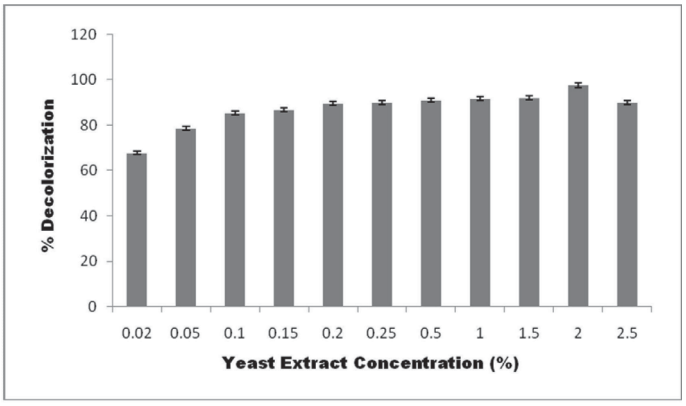

Fig. 2(b): Effect of initial yeast extract concentration on decolorization of Reactive Magenta-HB dye.

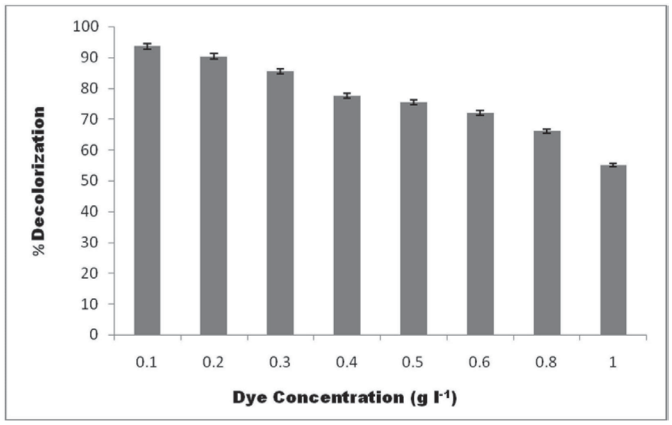

Fig. 2 (c): Effect of initial dye concentration on decolorization of Reactive Magenta-HB dye.

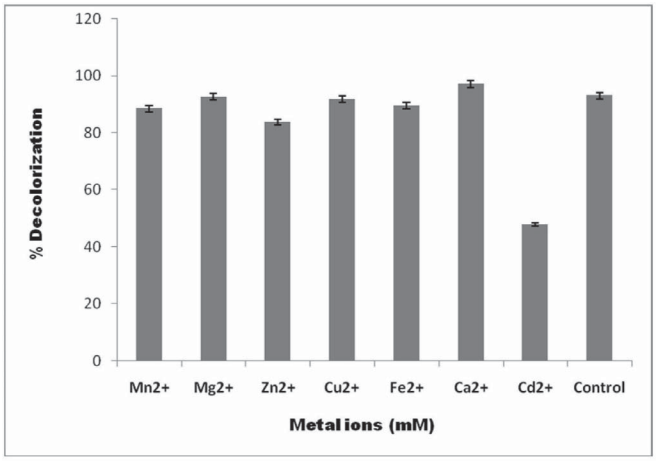

Fig. 2 (d): Effect of metal ions on decolorization of Reactive Magenta-HB dye.

well tolerated and decolorized by the *Ganoderma cupreum* AG-1 in all the tested dye concentrations. It was observed that at lower concentrations, the percent decolorization was high at 0.1 g l^{-1} (93.62 ± 1%) decolorization however, 55 ± 0.4% decolorization was also observed with 1 g l^{-1} dye concentration as shown in Fig. 2 (c). The result suggests a high concentration of dye show detrimental effect on the growth of the organism and as a result lowers down the efficiency of decolorization. Vaithanomast *et al.* (2010) reported more than 90 % decolorization of 1.0 g l^{-1} reactive dye RBBR and RB5 by *Datronia* species, whereas Shazia and Safia (2011) denoted 0.2 g l^{-1} of AR151 dye as a maximum limit to be decolorized (60–70 %) by three indigenous *Aspergillus* species. Kashif *et al.* (2011) reported 78 % decolorization of 0.5 g l^{-1} of solar golden yellow R dye by *Pleurotus ostreatus*.

Effect of metal ions on decolorization of Reactive Magenta-HB dye

The metal ions are general potent inhibitors of enzyme reactions but they also play a vital role as co-factors at low or moderate concentration and enhance the activity of enzymes. The metal ions have shown variable effect on decolorization efficiency. In the presence of metal ion sources like Cd^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}, the percent decolorization obtained were 47.89 %, 97 %, 83.77 %, 89.55 %, 91.70 %, 92.66 % and 88.48 %, respectively (Fig. 2 (d)). The results indicate percent decolorization increased with supplementation of Ca^{2+}, Mg^{2+} and Cu^{2+} ions. Similar kind of observation was described for decolorization of Reactive orange 16 by *Bacillus sp.* ADR in the presence of $CuSO_4$ and decolorization was greatly enhanced up to 97 % (Van der Zee *et al.*, 2001; Pearce *et al.*, 2003; Telke *et al.*, 2009). It is also well reported by Bras *et al.* (2001) that addition of metal ions apparently induces the reduction of azo bonds.

Effect of redox mediators on decolorization of Reactive Magenta-HB dye

The color removal efficacy was studied in the presence of various mediators; veratryl alcohol, *p*-coumaric acid, vanillin, vanillic acid, L-tyrosine, syringaldehyde, veratryaldehyde, 1-hydroxy benzotriazole, 4-hydroxy benzaldehyde and *p*-hydroxy benzyl alcohol. Various mediators showed variable effects on the initial rate of decolorization and significant result was observed with veratryl alcohol (2.01 mg/l/h), L-tyrosine (1.97 mg/l/h) and p-coumaric acid (1.96 mg/l/h) than non-mediated controls and the maximum rate of decolorization was achieved in the presence of veratryl alcohol. However, other mediators (HBD, HBT, HBA, vanillin, vallinic acid and veratryaldehyde) showed less or almost to the same extent of decolorization in comparison to decolorization in non mediated controls (Fig. 3 (a)).

The various ligninolytic enzyme activities were determined using the fungal culture after optimization. The enzyme profile revealed 311.22, 40.78 and 11.31 (U/ml) activity for laccase, MnP and MnIP, respectively. These enzyme activities increased in the presence of mediators like tyrosine and veratryl alcohol. 1.26 fold increases in laccase activity was observed in the presence of veratryl alcohol while no significant rise was observed in MnP and MnIP activities (Table 2). The production of laccase by white-rot fungi increased or induced when organic mediators (such as

Table 2: Effect of mediators on activities of ligninolytic enzymes of *Ganoderma cupreum* AG-1 and decolorization of Reactive Magenta-HB dye.

Mediators (1 mM)	Rate of decolorization (mg/l/hr)	Enzyme Activity (U/ml)		
		Laccase	MnP	MnIP
L-Tyrosine	1.96	338.55	46.14	15.49
Veratryl Alcohol	2.01	400.66	54.06	20.83
Control	1.93	311.22	40.78	11.31

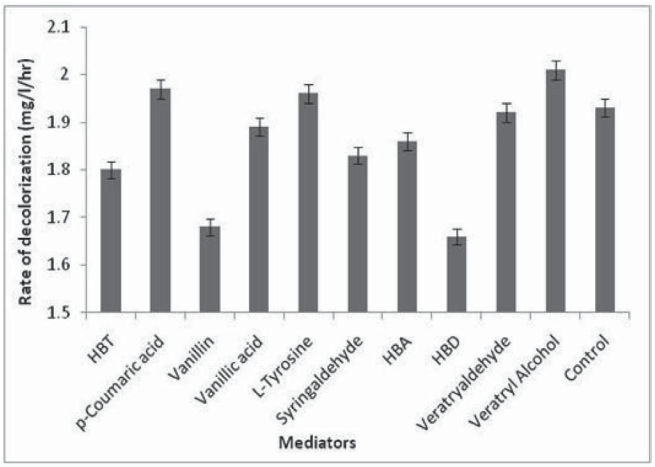

Fig. 3 (a): Effect of mediators on decolorization of Reactive Magenta-HB dye.

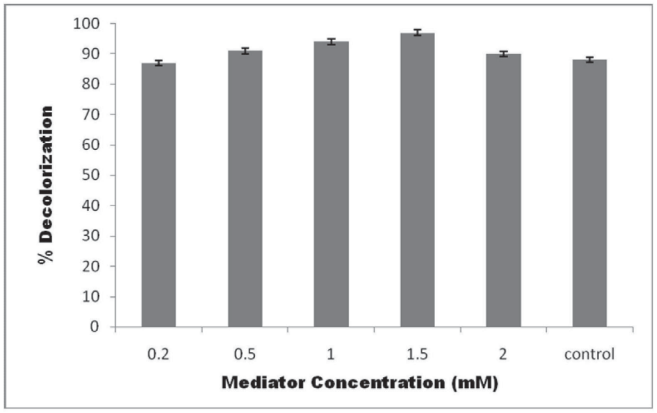

Fig. 3 (b): Effect of initial veratryl alcohol concentrations on decolorization of Reactive Magenta-HB dye.

veratryl alcohol, vanillic acid or syringaldehyde) were added to the growth medium. The inductive effect of veratryl alcohol on laccase production was observed in *Dichomitus squalens*, *Ganoderma lucidum* and *Trametes versicolor* has been reported by Patel *et al.* (2009).

To investigate an effective concentration of mediator (veratryl alcohol), variable concentration of veratryl alcohol (0.2, 0.5, 1.0, 1.5, 2.0 mM) was added in the optimized basal media. The percent decolorization increased with an increase in concentration of veratryl alcohol till 1.5 mM concentration. However, any further increase in the concentration of veratryl alcohol lowers the efficiency of decolorization shown in Fig. 3 (b). This may be due to the detrimental effect of veratryl alcohol at high concentration because high concentrations of mediators which are usually phenolic compounds can affect both the growth of the fungi and production of enzymes (Patel *et al.* 2009).

Repeated batch decolorization study of Reactive Magenta-HB dye

The repeated batch decolorization of Reactive Magenta-HB dye was studied under optimized condition. The main purpose of this study was to evaluate the efficiency of our fungal isolate to decolorize Reactive Magenta-HB dye by repeated addition of 0.1 g l^{-1} Reactive Magenta-HB dye. Fig. 4 shows that fungal culture was able to decolorize dye consecutively up to 9 cycles with gradual reduction in decolorization efficiency ranging from 92 to 83.5 ± 1.5% with the mediator (veratryl alcohol) and 88 to 82 ± 0.5% without mediator for 1–9 cycles, respectively. In the first cycle, mediated decolorization efficiency was 92 ± 1.5% while non-mediated decolorization efficiency was 88 ± 0.5% was within 48 h. The subsequent cycles took less time period (24 h) to achieve the same rate of decolorization and decolorization cycle runs efficiently till the 7th cycle with the mediator and 5th cycle without mediator. From 8th cycle onwards the decolorization efficiency of organism gradually declined and required more time for decolorization of dye. The eventual cessation of decolorization may be obtained due to the nutrient depletion, excessive growth of fungal mycelium and waste accumulation in flasks (Saratale *et al.* 2006). Jadhav *et al.* (2009) showed six cycles of decolorization process by *Comamonas sp* UVS with more

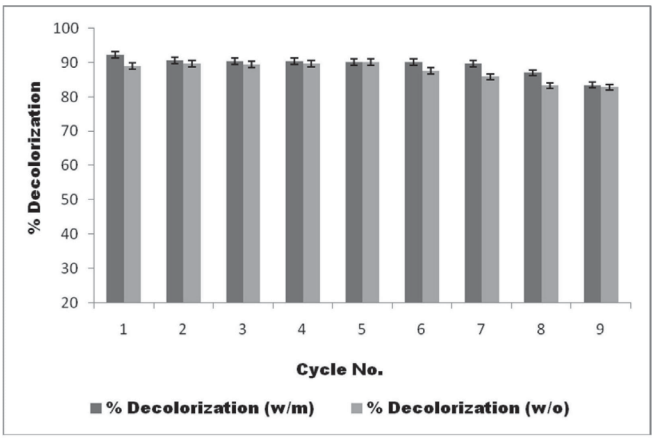

Fig. 4: Effect of repeated dye addition on decolorization of Reactive Magenta-HB dye.

than 70 % decolorization of direct blue GLL dye, whereas, Saratale *et al.* (2009) reported four cycles of Navy blue HER dye decolorization using *Trichosporon beigelii* fungal species.

In vitro decolorization study of various dyes in the presence and absence of mediator

In *in vitr*o decolorization study in the presence and absence of veratryl alcohol showed a variable response for different dyes. In case of Reactive Magenta-HB, the percent decolorization was increased by 1.36 fold in the presence of veratryl alcohol while percent decolorization remained similar in mediated and non-mediated decolorization of Reactive Violet 1. However, no in vitro decolorization was observed in case of Reactive Blue 3 R dye in the absence of veratryl alcohol (Table 3). *In vitro* decolorization was also evaluated by Trupkin *et al.* (2003) for various dyes by the addition of compounds such as veratryl alcohol and H_2O_2, HBT, phenol and tyrosine. Crude extracellular extracts of *Peniophora sp*. hpF-04 and *Phellinus sp.* hpF-17were also able to decolorize various dyes up to 60 % (Poojary H. *et al.* 2012).

Table 3: *In vitro* decolorization study of various dyes in the presence and absence of mediator

% Decolorization	Dyes		
	Reactive Magenta-HB	Reactive Violet 1	Reactive Blue- 3R
Without mediator	60%	25%	-
With mediator	82%	27%	17%

Analytical study of degradation of Reactive Magenta-HB dye

Fourier Transform Infrared (FTIR)

Fourier Transform Infrared (FTIR) Spectroscopy analysis was done for the original and the decolorized dye samples as shown in Fig. 5. The IR spectrum of the original dye showed various peaks. The peaks at 3439.22 cm^{-1}, 2930.32 cm^{-1} and 2856.55 cm^{-1} indicate the presence of hydroxyl groups (O-H stretching) and secondary amine salts due to N-H stretching, respectively. Peaks 1721.55 cm^{-1}, 1606.10 cm^{-1}, 1487.68 cm^{-1} and 1432.89 cm^{-1} may indicate the presence of C=O stretching of esters, N-O bond of Nitramine, C=S stretching and C-N stretching of thiocarbonyl group, respectively. It also showed the peaks at 1379.91 cm^{-1}, 1300.67 cm^{-1}, 1190.80 cm^{-1} and 1166.28 cm^{-1} which may indicate N-O stretching due to nitro compound and C-O stretching of esters (1300-1000 cm^{-1}), respectively. The peaks at 1045.29 may indicate the presence of C-H stretching of unsaturated tertiary or branched secondary alkanes, while 886.33 cm^{-1} and 829.31 cm^{-1} may indicate mononuclear aromatic hydrocarbons.

The FTIR of the degraded sample showed the peaks at 3415.29 cm^{-1}, 2959.01 cm^{-1}, 2110.65 cm^{-1} and 1638.50 indicating the presence of hydroxyl group (O-H stretching), dimer of carboxylic acids (O-H stretching), cumulative bond stretching due to thiocyanates and presence of nitrates or nitroso compounds, respectively. The peaks at 1456.44 cm^{-1} and 1404.99 cm^{-1}, indicates sulfonyl chlorides or sulfones due to S=O stretching. Various peaks at 1352.23 cm^{-1}, 1122.92 cm^{-1} and 1082.50 cm^{-1} may indicate symmetrical absorption of N-O bond of aromatic dimer (cis) nitro compounds, S=O stretching of sulfones (1160-1120 cm^{-1}) and C-O stretching of saturated secondary alcohol or phenol, respectively. A peak at 935.91 indicates the C-H bending of alkanes (650-1000 cm^{-1}). The result of the parent compound and the decolorized sample suggest the chemical structure of the dye is greatly influenced due to biological decolorization and degradation by Ganoderma cupreum AG-1.

High Performance Thin Layer Chromatography

High Performance Thin Layer Chromatography was done for the parental and the decolorized samples. The results of which showed various bands. Control sample showed a single band at R_f value 0.88 while experimental sample showed 3 bands at R_f value 0.82, 0.62, and 0.49. Formation of new bands indicates some transformation occurred in

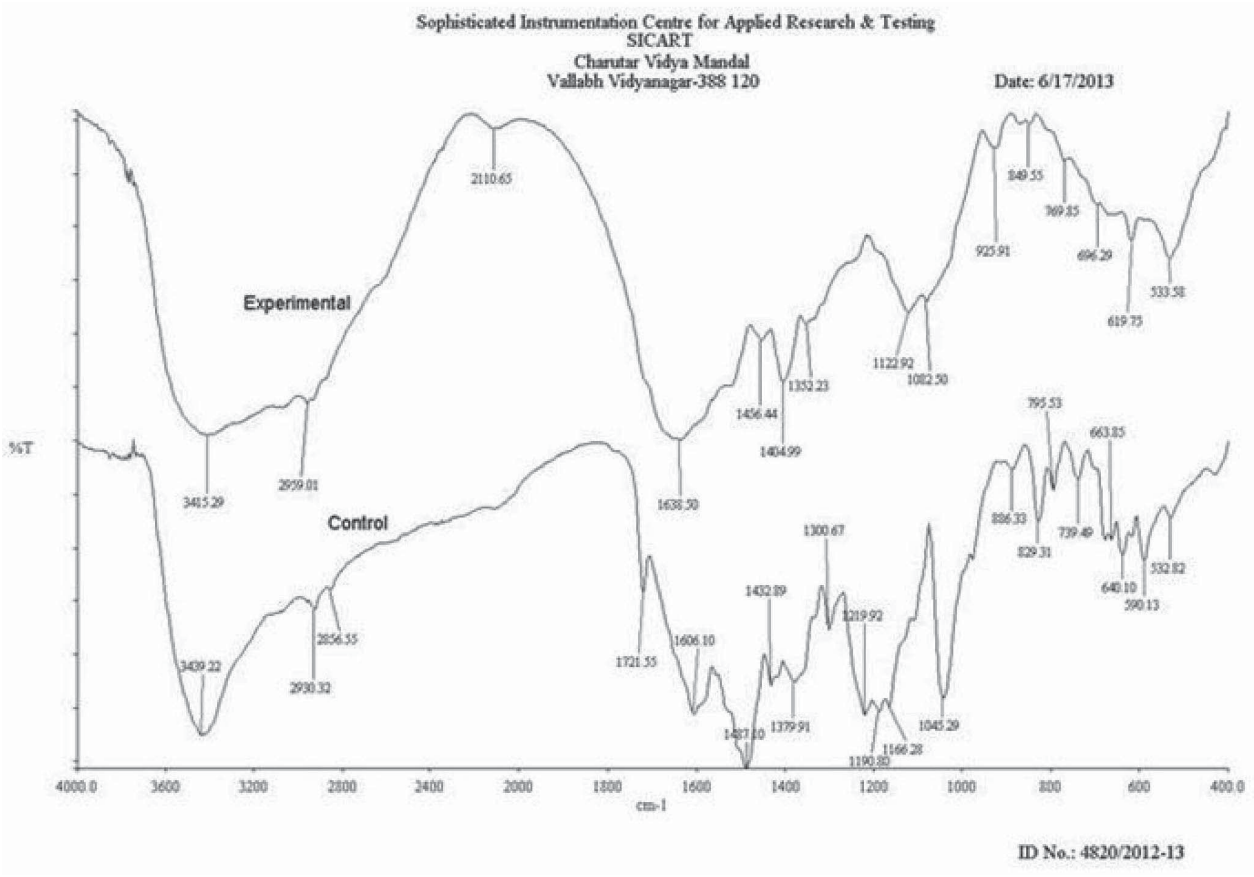

Fig. 5: FTIR spectral comparison of control Reactive Magenta-HB dye and degradation products.

the dye structure due to biological treatment using fungal growth. This can be studied in more detail using other analytical methods.

Conclusion

The study proposes the ability of *Ganoderma cupreum* AG-1 for decolorization of various dyes. The fungal isolate was found to decolorize 9 dyes within 65 – 94 % efficiency, suggesting its ability as a dye remediation organism. The overall study concludes optimization of physico-chemical parameters significantly increase the dye removal efficiency of the fungal culture. The metal ions and redox mediators show a positive influence on the dye decolorization. Thus, the fungi *Ganoderma cupreum* AG-1 can be exploited further for its various applications.

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