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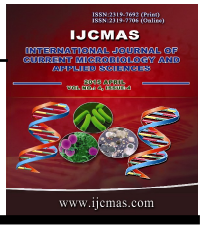

Original Research Article

Decolorisation and Detoxification of Widely Used Azo Dyes by Fungal Species Isolated from Textile Dye Contaminated Site

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Keywords

Aspergillus flavus, direct and reactive azo dyes degradation, fungi

A B S T R A C T

The fungal isolates (*Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporium* and *Penicillium notatum*) from textile dye contaminated site were screened for the first time in degradation and detoxification of widely used azo textile dyes (R Navy Blue M3R, R Red M8B, R Green HE4B, R Orange M2R, R RedM5B, Dt Orange RS, Dt Black BT, Dt Blue GLL and Dt Sky Blue FF). *Aspergillus flavus* exhibited consistently highest decolorization potential than other isolates. Complete removal of all the dyes under study was observed within three to seven days of incubation in agitated culture at 30^0C, pH 5, initial dye concentration of 40 mg/L. Moreover, the fungal biomass could be reused in weekly consecutive cycles to remove dyes and their mixture over a period of a year. Compared to control, in the presence of dyes, significant induction of the activity of biotransformation enzymes (lignin peroxidase>laccase>manganese peroxidase>tyrosinase) in the medium revealed their crucial role in the dye metabolism. The colorless nature of the fungal mycelia at the end of week indicated that decolorization occurred through degradation which was confirmed by significant decline in absorbance and disappearance of dye band in UV-visible spectrophotometric and HPTLC analysis respectively. The seed germination percentage and growth of seedlings of *Guizotia abbysinica* were more or less similar with water and decolorized dye sample which demonstrated non-toxic nature of the metabolites formed after degradation of dye. The results suggest that fungal isolate *A. flavus* has potential for cleaning up azo dyes containing effluents and development of user friendly, non-conventional, and low cost method of bioremediation.

Introduction

Today water pollution is one of the major global threats and the World Bank estimates that 17 to 20 percent of industrial water pollution comes from textile and other dyeing industry.

During synthesis and dyeing process, about 10-15% of dyes which is unused get added to the effluents (Sharma et al. 2009). Synthetic dyes are widely used in number of industries which include textile dyeing,

tanning, leather, paper printing, food, cosmetics with textile industry as the largest consumer (Kumar et al. 2014). However several such industries do not have adequate technology or facility to treat their effluents. So, huge volumes of partially treated and untreated textile effluents with suspended solids and toxic chemical compounds are released into the nearby water streams. This leads to adverse changes in physicochemical properties with high chemical oxygen demand (COD) and biological oxygen demand (BOD) (Casieri et al. 2008; Shedbalkar et al. 2008; Shedbalkar and Jadhav 2011).

The chemicals used in textiles that are in turn released in effluents even at very small concentrations, has mutagenic and carcinogenic effects on living organisms (Moorthi et al. 2007; Hu et al. 2009). The physical and chemical treatments are cost intensive, not highly effective and may have several disadvantages such as formation of toxic by-products leading to disposal problems (Robinson et al. 2001; Aubert and Schwitzguebel 2004).

In such situations, microorganisms play a very important role in the biodegradation, mineralization and detoxification of these dyes which is of great significance (Diaz 2004). There are several reports on isolation and application of microbial strains having the potential to decolorize a large variety of dyes belonging to different groups (Marimuthu et al. 2013).

However, dyes used in textile industry have a synthetic origin and complex aromatic molecular structures which make them stable and difficult to be biodegraded. It has been estimated that 80% of the commercial dyes used all over the world in textile industries are azo dyes (Prasad 2014).

Due to their intricate structure the azo dyes are poorly biodegradable and represent a potential class of organic pollutant. Most azo dyes unfortunately are recalcitrant to aerobic degradation by bacterial isolates (Junnarkar et al. 2006). However, the bacterial strains are capable to degrade some azo reactive dyes (Tan et al. 1999) but the process usually results in production of toxic, carcinogenic and/or mutagenic aromatic amines which may be more toxic than the parent azo dyes. (Hu and Wu 2001; Ambroosio and Campos-Takaki 2004).

The anaerobic bacterial degradation requires intracellular uptake of dyes, however, fungal organisms cause dye degradation via extracellular enzymes (Bagewadi et al. 2011). Fungal species are capable of metabolizing a wide range of compounds, particularly by demethylation and oxidation (Cha et al. 2001). In last few decades, there has been extensive research on the use of lignolytic fungal species for textile dye degradation but studies on non-ligninolytic fungi metabolizing dyes are minimal and are limited to few dyes (Blanquez et al. 2004; Gupta and Manisha 2012).

Against these background for the first time attempts have been made for degradation of nine azo dyes which are carcinogenic and mutagenic in nature by the four fungal isolates from textile dye effluent contaminated water samples and the responsible enzyme analysis of selected fungal species *Aspergillus flavus*. Furthermore, optimization of physico-chemical parameters to obtain maximum decolorization of azo dyes and confirmation of dye degradation using UV-Vis spectrophotometry and HPTLC method and the phytotoxicity of dyes and their resultant metabolites in degradation experiments was performed with crop plants.

Materials and Methods

Dyes and other chemicals

Dyes R Navy Blue M3R, R Red M8B, R Green HE4B, R Orange M2R, R RedM5B, Dt Orange RS, Dt Black BT, Dt Blue GLL and Dt Sky Blue FF were procured from Sheetal dye and pigment pvt Ltd at Thane, Maharashtra, India. ABTS (2,2-azino-bis, 3-ethylbenzothiazoline-6-sulfonic acid) was obtained from Sigma Aldrich. Tartaric acid, Dextrose and Agar were purchased from Himedia, India.

Isolation, establishment and identification of culture

The water samples from streams contaminated with textile dye effluents at Taloja MIDC area (N 19º 3' 59.1012'', E 73º 6' 34.3794'') in Maharashtra, India were collected in sterile bottles and brought to the Laboratory. These samples were inoculated using serial dilution and streak plate method on petri plates containing Potato Dextrose Agar (PDA) medium. The plates were incubated at 25 ± 2^0C and observed for fungal colonies. The inoculum from individual colonies was sub cultured and the pure cultures of fungal organisms were established and used for identification.

The pure isolated fungal strains were identified and the cultures of *Aspergillus flavus, Aspergillus niger, Fusarium oxysporium* and *Penicillium notatum* were deposited at Agarkar Research Institute, Pune, Maharashtra, India (Accession no: ARI/NFCCI/FIS/2013/Add Reg/ SI No. 6/1838). The strain of *Aspergillus flavus* was found to be promising for dye degradation and its identification was confirmed by Large Sub Unit (LSU) gene sequencing from geneOmbio, Baner, Pune. The individual cultures were maintained in PDA medium on subculture at 4 weeks interval over a period of 2 years.

Screening and selection of fungal strain

The suspension culture of *Aspergillus flavus, Aspergillus niger, Fusarium oxysporium* and *Penicillium notatum* mycelium along with spore was established in liquid Potato (20%) Dextrose (2%) medium. 10 ml volume of individual fungal strain (approx. 0.1gm) was inoculated in dye (40 mg/l) containing 200ml liquid medium in 300 ml capacity glass bottles and placed on an orbital agitator (120 rpm) at 25±2^0C. After every two days, absorbance was measured at λmax of each dye using UV-Vis spectrophotometer (Shimadzu) to determine the extent of dye degradation. The fungal isolate which was capable of complete degradation of dye in shorter duration was selected for the further optimaization of physico-chemical parameters for maximum dye degradation.

Dye decolorization analysis

The aliquots of control and treatment samples at two days interval were centrifuged at 10,000 rpm at room temperature for 15 min to remove the mycelium. The obtained supernatant was filtered using nylon filters of 0.45 µ pore size to remove the spores and any other particles.

The decolorization of the dyes by *Aspergillus flavus* was monitored by measuring the change in absorbance of the treatment supernatant against controls at the respective absorption maxima (λ max) of the dyes screened using UV–visible spectrophotometer (Shimadzu, Japan). Decolorization percentage of the respective dyes was calculated using the following equation:

% decrease = Absorbance of control- Absorbance at respective time of incubation X 100
in Absorbance of control

Optimization of Physical factors and dye decolorization

Degradation of nine azo textile dyes samples were optimized with the help of *Aspergillus flavus* under different physical parameters using the culture media constant ie Potato (20%) dextrose (2%) broth. The dye concentrations used for optimization of the degradation in the present investigation was between 20 to 100 mg/l. The optimization of pH was between 5 and 8. The incubation temperature for optimization was 20^0C, 25^0C, 30^0C and 35^0C. For optimization of light duration, the cultures were incubated in continuous dark, continuous light and under 12 hours of light and 12 hours of dark cycle. Light (40µmol m^{-2} s^{-1}) was provided using 40 watt fluorescent tube lights (Philips India Ltd.). The extent of degradation of the dye samples under different incubation condition were determined by measuring the absorbance at the respective absorption maxima of the dyes.

Continuous dye decolorization

To study the effect of repeated azo dye addition on the decolorization potential of *Aspergillus flavus*, the experiment was performed in 10 l glass jar with initial 200ml liquid medium containing 10 ml volume of individual fungal strain (approx. 0.1gm) and 40 mg/l dye. The aliquots were analyzed and within a week 100% of the dye was removed from the culture medium. Fresh medium (200ml) along with the dye was added at weekly interval for 4 weeks to the same culture. Every time the initial dye concentration was maintained at 40 mg/l (ie the dye added was 80 mg/l-after 1st week, 120 mg/l- after 2nd week and 160 mg/l- after 3rd week) and observed for the continuous removal of dye by the same culture of *Aspergillus flavus.*

The *Aspergillus flavus* biomass was tested for repeated use in decolorization of mixture of nine azo dyes over four cycles. The decolorization experiment was performed with the mixture containing 4.44 mg/l of each dye (R Navy Blue M3R, R Red M8B, R Green HE4B, R Orange M2R, R RedM5B, Dt Blue GLL, Dt Orange RS, Dt Black BT and Dt Sky Blue FF) in the medium. The dye mixture along with fresh medium was incorporated at weekly interval for four cycles. Every time the initial concentration of dye mixture was maintained at 40 mg/l (ie the mixture of dyes added was 80 mg/l-after 1st week, 120 mg/l- after 2nd week and 160 mg/l- after 3rd week) and observed for the continuous removal of dye by the same culture of *Aspergillus flavus.*

Determination of growth

The control and treatment samples along with fungal mycelium were centrifuged at 10,000 rpm for 10 min and the obtained residual mycelial mass was washed three times with sterile distilled water. Then the residual mycelial mass was placed in a fold of whatmann filter paper to remove the surface water and fresh weight (F.W) was recorded. The biomass was dried in an oven at 60^oC till constant weight and the dry weight (D.W) was recorded.

Enzyme assay

The filtrates obtained on centrifugation (10,000 rpm at 4^oC for 15 min) of aliquots were filtered using nylon filters with pore size of 0.45 µ and used as a source of enzymes. The activity of Lignin peroxidase (LiP) (EC 1.11.1.14), Laccase (Lac) (EC1.10.3.2), Tyrosinase (EC 1.14.18.1) and

Mn-dependent peroxidase (MnP) (EC 1.11.1.13) were assayed spectrophotometrically from filtered sample. Lignin peroxide activity was determined on oxidative conversion of veratryl alcohol to veratryl aldehyde (molar absorptivity ε 310=9300 M-1cm-1) at 310 nm as described by Kirk et al. (1990). Laccase activity was determined by measuring the oxidation of 2,2-azino-bis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) at 420 nm as described by Li et al. (2008). Tyrosinase activity was determined on enzymatic conversion of catechol to catechol quinone at 410 nm by following the Zhang and Flurkey (1997). Mn peroxidase activity was determined by oxidative conversion of ABTS at 420nm and Mn Peroxidase (MnP) activity was corrected for Mn independent peroxidase (MnIP) by subtracting MnIP activity obtained at pH 3.25 in absence of MnSO4 at 469 nm. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1µM of substrate per min at room temperature.

Analysis of dye transformation metabolites

The dyes and its metabolites in aliquots of control and treatment samples after centrifugation and filtration were extracted using equal volumes of ethylacetate and dried over anhydrous Na_2SO_4. The resulting residue was dissolved in small volume of HPLC grade methanol and used for analysis using High Performance Thin Layer Chromatography (HPTLC) (CAMAG, Switzerland) (Waghmode et al. 2012). The samples were loaded on pre-coated HPTLC plates (DC Kieselgel $60F_{254}$ silica gel plate, Merck, Germany), by spray gas nitrogen and TLC sample loading instrument (CAMAG Linomat V). Methanol: ethyl acetate in the proportion of 6:4 (v/v) was used as mobile phase. The developed TLC plate were visualized for dyes and their metabolites in UV chamber and scanned at 280 nm with slit dimension 5 X 0.45 mm by using TLC scanner (CAMAG). HPTLC software WinCATS 1.4.4.6337 was used for analysis of the results.

Phytotoxicity study

The obtained supernatant samples of control and with dye treatments from experiments with *Aspergillus flavus* cultures were filter sterilized and tested for their toxicity on germination of seeds of edible oil crop Niger (*Guizotia abbysinica Cass*) *CV IGP*-76 (obtained from MPKV Niger Research centre, Igatpuri, Nashik, India). The seeds were surface sterilized with 0.1% $HgCl_2$ for 4 mins then washed with sterile distilled water for 5 times. The surface sterilized seeds (32/ petri-plate) were arranged approximately at equal distance in disposable sterile petri plates. The filtered sterilized supernatant (10 ml) was added in petri plates everyday so that the seeds were partially suspended in supernatant. Initially the petri plates were placed in continuous dark for 48 hours and then subsequent 8 hours light (40 µmol $m^{-2}s^{-1}$light intensity) and 16 hour dark cycle for five days. Seed germination percentage, the shoot length and root length were measured after 7 days of treatment.

Experimental designs and statistical analysis

All experiments were organized using completely randomized designs (CRD) with minimum 40 replicates and were repeated at least thrice. Data were subjected to ANOVA and comparisons between the mean values of treatments were made by Duncan's multiple range test ($P< 0.05$) using SPSS Software version 9.

Result and Discussion

Screening and identification

The fungal strains *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporium* and *Penicillium notatum* were isolated from heavily contaminated textile dye effluent water bodies with the intension that these fungal organisms can have ability to grow and survive in the presence of textile dyes and that they could be a good candidate for textile dye degradation. All the four isolates tested showed relatively good mycelia growth in PDB medium containing 40 mg/l of dyes. They decolorized both reactive and direct azo dyes to variable extent in agitated culture (Table1). Among the tested isolates, *Aspergillus flavus* was the most superior as it completely decolorized most of the dye under study in 6 days. Ranking of the decolorization ability of the azo dyes under study by the isolated fungus strains is recorded in Table1 hereinafter in increasing ability order *Penicillium notatum*<*Fusarium oxysporium*<*Aspergillus niger*<*Aspergillus flavus*. In concurrence to this, Karthikeyan et al. (2012) reported that among 39 fungal isolates from soil samples previously polluted with textile dyes from textile hub of Tamilnadu, India, the *Aspergillus niger* HM11 was reported to be efficient to decolorize the Congo red and Reactive blue 140 dye.

Few other studies also emphasize that *Aspergillus flavus* decolorize Bromophenol blue and Congo red dye on Potato Dextrose Agar (PDA) medium (Singh and Singh 2010).Three isolates of *A.niger* from Egyptian soil decolorized two reactive dyes and reactive disperse dyes (Mohamed et al. 2014). The results of present investigation and the earlier reports confirm that the strains of *Aspergillus* are efficient for dye decolorization.

Optimization of Physical factors and dye decolorization

Optimization of culture (Physical) conditions is an important methodology which leads to cultural stability ultimately providing a favorable environment for fungal growth and maximum dye decolorization. The data depicted in table 2-7 showed that the various factors like concentration of dyes, pH and temperature, static and shaking conditions extensively affect the decolorization process. In the current study, the decolorization of both reactive and direct azo dyes was investigated with *Aspergillus flavus* using different concentrations of the dyes (20-100mg/l) (Table 2). On incorporation of lower dye concentration (20mg/l), 100% decolorization was observed within three days. Addition of increased concentration of dye (40 mg/l) also resulted in complete decolorization within 5 to 7 days after initiation of experiments. Dye concentration of 60 mg/l and above showed gradual inhibition of decolorization ability of the organism. In earlier reports, Banerjee and Chattopadhyaya (2013) suggested that the dye concentration affect the dye removal by number of factors including the toxicity of the dye at higher concentrations. In line with this, Bhimani et al. (2014) reported that the decrease in decolorization efficiency might be due to the toxic effect of dyes. According to Singh and Singh (2010) the strains of *Aspergillus flavus* showed maximum decolorization for Bromophenol Blue and Congo red at 1.0 % w/v (2010). The inhibition of dye decolorization at higher concentrations might be due to the inhibition of nucleic acid biosynthesis and the growth of fungus (Chen et al. 2003).

The hydrogen ion concentration is one of the significant factors influencing color removal, it might be at neutral, slightly

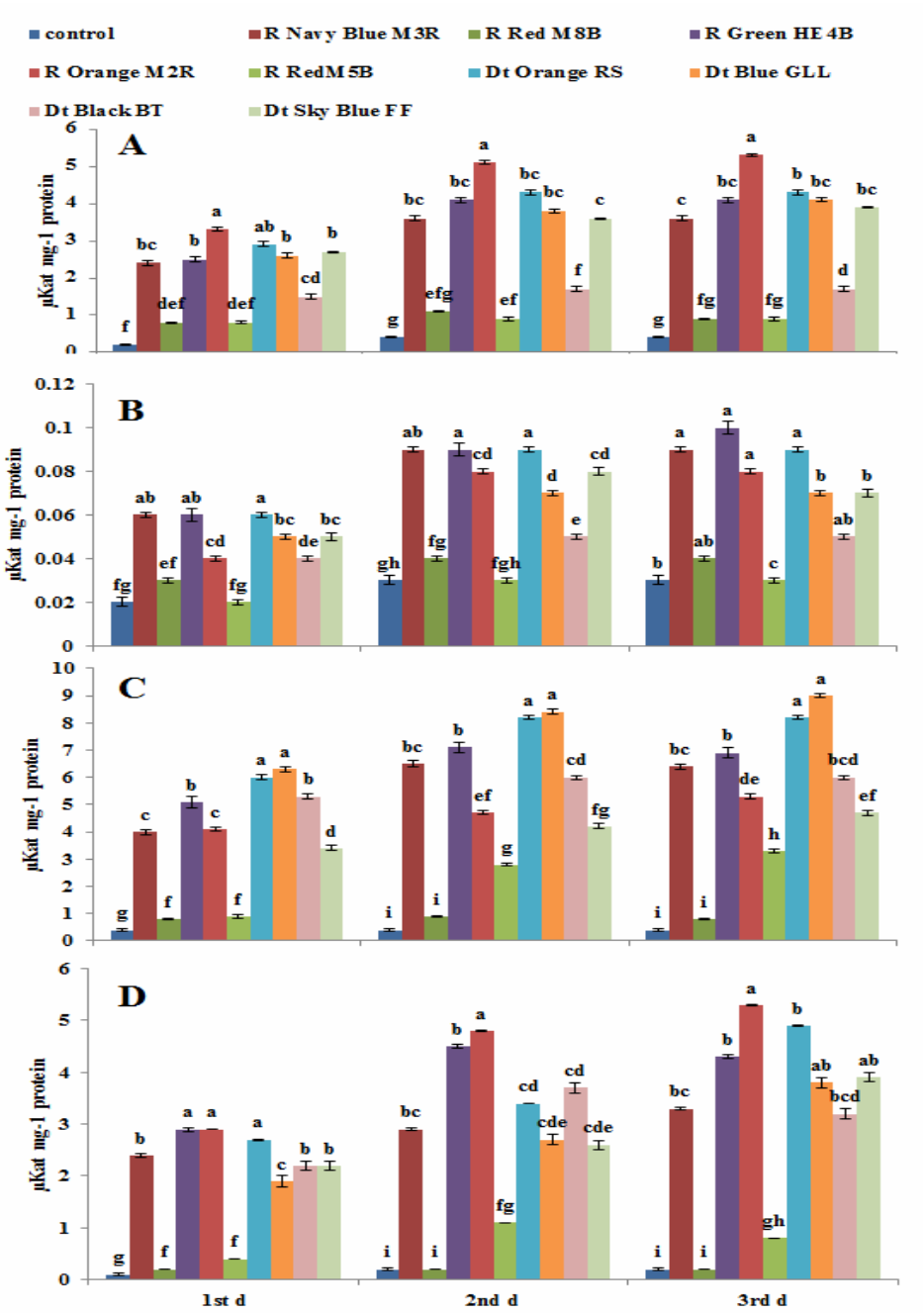
alkaline or acidic pH depending on the fungal species (Pearce et al. 2003). In the present investigation, the effect of initial pH (5 to 8) on decolorization of azo dyes (40 mg/l) by *A.flavus* after week duration is depicted in Table 3. The data clearly showed that the percentage removal of dye decreased with increase in pH of the medium. The maximum removal of dyes under study was found at pH 5 however the percentage of removal of color varies with the dye molecule under study. Further increase in the pH declined the percentage removal of dye by *A.flavus*. In consistent with our results, Radhika et al. (2014) reported that the fungal strain of *Pleurotus florida* LCJ65 and *P.ostreatus* LCJ183 showed maximum decolorization of dyes Bromophenol Blue and Brilliant Green at pH 5. Most of the white rot fungi decolorize dyes to maximum extent in pH range of 3–6 (Asgher et al. 2008). At extreme pH conditions, the dye removal efficiency of white rot fungus was hampered without any relationship to dye structure. (Young and Yu 1997)

In static culture, the growth of mycelium appeared at the end of the first week of incubation and the mass of mycelium become prominent during second and third week of incubation. There was no drastic change in the mycelium mat in the fourth week of incubation. The incorporation of dye R Green HE4B and Dt T Blue GLL in the medium promoted the growth by 13-20 % and 8-15.6% in terms of FW and DW over the control (Table 4). At the same time, there was exponential increase in the removal of color of both the dyes and 100% of decolorization appeared at the end of fourth week of incubation. However, inclusion of Dt. Orange RS, R. Red M8B, R. Orange M2R, R. Red M5B, Dt. Black BT and Dt. Sky Blue FF dye in the medium resulted in decrease in growth by 0-8.2% as compared to the control and the percent decolorization was in the range of 90-98.2 % at the end of the fourth week.

Compared to static culture, only a week duration was required for 100% decolorization of 40 mg/l of dyes in agitated culture. In control and treatment samples, pellet formation of inoculum mycelia was observed in agitated culture at the end of the first day which continued to increase in the subsequent days of culture. At the same time, the dye adsorbed on the surface of the mycelia and mycelial pellet gets the color of the respective dye which subsequently resulted in removal of color. The adsorbed dyes and dyes in the culture medium were completely decolorized within a week of incubation (Table 5). The growth of *A. flavus* in agitated culture was rapid in control as well as in dye containing medium and at the end of week it was in the range of 10.4-20.7 % higher compared to the growth observed in static culture at the end of fourth week. This is possible because *A.flavus* is obligate aerobe and as the nutrient and oxygen requirement for growth and maintenance of its viability was available readily in agitated culture (Swamy and Ramsay 1999). The fungi are aerobic organism; they show better degradation under agitated condition (Faison and Kirk 1985; Ge et al. 2004). In concurrent to this, there was only 45% decolorization of Orange II after 23 hours incubation in static condition. Whereas in shaking condition 97.5% of decolorization occurred using wood rotting fungus (Knapp et al. 1997).

Temperature is also a key environmental factor affecting the biodegradation of textile dyes (Vijaykumar et al. 2006). Therefore the experiments were performed in an incubator shaker set at constant temperature of 20, 25, 30 and 35^{o}C by keeping other conditions constant (dye concentration 40mg/L, pH 5).

Fig. 1 Influence of azo dyes on oxidoreducto enzyme activity of *Aspergillus flavus* in agitated culture after 7 days of incubation A) Mn peroxidase B) Tyrosinase C) Lignin peroxidase D) Laccase



The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT).

Fig. 2 Chromatogram of the dye and its metabolites produced after 7 days of incubation by *Aspergillus flavus* (A: R.Navy blue M2R, B: R.Red M8B, .C: R.Green HE4B D: R.Orange M2R, a- Pure dye (Control), b-derived Metabolite)

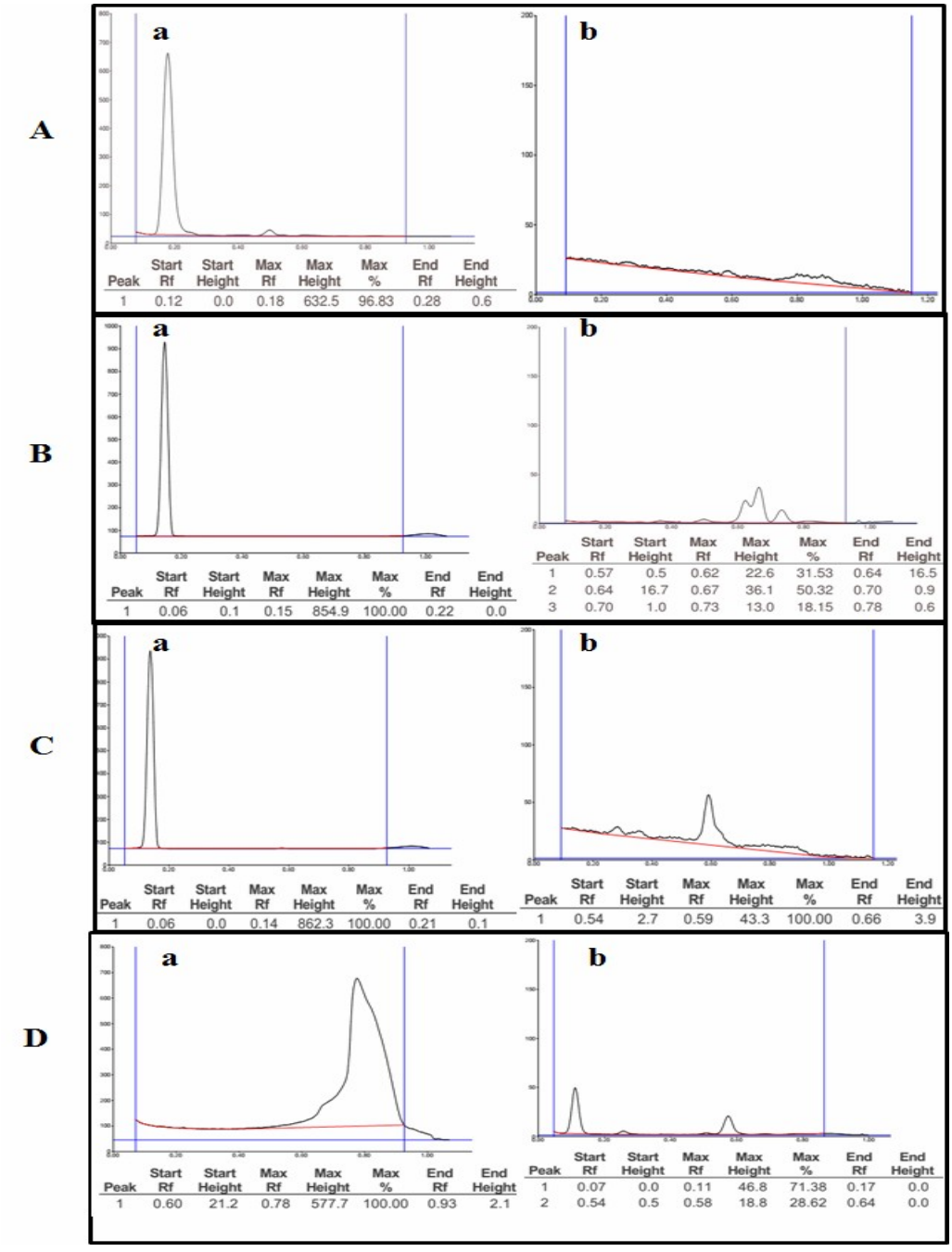


Fig. 3 Chromatogram of the dye and its metabolites produced after 7 days of incubation by *Aspergillus flavus* (E: R.Red M5B, F: Dt.Orange RS, G: Dt.T.Blue GLL, H: Dt Black B, I: Dt.Sky blue FF, a- Pure dye (Control), b- derived Metabolites)

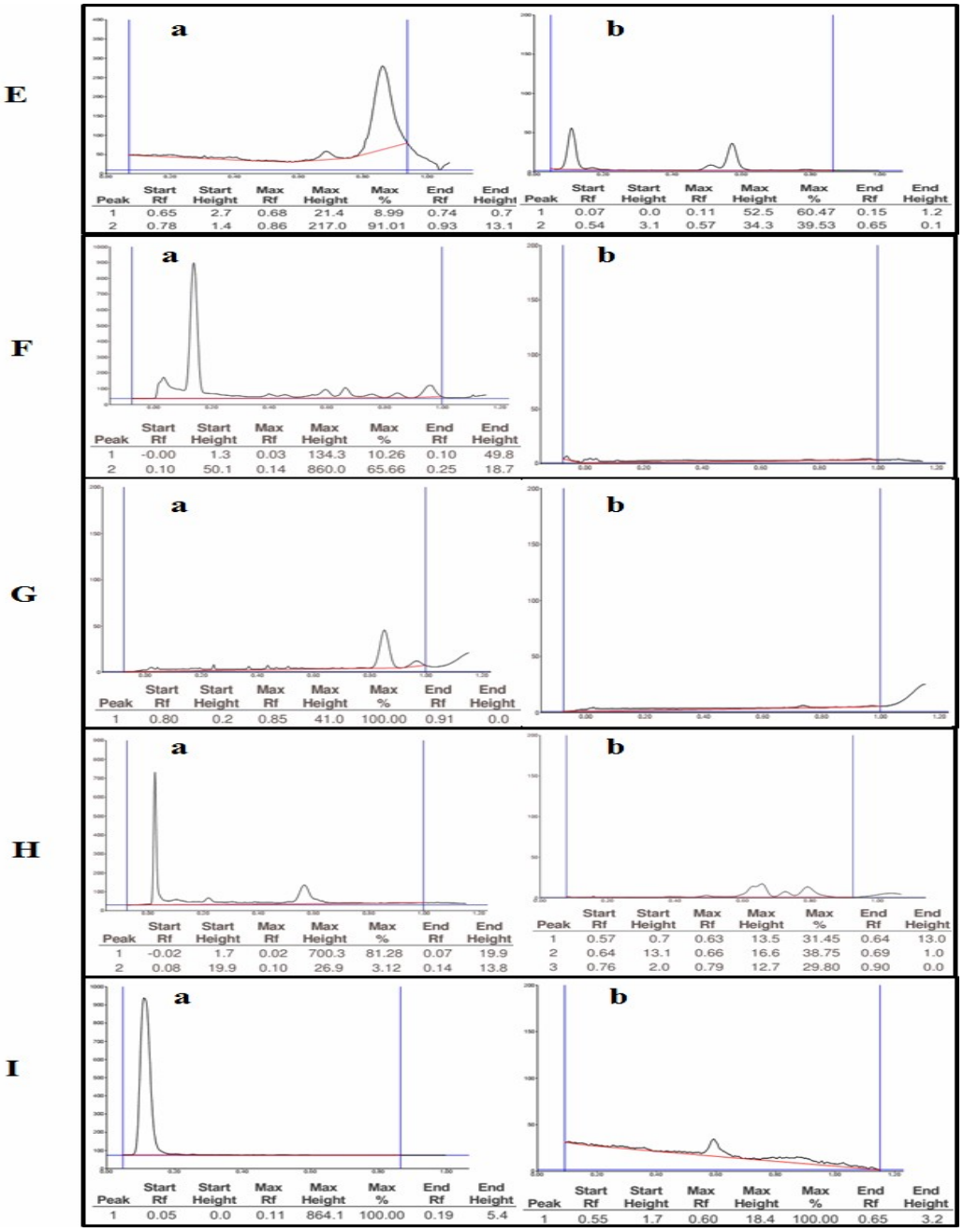


Fig.4 Repeated decolorization of azo dyes by *Aspergillus flavus* in four cycles of agitated culture

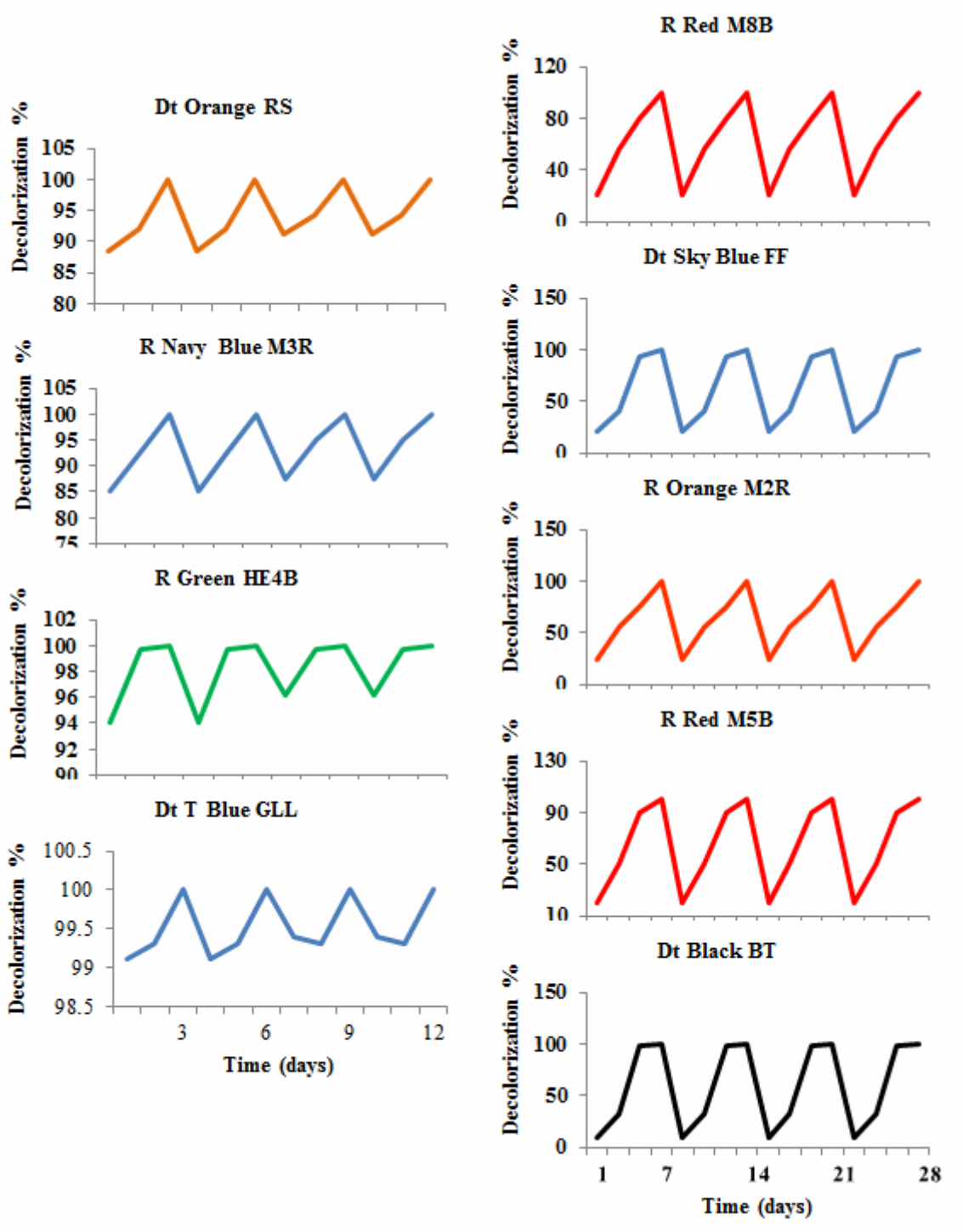


Table.1 Decolorization of Azo dyes by fungal strains isolated from textile dye contaminated industrial effluent

Azo Dye	% dye decolorization in 7 days using			
	Aspergillus flavus	*Aspergillus niger*	*Fusarium oxysporium*	*Penicillium notatum*
R Navy Blue M3R	100±0.0^{a}	70.3±1.2^{b}	68.0±1.7^{b}	65.0±1.2^{b}
R Red M8B	100±0.0^{a}	63.7±0.9^{c}	58.7±1.2^{c}	54.7±0.9^{cd}
R Green HE4B	100±0.0^{a}	40.3±1.2^{f}	37.7±0.9^{e}	36.0±1.5^{fg}
R Orange M2R	100±0.0^{a}	60.3±1.2^{d}	56.7±0.9^{c}	52.3±0.9^{d}
R Red M5B	100±0.0^{a}	42.7±0.3^{f}	39.0±1.2^{e}	37.7±0.9^{f}
R Yellow M4G	100±0.0^{a}	70.7±1.5^{b}	67.3±1.2^{b}	63.3±0.9^{b}
Dt Orange RS	100±0.0^{a}	56.0±2.1^{e}	50.3±1.2^{d}	47.3±0.7^{e}
Dt T Blue GLL	100±0.0^{a}	82.0±0.6^{a}	78.0±1.5^{a}	75.0±2.3^{a}
Dt Red 12 B	100±0.0^{a}	59.7±0.9^{d}	58.0±1.7^{c}	54.7±0.9^{cd}
Dt Sky Blue FF	100±0.0^{a}	34.0±1.2^{g}	31.3±0.3^{f}	33.0±0.6^{g}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT).

Table 2 Effect of initial dye concentration on decolorization of azo dyes after 7 days of incubation by *Aspergillus flavus*

Dye	% decolorization of dye at concentration of				
	20 (mg/L)	40 (mg/L)	60 (mg/L)	80 (mg/L)	100 (mg/L)
R Navy Blue M3R	100±0.0^{a}	100±0.0^{a}	90.5±0.6^{a}	83.3±0.6^{bc}	73.1±0.9^{c}
R Red M8B	100±0.0^{a}	100±0.0^{a}	73.7±0.9^{d}	70.5±0.5^{e}	58.7±1.0^{f}
R Green HE4B	100±0.0^{a}	100±0.0^{a}	90.5±0.5^{a}	85.6±0.6^{b}	76.7±0.5^{b}
R Orange M2R	100±0.0^{a}	100±0.0^{a}	89.5±0.5^{ab}	82.6±0.7^{bc}	71.6±0.6^{cd}
R RedM5B	100±0.0^{a}	100±0.0^{a}	73.4±0.5^{d}	69.4±0.6^{e}	44.7±0.6^{h}
Dt Orange RS	100±0.0^{a}	100±0.0^{a}	90.6±0.5^{a}	84.5±0.7^{ab}	77.6±0.8^{ab}
Dt Blue GLL	100±0.0^{a}	100±0.0^{a}	90.6±0.6^{a}	86.4±0.7^{a}	78.7±0.6^{a}
Dt Black BT	100±0.0^{a}	100±0.0^{a}	87.6±0.5^{c}	81.5±0.7^{cd}	61.6±0.7^{e}
Dt Sky Blue FF	100±0.0^{a}	100±0.0^{a}	88.7±0.4^{bc}	83.3±0.8^{bc}	64.3±0.6^{e}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT).

Table 3 Influence of initial medium pH on decolorization of azo dyes after 7 days of incubation by *Aspergillus flavus*.

Dyes	% Decolorization of dye at pH			
	5	6	7	8
R Navy Blue M3R	100±0.0^{a}	96.0±0.6^{a}	92.0±0.6^{a}	90.3±0.9^{c}
R Red M8B	100±0.0^{a}	77.7±1.5^{e}	74.7±0.9^{e}	73.0±1.2^{d}
R Green HE4B	100±0.0^{a}	95.0±0.6^{a}	92.0±0.6^{a}	90.0±0.6^{c}
R Orange M2R	100±0.0^{a}	96.0±0.6^{a}	92.7±1.2^{a}	89.3±0.3^{c}
R RedM5B	100±0.0^{a}	78.7±1.9^{e}	76.0±1.7^{e}	73.7±1.5^{d}
Dt Orange RS	100±0.0^{a}	95.0±0.6^{ab}	93.0±0.6^{ab}	90.7±0.9^{a}
Dt Blue GLL	100±0.0^{a}	94.0±0.6^{bc}	91.7±0.3^{ab}	90.7±0.3^{ab}
Dt Black BT	100±0.0^{a}	91.7±0.3^{cd}	89.0±1.2^{cd}	87.0±1.5^{b}
Dt Sky Blue FF	100±0.0^{a}	93.0±0.6^{bc}	90.0±0.6^{bc}	88.3±0.9^{b}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT). Medium used for the experiment was PDB medium.

Table 4 Growth of *Aspergillus flavus* and dye decolorization in static culture (F.W and D.W were monitored at the end of fourth week of incubation and dye decolorization at weekly interval)

Treatment	FW (gms)	DW (gms)	% Decolorization of dye at the end of			
Control	8.13±0.17^{c}	1.40±0.02^{c}	1st week	2nd week	3rd week	4th week
R Red M8B	3.95±0.09^{h}	0.55±0.03^{g}	22.4±1.3^{bc}	56.5±0.7^{a}	76.3±1.2^{b}	90.3±1.3^{c}
Dt Sky Blue FF	4.45±0.19^{g}	0.52±0.02^{g}	22.5±1.7^{bc}	41.5±1.7^{d}	71.3±1.0^{c}	91.8±1.0^{c}
R Orange M2R	5.28±0.09^{f}	0.59±0.01^{g}	26.4±1.8^{ab}	54.4±1.0^{ab}	62.4±1.3^{de}	92.4±1.1^{c}
R Red M5B	6.23±0.08^{e}	0.81±0.05^{f}	21.7±1.9^{cd}	49.4±2.3^{c}	55.2±1.2^{f}	93.4±1.3^{bc}
Dt Black BT	7.07±0.12^{d}	1.15±0.01^{e}	10.5±1.2^{f}	32.6±1.7^{e}	74.6±1.2^{bc}	97.3±1.3^{a}
Dt Orange RS	8.05±0.19^{c}	1.24±0.06^{d}	27.5±1.9^{a}	56.5±0.9^{a}	34.6±1.1^{g}	97.4±1.1^{a}
R Navy Blue M3R	8.13±0.17^{c}	1.40±0.02^{de}	18.5±0.6^{cde}	22.4±1.8^{f}	84.8±0.8^{a}	98.2±1.1^{a}
R Green HE4B	9.39±0.03^{b}	1.53±0.07^{b}	30.3±1.6^{a}	57.8±1.5^{a}	82.4±1.2^{a}	100±0.0^{a}
Dt T Blue GLL	10.21±0.09^{a}	1.66±0.08^{a}	28.4±1.0^{a}	57.3±0.6^{a}	64.3±1.2^{d}	100±0.0^{a}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT).

Table 5 Growth of *Aspergillus flavus* and dye decolorization in agitated culture (F.W and D.W was monitored at the end of a week of incubation and dye decolorization at the end of each day)

Dye	FW (gms)	DW (gms)	% Decolorization of dye at the end of						
Control	10.8±0.7^{a}	2.1±0.02^{b}	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
R Red M8B	6.8±0.3^{f}	0.9±0.04^{g}	20.5±1.0^{c}	22.6±1.3^{c}	56.4±1.7^{b}	76.2±1.7^{d}	80.0±2.9^{d}	93.4±1.7^{c}	100±0.0^{a}
Dt Sky Blue FF	7.0±0.1^{ef}	1.1±0.03^{f}	21.5±1.2^{c}	22.4±2.4^{c}	41.3±1.5^{d}	91.2±1.2^{b}	93.5±1.8^{bc}	100±0^{a}	100±0.0^{a}
R Orange M2R	7.7±0.1^{de}	0.9±0.03^{g}	24.4±2.3^{c}	26.2±1.8^{c}	54.9±2.2^{b}	55.3±1.8^{e}	74.5±1.9^{e}	96.2±1.7^{b}	100±0.0^{a}
R Red M5B	8.4±0.1^{cd}	1.3±0.02^{e}	19.6±1.2^{c}	21.5±1.8^{c}	49.8±1.9^{c}	84.7±2.1^{c}	89.6±2.2^{c}	97.6±1.3^{ab}	100±0.0^{a}
Dt Black BT	8.6±0.2^{c}	1.5±0.03^{d}	9.5±0.8^{d}	10.5±1.1^{d}	32.3±1.1^{e}	90.2±2.3^{b}	97.4±1.6^{ab}	100±0^{a}	100±0.0^{a}
Dt Orange RS	8.7±0.2^{c}	1.4±0.01^{e}	88.2±2.3^{b}	92.0±1.7^{b}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0.0^{a}
R Navy Blue M3R	9.5±0.3^{b}	1.8±0.03^{c}	85.2±1.8^{b}	92.6±1.3^{b}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0.0^{a}
R Green HE4B	11.4±0.2^{a}	2.3±0.03^{a}	94.2±1.8^{a}	99.6±0.3^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0.0^{a}
Dt T Blue GLL	11.5±0.1^{a}	2.2±0.03^{b}	96.8±1.9^{a}	99.4±0.3^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0.0^{a}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT).

Table 6 Influence of temperature on decolorization of azo dyes after 7 days of incubation by *Aspergillus flavus*

Dyes	% decolorization of dye at temperature			
	20^{o}C	25^{o}C	30^{o}C	35^{o}C
R Navy Blue M3R	87.0±0.6^{abc}	90.3±0.9^{a}	100±0.0^{a}	96.0±0.6^{a}
R Red M8B	70.7±0.3^{f}	73.0±1.2^{d}	100±0.0^{a}	77.7±1.5^{d}
R Green HE4B	86.3±0.3^{bcd}	90.0±0.6^{ab}	100±0.0^{a}	95.0±0.6^{a}
R Orange M2R	86.0±0.6^{cd}	89.3±0.3^{ab}	100±0.0^{a}	96.0±0.6^{a}
R RedM5B	71.3±0.9^{f}	73.7±1.5^{d}	100±0.0^{a}	78.7±1.9^{d}
Dt Orange RS	89.3±1.2^{a}	90.7±0.9^{a}	100±0.0^{a}	95.0±0.6^{a}
Dt Blue GLL	89.0±0.6^{ab}	90.7±0.3^{a}	100±0.0^{a}	94.0±0.6^{ab}
Dt Black BT	84.0±1.0^{de}	87.0±1.5^{bc}	100±0.0^{a}	91.7±0.3^{bc}
Dt Sky Blue FF	86.3±0.9^{bcd}	88.3±0.9^{abc}	100±0.0^{a}	93.0±0.6^{abc}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT).

Table 7 Phytotoxicity of azo dyes and their extracted metabolites produced on degradation by *A.flavus* on seed germination and growth of *Triticum aestivum*

Azo dyes	Germination %		Plumule length (cm)		Radicle length (cm)		Protein (mg/g)		carbohydrate (mg/g)	
	Dye*	Dye metabolite**	Dye*	Dye metabolite**	Dye*	Dye metabolite**	Dye*	Dye metabolite**	Dye*	Dye metabolite**
Control	98.3±0.7^{a}	98.3±0.7^{b}	8.4±0.2^{a}	8.4±0.2^{b}	4.4±0.1^{a}	4.4±0.1^{b}	34.4±0.4^{ab}	34.4±0.4^{a}	72.1±0.7^{a}	72.1±0.7^{a}
R Navy Blue M3R	54.5±0.9^{c}	97.4±0.7^{b}	3.1±0.3^{b}	8.3±0.2^{b}	2.3±0.1^{bc}	4.3±0.1^{b}	18.3±0.3^{c}	33.3±0.2^{b}	37.5.3±0.4^{b}	71.8±0.4^{b}
R Red M8B	39.2±1.2^{g}	95.2±0.6^{c}	1.2±0.1^{e}	8.3±0.3^{b}	1.1±0.1^{g}	4.3±0.2^{b}	13.4±0.3^{f}	33.1±0.4^{b}	18.3±0.2^{c}	70.1±0.1^{b}
R Green HE4B	61.2±1.2^{b}	99.4±0.6^{ab}	3.2±0.1^{b}	8.6±0.3^{a}	1.8±0.1^{bcd}	4.6±0.1^{a}	20.5±0.2^{b}	34.3±0.5^{a}	31.5±0.3^{a}	69.2±0.2^{a}
R Orange M2R	58.8±0.9^{b}	97.4±0.4^{b}	2.9±0.1^{c}	8.3±0.4^{b}	1.5±0.1^{bcd}	4.4±0.1^{b}	17.3±0.6^{d}	33.7±0.4^{b}	28.1±0.3^{b}	71.5±0.4^{b}
R RedM5B	31.4±0.9^{h}	97.6±0.4^{b}	1.4±0.2^{e}	8.4±0.5^{b}	1.7±0.3^{ef}	4.3±0.1^{b}	13.0±0.4^{f}	33.1±0.1^{b}	17.3±0.3^{c}	70.2±0.1^{b}
Dt Orange RS	58.3±0.6^{b}	97.0±0.6^{b}	3.4±0.6^{b}	8.4±0.5^{b}	2.0±0.3^{b}	4.4±0.1^{b}	18.3±0.2^{c}	33.2±0.5^{b}	29.4±0.3^{b}	72.1±0.3^{a}
Dt Blue GLL	53.6±0.9^{cd}	99.6±0.3^{a}	3.0±0.3^{c}	8.6±0.2^{a}	1.3±0.2^{ef}	4.6±0.2^{a}	21.1±0.1^{b}	34.2±0.2^{a}	32.4±0.4^{b}	71.5±0.4^{b}
Dt Black BT	50.5±1.2^{ef}	96.3±1.1^{c}	2.0±0.5^{d}	8.4±0.3^{b}	1.7±0.7^{bcd}	4.4±0.4^{b}	17.2±0.4^{de}	33.2±0.5^{b}	27.4±0.2^{c}	70.5±0.6^{b}
Dt Sky Blue FF	51.5±0.9^{de}	97.8±0.3^{b}	2.8±0.4^{cd}	8.3±0.3^{b}	1.4±0.8^{de}	4.3±0.3^{b}	18.4±0.2^{c}	33.1±0.3^{b}	28.1±0.1^{b}	69.8±0.4^{cb}

The values represent the mean ± SE. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT)

*= 40 mg/l ,**= metabolites produced from 40 mg/l dye after 7 days of incubation by *Aspergillus flavus*

As shown in Table 6, the efficiency of decolorization was enhanced with rising temperature upto 30^0C suggesting that biosorption of dyes by *A.flavus* was an endothermic process. While higher temperature (35^oC) was inhibitory for both growth of *A.flavus* and dye decolorization (Table 6). The finding is in agreement with Sharma et al. (2009) that the degradation of orange II dye by *Phanerochaete chrysosporium* was optimum between 25 and 30°C. But optimum growth of *Irpex lacteus* and decolorization of reactive Levafix Blue E-RA dye was found at 35^0C (Kalpana et al. 2012). Thus temperature plays vital role in the growth of fungus and the dye degradation which may vary with the species.

The experiment performed in continuous light, continuous dark and with exposure to 12 hours of light and 12 hours of dark cycle showed no distinct variation in percentage of dye decolorization (Table 7). The results revealed that light is not playing any role for dye degradation in the presence of fungus *A.flavus.* This might be possible as *A.flavus* is heterotrophic organism and the dyes under study may be photoresistent. Such photo resistant dye creates potential risk of bioaccumulation and respectively serious threats for human health through the food chain transport (Stoyanova and Christoskova 2011). *A flavus* was able to remove all the dyes under study within three to seven days of incubation in agitated culture at 30^0C, pH 5, initial dye concentration of 40 mg/L. Thus, here the role of *Aspergillus flavus* in decolorization of nine photo resistant azo dyes is significant.

Effect of azo dyes on oxidoreducto enzymes in agitated culture

The mechanism of mycoremediation of textile dyes predominantly involves extracellular biophysical and biochemical processes like adsorption, precipitation, entrapment in inner spaces of fungal mycelium, increased cell-to-surface ratio, ion exchange due to surface ionization, by formation of hydrogen bonds, transformation and mineralization by enzymes (Park et al. 2006). In the present study, adsorption of dyes was observed on pellets followed by its concurrent decolorization/degradation by the extracellular secretion of enzymes. The mycelium observed under light microscope (1500 x) showed the dye absorption. It revealed that the *A. flavus* decolorize the synthetic dyes by both extracellular and intra cellular metabolism. Concurrent to this, Kashik and Malik (2009) reported that the degradation of Congo red and crystal violet by the soil fungi with their mycelia by adsorption/ absorption. As well some species demonstrate both enzyme-mediated degradation and biosorption in the decolorization of textile dyes (Park et al. 2007).

The first attempts on textile dye degradation initiated with white rot fungus *Phanerochate chrysosporium* which were characterized mainly with ligninolytic peroxidases called lignin peroxidase (LiP) (Glenn et al. 1983) and manganese peroxidase (MnP) (Kuwahara et al. 1984; Wariishi et al. 1988). Several ligninolytic fungi are well-known to degrade textile dyes by producing the extracellular enzymes laccase, tyrosinase, lignin peroxidase, Manganese- dependent Peroxidase. Very recently, few non ligininolytic fungi have also been reported to produce extracellular enzymes involved in dye degradation (Ademola and Ogunjobi 2012). In the present investigation, the results on activity of enzyme lignin peroxidase, laccase, MnP and tyrosinase detected in the cultures of non ligninolytic fungus *A. flavus* grown in the medium

lacking dye and containing the reactive and direct azo dyes are depicted in Fig.1. The data revealed that at the end of first day and at the end of every day for a week of incubation the activity of enzymes remained more or less similar in control. However, in presence of dyes pronounced increase in the enzyme activity of lignin peroxidase, laccase, MnP and tyrosinase in the medium was observed by 52, 87, 29, 30, and 37 %, respectively over the control indicating their possible role in the degradation of azo dyes (Fig. 1). The activity of enzymes Lignin peroxidase and Laccase was higher to that of Tyrosinase and MnP, it suggests that compared to others lignin peroxidase and laccase may be more responsible for dye degradation. The ranking of 100 % decolorization of dyes in decreasing order in the duration of 3-7 days observed was R Navy Blue M2R >R Magenta MB>R Orange M2R>R Yellow M4G>Dt Orange RS> Dt Blue GLL>R Green He4B> Dt Black BT> Dt Sky Blue ff. This variation in time duration of decolorization might be due to the interaction between enzymes and functional groups present in the dye structure.

Degradation product analysis

HPTLC spectral comparison between control and dye degradation experiments were carried out in addition to spectrophotometric analysis to confirm the biotransformation of the dye into different metabolites (Fig. 2, 3). The HPTLC chromatogram showed the absence of control dye band in the samples of dye degradation metabolites lane, which indicates their complete transformation. The two dimensional graph of Rf value versus absorbance generated after scanning of HPTLC plate at 280 nm showed a prominent single peak for dye in control. There was absence of peak corresponding to dye and appearance of different one to three peaks indicates partial degradation of dyes or there were no peak observed depending on the type of reactive and direct azo dye suggest complete mineralization of dyes (Fig.2, 3,4,5). Similarly Chaube et al. (2010) reported different Rf values of the dye metabolites from that of dye. In line with this, difference in Rf value of the control dye (0.88) and the formed metabolite (0.60, 0.65, 0.74, and 0.82) after treatment with *Galactomyces geotrichum* MTCC 1360 indicate the biodegradation of Remazol Red (Waghmode et al. 2011).In line with this, different Rf values of the dye metabolites was reported from dye Green HE4B by *Pseudomonas* sp. LBC1 (Joshi et al. 2013).

Continuous dye decolorization

The potential of microorganisms for continuous removal of textile dyes has been considered as an important criterion in the selection of capable species for biodegradation and from commercial point of view. This study was carried out in 10 l glass bottle to examine the ability of *A. flavus* to decolorize repeated addition of azo dyes (40 mg/L) and their mixture at weekly interval under agitated condition. Along with dyes, 200 ml fresh medium was also incorporated to fulfill the nutrient requirement of fungus. The isolate had an ability to decolorize 100% individual dye and their mixture over a period of four weeks as shown in Fig. 4 and after that the inoculum from harvested biomass in subsequent cycle also showed continuous decolorization over a period of one year. In addition, the growth of isolate was not affected in dye lacking, individual dye and dye mixture containing medium. Since the dyes are present in the effluent in mixture, therefore the results were interesting that the *A.flavus* decolorize the nine azo dyes mixture simultaneously. The significant

differences were not observed in activity of enzymes with repeated addition of individual azo dyes (40 mg/L) and their mixture at weekly interval under agitated condition (data not shown). In fact the individual dyes R Navy Blue M3R and Dt Blue GLL showed the promotive effect on growth and biomass production of fungus. On incorporation of dyes in mixture, there was also promotion in growth of *Aspergillus flavus* that may be attributed to either promotive effect or utilization of dyes R Navy Blue M3R and Dt Blue GLL and their metabolites for growth. Thus *A flavus* showed the ability to decolourize repeated incorporation of azo dyes and their mixture which is significant for its commercial applications in development of continuous dye removal system. Gahlout et al. (2013) reported that *G. cupreum* AG-1 was able to decolorize RV 1 dye with gradual reduction in decolorization efficiency ranging from 96 to 70 % for 1–11 cycles. In consecutive four cycles the decolorization of Navy blue HER dye by *Trichosporon beigelii* was possible (Saratale et al. 2009) and suggested that, the gradual decline of decolorization may be obtained due to the nutrient depletion in system flasks (Saratale et al. 2006 , Gahlout et al. 2013).

Phytotoxicity

The different metabolites formed from parent dye after degradation is reported to be of toxic or non toxic nature. Moreover, things get worse when the dye contaminated water reaches agricultural fields or water streams (Isik and Sponza 2004; Przytas et al. 2013). For practical applications, the treated textile dye solutions along with newly formed compounds must be of non toxic nature (Puvaneswari et al. 2006; Franciscon et al. 2012). In this study, phytotoxicity of samples were tested using seed germination and plant growth bioassays in seeds of edible oil crop Niger (*Guizotia abbysinica Cass*) *CV IGP*-76. The % germination, plumule and radicle length as well as the protein and total carbohydrate content drastically reduced in the presence of pure dyes indicating that pure dyes were toxic in nature. However, in the *A.flavus* dye decolorized samples, the % germination, plumule and radicle length as well as the protein and total carbohydrate content were not significantly different from that of the control (Table 7). The metabolites produced from dye R Navy Blue M2R and Dt Blue Gll showed the growth promoting effect in terms of increased length of plumule and radicle .These results reveal that metabolites of the dye produced in the presence of *A.flavus* were found to be nontoxic to the growth of the plants. In line with our finding, the phytotoxicity studies on *Triticum aestivum* and *Ervum lens* Linn. revealed high germination (%) and significant growth in shoots and roots of both the crop plants grown in decolorized dye (malachite green) samples obtained with fungus *Penicillium ochrochloron* (Shedbalkar and Jadhav 2011). The toxicity of post-degradation compounds derived from dye reactive Levafix Blue E-RA by *Irpex lacteus* fungi on seeds of *V. radiata and B. juncea* was reduced as compared to the pure dyes (Kalpana et al. 2012). Similarly, Kalyani et al. (2008) reported that Sorghum vulgare and Phaseolus mungo showed good germination rate as well as significant growth in the plumule and radical of both plants in the Red BLI metabolites extracted after decolorization as compared to dye sample.

The importance of extensive growth, biosorption and high levels of enzyme production during textile dye degradation should be taken into account when bioremediation strategies are evaluated. At

the same time, it is also important that the resultant products of dye degradation should be non toxic. The present study demonstrates that the single fungal strain *Aspergillus flavus* has an efficient potential to completely decolorize and degrade the variety of recalcitrant azo dyes and their mixture over a period of one year. The information about the optimum environmental factors/ conditions, significant levels of extracellular ligninolytic enzymes in liquid media especially lignin peroxidase and laccase can be useful for employing this fungal organism to develop system to clean up the water polluted by azo dyes discharged from textile and dyeing industry. Spectrophotometric and HPTLC studies confirmed the dye degradation and formation of new compounds. The phytotoxicity study suggested the non toxic and growth promoting nature of the metabolites of toxic azo dye degradation by *A.flavus*. Thus *A flavus* from textile industry contaminated effluent was found to cover wide range of recalcitrant azo dye degradation and could be a better choice because of its nature of degradation.

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