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Differential fate of metabolism of a sulfonated azo dye Remazol Orange 3R by plants *Aster amellus* Linn., *Glandularia pulchella* (Sweet) Tronc. and their consortium

Akhil N. Kabra[a], Rahul V. Khandare[a], Tatoba R. Waghmode[b], Sanjay P. Govindwar[b,*]

[a] *Department of Biotechnology, Shivaji University, Kolhapur, India*
[b] *Department of Biochemistry, Shivaji University, Kolhapur, India*

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ABSTRACT

Plant consortium-AG of *Aster amellus* Linn. and *Glandularia pulchella* (Sweet) Tronc. showed complete decolorization of a dye Remazol Orange 3R in 36 h, while individually *A. amellus* and *G. pulchella* took 72 and 96 h respectively. Individually *A. amellus* showed induction in the activities of enzymes veratryl alcohol oxidase and DCIP reductase after degradation of the dye while *G. pulchella* showed induction of laccase and tyrosinase, indicating their involvement in the dye metabolism. Consortium-AG showed induction in the activities of lignin peroxidase, veratryl alcohol oxidase, laccase, tyrosinase and DCIP reductase. Two different sets of induced enzymes from *A. amellus* and *G. pulchella* work together in consortium-AG resulting in faster degradation of the dye. The degradation of the dye into different metabolites was confirmed using High Performance Liquid Chromatography and Fourier Transform Infra Red Spectroscopy. Gas Chromatography Mass Spectroscopy analysis identified four metabolites of dye degradation by *A. amellus* as acetamide, benzene, naphthalene and 3-diazenylnaphthalene-2-sulfonic acid, four metabolites by *G. pulchella* as acetamide, 3-diazenyl-4-hydroxynaphthalene-2-sulfonic acid, naphthalen-1-ol and (ethylsulfonyl)benzene, while two metabolites by consortium-AG as 2-(phenylsulfonyl)ethanol and N-(naphthalen-2-yl)acetamide. The non-toxic nature of the metabolites of Remazol Orange 3R degradation was revealed by phytotoxicity studies.

1. Introduction

Population growth and urbanization together with parallel global industrialization have placed major pressure on the environment, potentially threatening the environmental sustainability. This has resulted in the buildup of chemical and biological pollutants throughout the biosphere causing threat to the environment and human health. Synthetic dyes and dye stuffs are one of the major classes of recalcitrant compounds used extensively in textile industries for dyeing process. During dyeing process up to 15% of the used dyestuffs is lost in the industrial effluents [1]. Textile industries release millions of liters of such dye containing effluents directly into the water resources every day. Presence of these dye stuffs in the water bodies reduces the penetration of sunlight which decreases the photosynthetic activity, dissolved oxygen concentration, water quality and depicts toxic effects on the aquatic flora and fauna [2]. Moreover the dyes and dyestuffs released from the textile industries have carcinogenic and mutagenic effects and are potentially toxic to all life forms [3]. Thus it is very essential to treat the textile effluent before released into the environment.

Dyes have complex aromatic structures and exhibit wide structural diversity. Properties of the dyes are enhanced to provide resistance to fading, to improve delivery to the fabrics and to have variety of shades, which makes them highly resistant to degradation [4]. Different physico-chemical methods like ozonation, electrolysis, adsorption on activated charcoal, precipitation by alum, etc., are being used for color removal from wastewater [5] but these methods have drawbacks of generating large amount of sludge that may result in secondary pollution, unable to remove recalcitrant azo dyes completely and involve complicated procedures which are economically unfeasible [6]. Bioremediation can be used as an alternative technology for treating the textile effluent. Bioremediation is the use of living organisms for the recovery or cleaning up of contaminated sites (soil, sediment, air, water). Many microorganisms are capable of degrading textile dyes, including bacteria [7], fungi [8], yeast [9], actinomycetes [10] and algae [11]. Scientists have now realized that plants also have complex

* Corresponding author at: Department of Biochemistry, Shivaji University, Kolhapur 416004, India. Tel.: +91 231 2609152; fax: +91 231 2691533.
E-mail addresses: spg_biochem@unishivaji.ac.in, spgovindwar@rediffmail.com (S.P. Govindwar).

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systems and can be used as a remediating tool. Phytoremediation is the use of vegetation to contain, sequester, remove or degrade organic and inorganic contaminants from soil, sediments, surface water and ground water. Phytoremediation is advantageous as it is a cost effective and aesthetically pleasing technology, which is easier to manage being an autotrophic system of large biomass and requires little nutrient input.

Few cases have been reported that demonstrate the potential of plants to degrade textile dyes. The potential of *Typhonium flagelliforme* to degrade the dye Brilliant Blue R has been reported [12]. Accumulation and transformation of sulfonated anthraquinones the starting material for many dyes was observed in *Rheum rabarbarum* [13]. *Tagetis petula* hairy roots are capable of degrading Reactive Red 198 [14]. Decolorization of textile effluent by *Brassica juncea* [15] and the potential of narrow leaved cattails (*Typha angustifolia* Linn.) to accumulate and remove the dye Reactive Red 141 from synthetic dye mixture was demonstrated [16]. Adsorptive removal of the dye Malachite Green up to 45% by *Blumea malcolmii* [17] and the ability of *Aster amellus* Linn. to degrade a sulfonated azo dye Remazol Red have been reported recently [18].

Although some plants do have potential to degrade the textile dyes, they consume considerable time for degradation. Thus the plants having potential to degrade the dyes can be used to develop a consortium to achieve faster degradation. Present study deals with the differential degradation of Remazol Orange 3R dye by a consortium-AG (*A. amellus* and *Glandularia pulchella*) and individual plant species.

2. Experimental

2.1. Materials

2,2 Azino-bis (3-ethylbenzothiazoline) 6-sulfonic acid (ABTS) and riboflavin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Nicotinamide adenine dinucleotide (di-sodium salt), *n*-propanol, 2,6-dichlorophenol indophenol (DCIP), veratryl alcohol and catechol were purchased from Sisco Research Laboratories, Mumbai, Maharashtra, India. Tartaric acid was obtained from BDH chemicals (Mumbai, Maharashtra, India). All the chemicals used were of the highest purity available and of an analytical grade. The dyes were procured from Laxmi Dyeing industry, Ichalkaranji, Maharashtra, India. The plants of *A. amellus* and *G. pulchella* for experiments were purchased from Sajeev Nursery, Kolhapur, India.

2.2. Decolorization experiments

2.2.1. Decolorization of various textile dyes by consortium-AG

Decolorization experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of (20 mg l^{-1}) dye solutions in plain distilled water. For consortium studies, one plant each (average wet weight of the entire plant 10.50 g and average dry weight 0.352 g) of *A. amellus and G. pulchella* were used together as consortium-AG. Roots of the plants were washed with running tap water to remove the adherent soil followed by treatment of the roots with 0.1% $HgCl_2$ (w/v) for 3 min after which the plants were thoroughly washed with distilled water. Plants of *A. amellus, G. pulchella* and consortium-AG were dipped separately in each of the 250 ml Erlenmeyer flasks containing solutions of various dyes namely Remazol Orange 3R, Red HE8B, Red HE3B, Blue GLL and Methyl Orange in distilled water (20 mg l^{-1}). Aliquots (5 ml) of the dye solutions were withdrawn at different time intervals, centrifuged (5000 rpm for 10 min) and supernatant was separated. Decolorization of various dyes was monitored by measuring the absorbance of the supernatant of dye solutions at their respective λ_{max}. The decolorization percentage was calculated as:

$$\%\,\text{decolorization} = \frac{(\text{initial absorbance} - \text{observed absorbance})}{\text{Initial absorbance}} \times 100$$

All the decolorization experiments were performed in triplicates and the average values were determined.

2.2.2. Effect of increasing Remazol Orange 3R dye concentration on the decolorization potential of consortium-AG

To study the effect of increasing dye concentration on the decolorization potential, the consortium-AG were dipped in each of the 250 ml Erlenmeyer flasks containing 100 ml of Remazol Orange 3R dye solutions of 20, 40, 80, 160 and 320 mg l^{-1} concentrations respectively and the decolorization percentage were calculated at regular intervals.

2.2.3. Effect of repeated Remazol Orange 3R dye addition on the decolorization potential of consortium-AG

To study the effect of repeated dye addition on the decolorization potential, the consortium-AG was initially dipped in 100 ml solution of Remazol Orange 3R (20 mg l^{-1}) and when the dye solution was completely decolorized, dye was added to the decolorized solution to establish a dye solution of initial concentration (20 mg l^{-1}). This was repeated for three more decolorization cycles. The duration of all the decolorization cycles were recorded.

2.3. Preparation of cell free extract

Roots of the control plants (those suspended in plain distilled water) and roots of the test plants (those suspended in the Remazol Orange 3R dye solution for 36 h) were used for the preparation of cell-free extracts. The roots were initially detached from the shoot system, weighed, and then cut into fine pieces after which they were suspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) and ground finely in a mortar and pestle. The tissue was then homogenized in a glass homogenizer with intermittent cooling, and then the extract was centrifuged at 8500 × *g* for 20 min at 4 °C. The supernatant thus obtained after centrifugation was used as an intracellular enzyme source. After removal of plants, the distilled water (control) and the remaining dye solution (test) were used as a source of extracellular enzyme after centrifugation. In case of consortium-AG, after decolorization the plants of *A. amellus* and *G. pulchella* were separately subjected to cell free extraction and enzymatic analysis.

2.4. Enzymatic analysis

The activities of the enzymes laccase, lignin peroxidase and tyrosinase were assayed spectrophotometrically, in cell free extract (intracellular) as well as in the supernatant (extracellular). Laccase activity was determined using 2,2 azino-bis (3-ethylbenzothiazoline) 6-sulfonic acid (ABTS) (10%) as a substrate in 0.1 M acetate buffer (pH 4.9). Oxidized ABTS was measured at 420 nm [19]. Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm. The reaction mixture of 2.5 ml contained, 0.5 ml 100 mM *n*-propanol, 0.5 ml 250 mM tartaric acid, 1.3 ml distilled water and 0.2 ml 10 mM H_2O_2 [20]. The tyrosinase activity was determined by the formation of *o*-benzoquinone and dehydro-ascorbic acid in 3 ml reaction mixture containing 50 mM of catechol and 2.1 mM of ascorbic acid in 50 mM potassium phosphate buffer (pH 6.5). The decrease in absorbance at 265 nm was measured [21]. All enzyme assays were carried out at 30 °C with reference blanks that contained all components except

Table 1
Decolorization of various textile dyes by *A. amellus*, *G. pulchella* and consortium-AG.

Name of the dyes	Time (h)	Decolorization (%)		
		Consortium-AG	*A. amellus*	*G. pulchella*
Remazol Orange 3R	36	100	42 ± 2.26*	37.2 ± 0.85*
Red HE3B	60	100	88.26 ± 1.59*	72.6 ± 1.35*
Blue GLL	96	85.2 ± 0.97	72.25 ± 0.94*	65.29 ± 1.76*
Methyl Orange	96	72.58 ± 1.25	25.15 ± 1.36*	36.5 ± 1.48*
Red HE8B	96	56.27 ± 0.85	33.3 ± 1.57*	42 ± 2.15*

Values are a mean of three experiments ± SEM. Significantly different from Consortium-AG at $^{*}P < 0.001$ by one-way ANOVA with Tukey–Kramer comparison test.

the enzyme and were run in triplicates, average rates were calculated and one unit of enzyme activity was defined as a change in absorbance unit per minute per milligram of protein.

NADH-DCIP reductase activity was determined by using the procedure reported earlier [22]. The assay mixture contained 50 μM DCIP, 1.142 mM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution in a total volume of 5.0 ml. The DCIP reduction was calculated using the extinction coefficient of 19 mM^{-1}. Veratryl alcohol oxidase activity was determined by using reports by Bourbonnais and Paice [23] while riboflavin reductase activity was determined by the method of Russ et al. [24]. The protein content was determined using Lowry method.

2.5. Extraction of the metabolites of Remazol Orange 3R degradation

After decolorization of the dye solution by *A. amellus*, *G. pulchella* and consortium-AG respectively, the plants were removed from their respective solutions. The decolorized solutions were centrifuged at 10,000 × *g* for 15 min to remove any solid matter if present like root hairs. Extraction of products from the supernatants was carried out using equal volume of ethyl acetate and the extract was then evaporated in vacuum over anhydrous Na_2SO_4 and dried. The crystals obtained were dissolved in a small volume of High Performance Liquid Chromatography (HPLC) grade methanol and the samples were then used for analytical studies.

2.6. Analysis of the metabolites of Remazol Orange 3R degradation

Decolorization of the dye was monitored using UV–vis spectroscopic analysis (Hitachi U-2800; Hitachi, Tokyo, Japan), using the centrifuged supernatant of the dye solutions. Degradation of the dye Remazol Orange 3R was confirmed by comparing the results of High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR) of the individual dye and the test samples (Remazol Orange 3R dye solution in which plants were dipped). Identification of the metabolites of dye degradation was carried out using Gas Chromatography Mass Spectroscopy (GC–MS).

HPLC analysis was carried out (Waters model no. 2690; Waters Corp., Milford, MA) on C_8 column (symmetry, 4.6 mm × 250 mm) by using gradient of methanol with flow rate of 1 ml min^{-1} for 10 min and UV detector at 254 nm. The degraded dye Remazol Orange 3R was characterized by FTIR (PerkinElmer, Spectrum one B; Shelton, WA). The FTIR analysis was performed in the mid-IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analysis was carried out.

GC–MS analysis of metabolites was carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm i.d., nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280 °C with oven conditions as: 80 °C kept constant for 2 min, then increased up to 200 °C with 10 °C min^{-1} and finally raised up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the NIST library.

2.7. Phytotoxicity studies

The ethyl acetate extracted metabolites of Remazol Orange 3R degradation by *A. amellus*, *G. pulchella* and consortium-AG respectively, were dried and dissolved in 50 ml of distilled water to make a final concentration of 1500 ppm. *Phaseolus mungo* and *Sorghum vulgare* seeds were used for phytotoxicity studies and the experiments were carried out at room temperature by placing ten seeds in separate 5 ml solutions containing the dye, metabolites of degradation of the dye, and distilled water. The samples were watered every day. Germination (%) and the length of plumule (shoot) and radicle (root) were recorded after 8 days.

2.8. Statistical analysis

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

3. Results and discussion

3.1. Decolorization experiments

3.1.1. Decolorization of various textile dyes by A. amellus, G. pulchella and consortium-AG

A. amellus, *G. pulchella* and consortium-AG showed decolorization of the dyes to varying extent (Table 1). It was observed that in case of all the dyes, consortium-AG had a greater potential of decolorization compared to *A. amellus* and *G. pulchella.* Two different systems of *A. amellus* and *G. pulchella* work together in the consortium-AG resulting in greater potential giving faster decolorization. Consortium-AG showed 100% decolorization of Remazol Orange 3R and Red HE3B in 36 and 60 h respectively, while it showed 85.2, 72.58 and 56.27% decolorization in 96 h for the dyes Blue GLL, Methyl Orange and Red HE8B respectively. As the dye Remazol Orange 3R took minimum time for decolorization, it was used for performing further studies. Individually *A. amellus* and *G. pulchella* took 72 and 96 h respectively to completely decolorize the dye Remazol Orange 3R (data not showed).

3.1.2. Effect of increasing dye concentration on decolorization potential of consortium-AG

With an increase in the dye concentration the decolorization potential of consortium-AG was observed to be decreased (Fig. 1). A decrease in the decolorization potential of *B. malcolmii* Hook. with an increase in the concentration of dye Direct Red 5B has been reported earlier [17]. Higher dye concentration might be having toxic effect on the plants hampering the enzymatic machin-

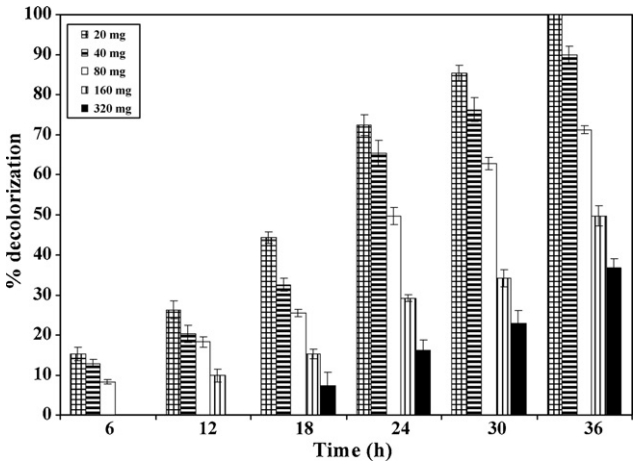

Fig. 1. Effect of increasing Remazol Orange 3R dye concentration on the decolorization potential of consortium-AG in 36 h.

ery, resulting in the decrease of the decolorization potential of consortium-AG.

3.1.3. Effect of repeated dye addition on decolorization potential of consortium-AG

In case of repeated dye addition experiment, consortium-AG showed complete decolorization in 36, 28 and 30 h in the first, second and third decolorization cycles respectively. The decrease in the time for decolorization in the second and the third cycle might be due to the presence of the active enzymatic system of the plants during the first decolorization cycle, leading to faster decolorization. During the first three decolorization cycles the plant leaves, stem and roots were as healthy as in the soil. The plants started drying during the fourth decolorization cycle, so it was not considered. The drying of the plants could be because of nutrient deficiency and the repeated dye stress, as the plants were uprooted.

3.2. Enzymatic analysis

Roots of the plants *A. amellus*, *G. pulchella* as well as of consortium-AG dipped in distilled water (control) and in the dye solution (test), were subjected for determination of the activities of the enzymes lignin peroxidase, laccase, tyrosinase, NADH-DCIP reductase, riboflavin reductase and veratryl alcohol oxidase. Individually *A. amellus* showed induction in the activities of veratryl alcohol oxidase (110%) and NADH-DCIP reductase (57%) (Table 2). *G. pulchella* showed induction of laccase (180%) and tyrosinase (69%) (Table 2). Within the consortium-AG, *A. amellus* showed induction of lignin peroxidase (288%), veratryl alcohol oxidase (350%) and NADH-DCIP reductase (50%) while *G. pulchella* showed induction of lignin peroxidase (260%), laccase (295%) and tyrosinase (135%) (Table 2). No extracellalar activities were observed.

The major mechanism of biodegradation in living cells is realized because of the lignin modifying enzymes, laccase, manganese peroxidase, lignin peroxidase, tyrosinase and to some extent by N-demethylase to mineralize synthetic dyes [25]. The induction of the enzymes peroxidase, tyrosinase and DCIP reductase have been reported during the decolorization of the dye Direct Red 5B in *B. malcolmii* [17]. Roots of *B. juncea* showed induction in the intracellular activity of laccase enzyme on exposure to the dye Reactive Red 2 [15]. During Remazol Red decolorization induction in the activities of lignin peroxidase, tyrosinase, veratryl alcohol oxidase and riboflavin reductase were reported suggesting their involvement in the decolorization process [18]. Induction of the enzymes in the roots of the plants of *A. amellus*, *G. pulchella* and consortium-AG

Table 2
Enzyme activities in the roots of *G. pulchella* and *A. amellus*, individually as well as in consortium-AG in the control tissue (0 h) and after 36 h of Remazol Orange 3R dye addition.

Enzyme	Individual				Consortium-AG			
	A. amellus		*G. pulchella*		*A. amellus*		*G. pulchella*	
	Control	Test	Control	Test	Control	Test	Control	Test
Lignin peroxidase[a]	0.07 ± 0.01	0.10 ± 0.02	0.09 ± 0.02	0.17 ± 0.03	0.09 ± 0.03	0.35 ± 0.01*	0.10 ± 0.04	0.36 ± 0.01*
Veratryl alcohol oxidase[a]	2.90 ± 0.01	6.11 ± 0.07*	–	–	2.70 ± 0.07	12.15 ± 0.25*	–	–
Laccase[a]	–	–	2.14 ± 0.06	6.06 ± 0.03*	–	–	3.16 ± 0.65	12.49 ± 0.74*
Tyrosinase[a]	–	–	12.17 ± 0.39	20.65 ± 0.45*	–	–	12.49 ± 0.67	29.47 ± 0.72*
DCIP reductase[b]	296.46 ± 7.23	465.90 ± 6.07*	13.51 ± 0.51	11.51 ± 0.65	282.58 ± 8.81	424.38 ± 6.17*	14.71 ± 3.12	6.19 ± 1.66
Riboflavin reductase[c]	17.84 ± 0.40	17.16 ± 0.40	–	–	17.65 ± 0.02	18.62 ± 0.26	–	–

Values are a mean of three experiments ± SEM. Significantly different from control (0 h) at $^{*}P < 0.001$ by one-way ANOVA with Tukey– Kramer comparison test.

[a] Activity in units min^{-1} mg^{-1}.

[b] μg of DCIP reduced min^{-1} mg $protein^{-1}$.

[c] μg of riboflavin reduced min^{-1} mg $protein^{-1}$.

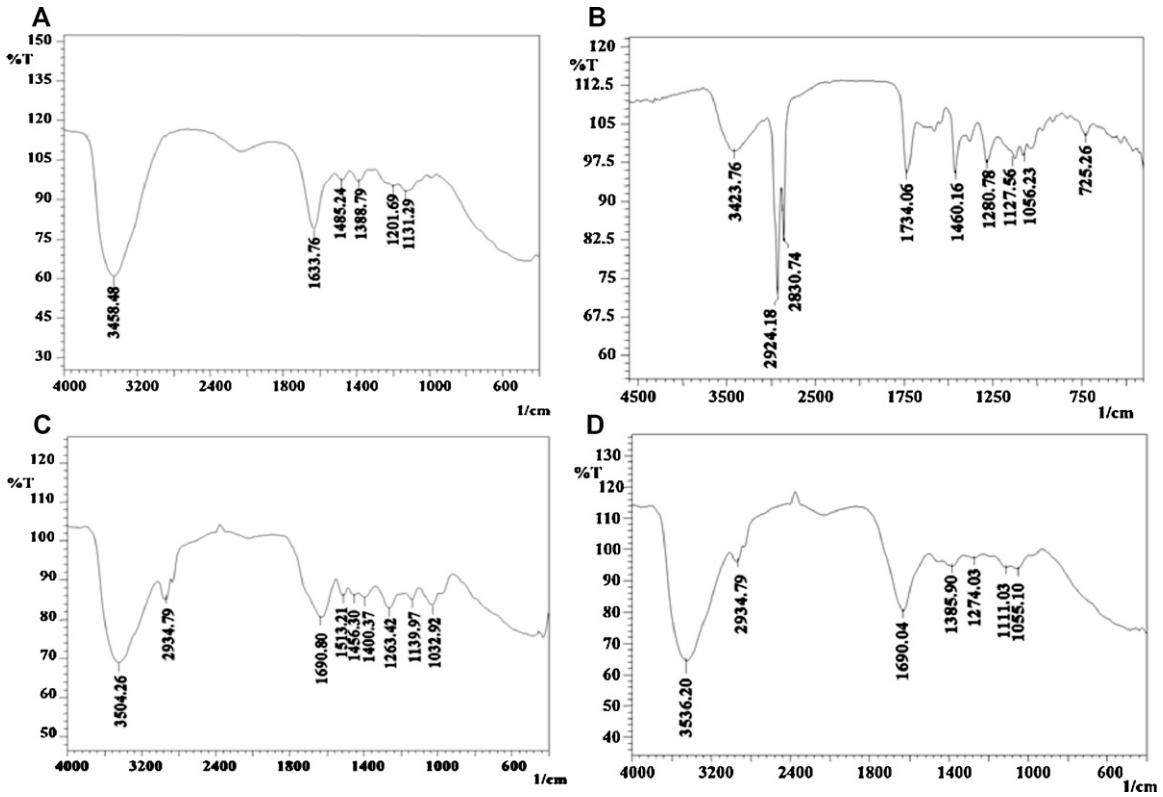

Fig. 2. FTIR spectra of (A) Remazol Orange 3R, (B) Remazol Orange 3R dye solution decolorized by consortium-AG, (C) Remazol Orange 3R dye solution decolorized by *G. pulchella*, (D) Remazol Orange 3R dye solution decolorized by *A. amellus*.

during the decolorization of the dye Remazol Orange 3R indicates their involvement in the dye metabolism.

Within the consortium-AG two different enzymatic systems of *A. amellus* and *G. pulchella* work together resulting in faster degradation of the dye compared to the individual species. Along with these two enzymatic systems working together in the consortium, the level of induction of the enzymes within the consortium is also greater than in the individual species, which adds to the faster degradation of the dye. Different set of enzymes were induced in the plants of *A. amellus* and *G. pulchella* when exposed to the dye Remazol Orange 3R individually as well as in consortium-AG. This indicates that the same dye induces different enzymatic system in different plants leading to the differential fate of metabolism of the dye.

3.3. Analysis of the metabolites of Remazol Orange 3R degradation

The FTIR spectra of the decolorized solution of Remazol Orange 3R by consortium-AG, *A. amellus* and *G. pulchella* respectively differed significantly from the FTIR spectra of the individual dye (Fig. 2). The FTIR spectra of the individual dye (Fig. 2A) showed the presence of different peaks like 3458.48 cm^{-1}, 1633.76 cm^{-1} and 1485.24 cm^{-1} representing N–H trans stretching of secondary amides, N=N stretching as in azo compounds and C–H deformation as seen in alkanes ($-CH_2-$) respectively. The dye spectra also showed peaks at 1388.79 cm^{-1}, 1201.69 cm^{-1} and 1131.29 cm^{-1} representing O–H deformation as in phenols, S=O stretching of sulfonic acids and S=O asymmetric stretching of sulfones respectively. The FTIR spectra of decolorized dye solution by the consortium-AG (Fig. 2B) showed appearance of new peaks compared to the dye spectra at 3423.76 cm^{-1}, 2924.18 cm^{-1}, 1460.16 cm^{-1} and 1280.78 cm^{-1} representing N–H cis stretching of secondary amides, C–H stretching of alkanes ($-CH_2-$), C–H deformation as in case of alkanes ($-CH_3$) and O–H deformation as for primary alcohols respectively. The spectra also showed peaks at 1056.23 cm^{-1}, 1127.56 cm^{-1} and 2830.74 cm^{-1} representing S=O asymmetric stretching as in sulfonic acids, S=O stretching of sulfones and C–H stretching as in ethers respectively. The FTIR spectra of decolorized dye solution by *G. pulchella* (Fig. 2C) showed new peaks at 3504.26 cm^{-1}, 2934.79 cm^{-1}, 1456.30 cm^{-1} and 1400.37 cm^{-1} representing N–H stretching of primary amides, C–H stretching of alkanes ($-CH_2-$), C–H stretching as in alkanes ($-CH_3$) and O–H deformation as in phenols respectively. The spectra also showed peaks at 1139.97 cm^{-1} and 1032.92 cm^{-1} representing S=O stretching as in sulfones and S=O stretching as in case of sulfonic acid respectively. The peak at 1690.80 cm^{-1} represents acetamide which supports the removal of acetamide from the dye molecule in the proposed pathway for the degradation of the dye by *G. pulchella*. The FTIR spectra of decolorized dye solution by *A. amellus* (Fig. 2D) showed new peaks at 3536.20 cm^{-1}, 2934.79 cm^{-1}, 1385.90 cm^{-1} 1111.05 cm^{-1} and 1055.10 cm^{-1} representing N–H stretching of primary amides, C–H stretching as in case of alkanes ($-CH_2-$), benzene, S=O stretching as in case of sulfones and S=O stretching as in sulfonic acids respectively. As seen in *G. pulchella*, *A. amellus* also showed peak at 1690.04 cm^{-1} representing acetamide which supports acetamide removal in the proposed pathway for the degradation of the dye by *A. amellus*. The difference in the FTIR spectra of the dye and the decolorized solutions supports the conformation of the biodegradation of the dye into different metabolites.

The HPLC analysis of the individual dye showed a single peak at 1.94 min (Fig. 3A). The decolorized dye solution by consortium-AG showed peaks at 1.953, 2.882, 4.044 and 7.561 min (Fig. 3B). Decolorized dye solution by *G. pulchella* showed peaks at 2.357, 2.844, 3.111, 4.392, 5.029 and 7.492 min (Fig. 3C). While the decolorized dye solution by *A. amellus* showed peaks at 2.866, 2.096 and 4.519 min (Fig. 3D). The appearance of new peaks and disappear-

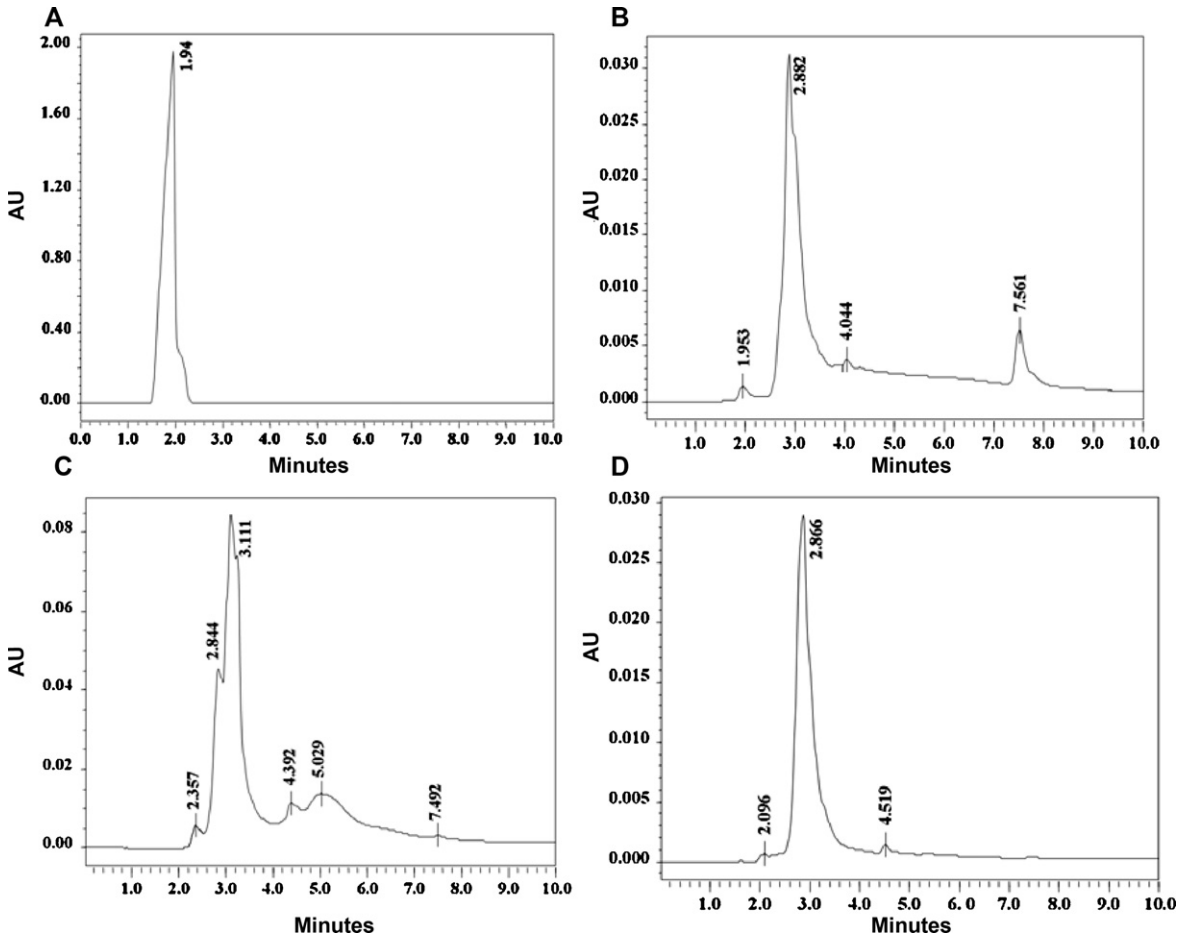

Fig. 3. HPLC analysis of (A) Remazol Orange 3R, (B) Remazol Orange 3R dye solution decolorized by consortium-AG, (C) Remazol Orange 3R dye solution decolorized by *G. pulchella*, (D) Remazol Orange 3R dye solution decolorized by *A. amellus*.

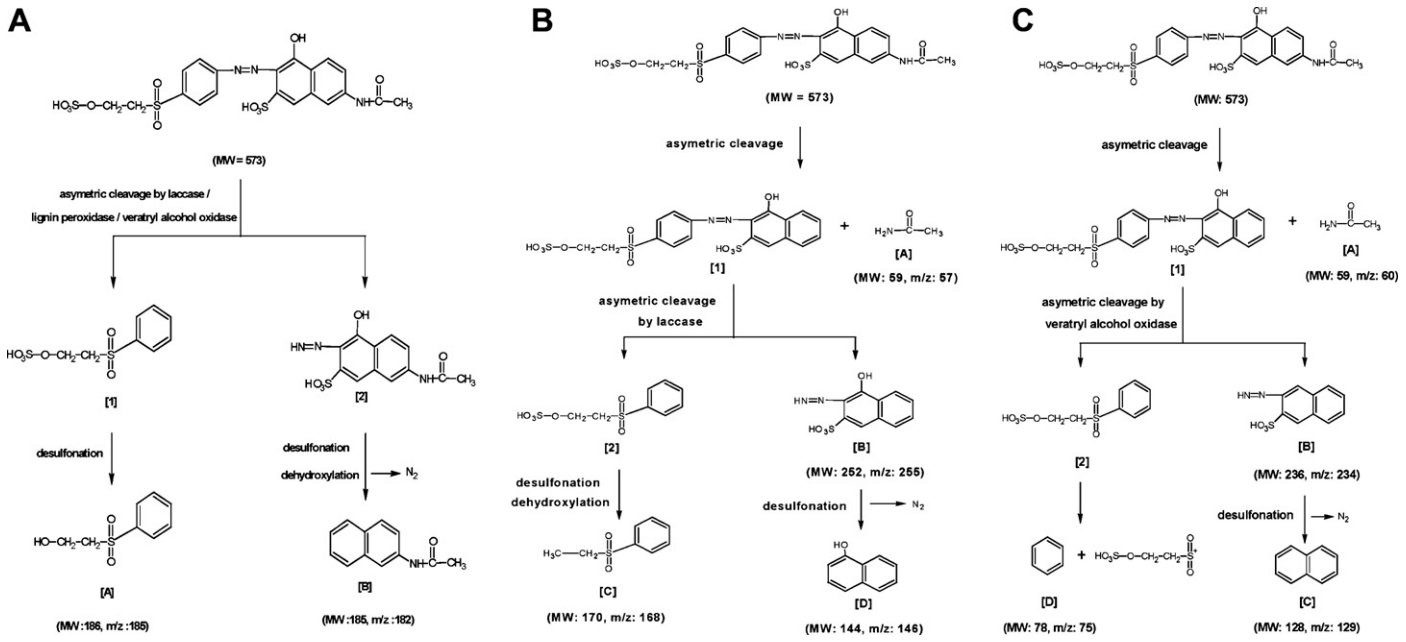

Fig. 4. Proposed pathway for the degradation of the dye Remazol Orange 3R by (A) consortium-AG, (B) *G. pulchella*, (C) *A. amellus*.

Table 3
Phytotoxicity studies of the dye Remazol Orange 3R and its metabolites formed after degradation by consortium-AG, *A. amellus* and *G. pulchella* respectively..

Parameters studied	*Phaseolus mungo*					*Sorghum vulgare*				
	(A)	(B)	(C)	(D)	(E)	(A)	(B)	(C)	(D)	(E)
Germination (%)	90	30	90	90	80	90	40	90	80	80
Plumule (cm)	8.42 ± 0.03	3.75 ± 0.21*	7.62 ± 0.08$	7.02 ± 0.05$	7.55 ± 0.08$	5.44 ± 0.28	2.39 ± 0.16*	5.16 ± 0.13$	4.79 ± 0.12$	4.96 ± 0.35$
Radicle (cm)	3.30 ± 0.07	0.93 ± 0.05*	3.42 ± 0.09$	2.29 ± 0.14$	3.01 ± 0.14$	3.47 ± 0.14	1.28 ± 0.05*	3.31 ± 0.12$	3.13 ± 0.10$	2.94 ± 0.04$

(A) Water, (B) Remazol Orange 3R, (C) extracted metabolites of dye degraded by Consortium-AG, (D) extracted metabolites of dye degraded by *A. amellus*, (E) extracted metabolites of dye degraded by *G. pulchella*. Values are a mean of three experiments ± SEM. Root and shoot lengths of plants grown in Remazol Orange 3R are significantly different from that of plants grown in distilled water by * $P < 0.001$. Root and shoot lengths of plants grown in the extracted metabolites are also significantly different from that of plants grown in Remazol Orange 3R by $ $P < 0.001$.

ance of the peaks in the decolorized dye solution compared to the individual dye support the biodegradation of the dye into different metabolites.

The GC–MS analysis results were used to determine the probable metabolites produced and also to propose the pathways for degradation of the dye by *A. amellus*, *G. pulchella* and consortium-AG respectively. Azo dyes can be cleaved symmetrically and asymmetrically, with an active site available for an enzyme to excite the molecule [26]. In case of consortium-AG (Fig. 4A), asymmetric cleavage of Remazol Orange 3R molecule mediated by enzyme laccase or lignin peroxidase or veratryl alcohol oxidase yields intermediate [1] and [2]. Intermediate [1] undergoes desulfonation to give [A] 2-(phenylsulfonyl)ethanol while the intermediate [2] undergoes desulfonation, dehyroxlation and on removal of N_2 gives [B] N-(naphthalen-2-yl)acetamide. In *G. pulchella* (Fig. 4B) removal of [A] acetamide through asymetric cleavage of the dye molecule gives an intermediate [1]. Further asymmetric cleavage of intermediate [1] by laccase gives [B] 3-diazenyl-4-hydroxynaphthalene-2-sulfonic acid and an intermediate [2], which further undergoes desulfonation and dehydroxylation to give [C] (ethylsulfonyl)benzene. The metabolite [B] on removal of N_2 and desulfonation gives [D] naphthalen-1-ol. In *A. amellus* (Fig. 4C) asymmetric cleavage of the dye molecule yields [A] acetamide and an intermediate [1]. Intermediate [1] on asymmetric cleavage by veratryl alcohol oxidase yields [B] 3-diazenylnaphthalene-2-sulfonic acid and an intermediate [2]. The metabolite [B] on N_2 removal and desulfonation gives [C] naphthalene while the intermediate [2] is cleaved to yield [D] benzene. The structures of the detected compounds were assigned from the *m*/*z* value obtained. The dye Remazol Orange 3R is shown to be metabolized differentially by *A. amellus*, *G. pulchella* and consortium-AG respectively.

3.4. Phytotoxicity studies

Mostly the textile effluent after the treatment is utilized for irrigation in the developing countries; therefore the seeds of agricultural crops of *P. mungo* and *S. vulgare* were selected to study the toxicity of the dye and the metabolites formed after degradation of the dye.

The results of phytotoxicity experiments showed that there was an inhibition of germination in solutions containing 1500 ppm of the dye Remazol Orange 3R for both *P. mungo* and *S. vulgare* by 66.6 and 55.5% respectively. The seeds of *P. mungo* and *S. vulgare* showed a higher percentage of germination in both distilled water and the extracted metabolites (Table 3). At the same time the length of the plumule and radicle was found to be lower in seeds germinated in the dye than those germinated in water and in metabolites of the dye (Table 3). The lower percentage of germination of *P. mungo* and *S. vulgare* with lower lengths of the plumule and radicle in dye solution of 1500 ppm concentration, indicate that the dye was toxic to these plants, while the metabolites formed after the degradation of the dye by *A. amellus*, *G. pulchella* and consortium-AG respectively were almost as non-toxic as distilled water.

Mostly the textile effluent after the treatment is utilized for irrigation purpose in agricultural practices, thus the seeds of agricultural crops of *P. mungo* and *S. vulgare* were selected to study the toxicity of the dye and the degraded dye solution.

4. Conclusion

The combined activities of enzymes in consortium-AG consisting of *A. amellus* and *G. pulchella* resulted in an increase in the decolorization efficiency of Remazol Orange 3R compared to the individual plants. The consortium-AG could effectively decolorize

various dyes. *A. amellus* and *G. pulchella* showed different pattern of induction of veratryl alcohol oxidase, DCIP reductase, laccase and tyrosinase for the degradation of same dye resulting different fate of metabolism. The potential of consortium-AG for practical phytoremediation is yet to be scrutinized and studies on survival of plants under real-life conditions are underway.

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