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Bacterial-yeast consortium as an effective biocatalyst for biodegradation of sulphonated azo dye Reactive red 198

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Abstract

A novel developed bacterial-yeast consortium (*Brevibacillus laterosporus* and *Galactomyces geotrichum*) has been acted as a proficient biocatalyst. It decolorized 92% of sulphonated azo dye-Reactive red 198 (RR 198) within 18h at a dye concentration of 50 mg L^{-1} as compared to 58 and 42% decolorization using *Brevibacillus laterosporus* and *Galactomyces geotrichum* alone respectively in the same experimental conditions (pH-7, 40 $^{\circ}$C, in static condition). The cumulative action of enzymes such as veratryl alcohol oxidase, laccase, NADH-DCIP reductase and azoreductase in the culture was responsible for dye degradation. Fourier transform infrared spectroscopy and High performance thin layer chromatography analysis of dye and its extracted metabolites suggested the biotransformation of RR 198 into simple metabolites; whereas the biotransformation of the same by individual microorganism was different than consortial biodegradation. According to Gas chromatography-Mass spectroscopy studies, RR 198 was biotransformed into much simpler compounds such as (ethylsulfonyl)benzene and 1,3,5-triazine by the bacterial-yeast consortium. This metabolic fate of dye was entirely different in consortium when compared to individual microbial treatment. Single microbial species could only lead to partial mineralization of intact dye molecule; whereas, nearly complete degradation of dye molecule was achieved using consortium culture. This study clearly suggests that the consortium has an enormous strength to catalyze RR 198 within a short period as compared to individual microbial cultures.

Keywords: Biocatalysis; Biodegradation, Biotransformation; Consortium, Decolorization; High performance thin layer chromatography; Phytotoxicity

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1.0. Introduction

Among 12 classes of chromogenic groups of dyes, the azo dyes including sulphonated and reactive dyes make up to 70% of all textile dyestuff produced. This leads to effluent streams containing intense color due to the presence of azo dyes. Normally colors are noticeable at a dye concentration of more than 1 mg L^{-1} and an average concentration of 300 mg L^{-1} has been reported in effluents from textile manufacturing processes.[1] Azo dyes are of concern in wastewater treatment because of their intense coloration, bio-recalcitrance and potential toxicity to animals and humans as it contains mutagenic and carcinogenic properties.[2,3] Several physicochemical techniques have been used for treating wastewater containing dyes but the methodologies appear to face several technical and economic limitations and lead to the generation of toxic by-products.[4-7]

On the other hand, a number of biotechnological approaches have been suggested by recent research as of potential interest in combating this pollution source in an eco-efficient manner.[8,9] Lower cost of treatment and amenability to scale up easily are the merits of biological methods. Numerous studies have been carried out with isolated, selected, mutated and genetically engineered microorganisms for effective bioremediation to achieve enhanced rates of degradation of recalcitrant target compounds.[10-14] However, the isolated/single cultures are often specific to type of dye under consideration. Moreover, pure cultures cannot be scaled up easily and maintained in large-scale operations typical of effluent treatment systems.[15] Instead, microbial consortium could be an attractive tool to improve the rate of decolorization process with better suitability in large-scale applications.

Recent studies have suggested that mixed culture may be more appropriate for decolorization of azo dyes.[16-18] Different strains may attack dye molecule at different positions or may use decomposition products produced by another strain for further decomposition.[19,20] In recent years, mixed microbial consortia are believed to have more

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superiority over pure isolates in some biorefractory wastewater treatment. Recently, a bacterial consortium (*Enterobacter dissolvens and Pseudomonas aeruginosa*) was used for the treatment of di-azo dye Acid Maroon V, which showed 2.15 fold increase in decolorization efficiency compared to individual microorganisms.[21] Fungal species *Aspergillus terreus* and *Rhizopus oryzae* in consortium enhanced the removal of metals and dyes.[22] Yeast species including *Galactomyces geotrichum, Candida tropicalis and Saccharomyces cerevisiae* were effectively used for dye degradation.[14,23-25]; however, as far as our knowledge is concerned, there is still no report on yeast consortium for dye decolorization.

Although studies carried out in the past have used undefined microbial consortium[1,26] or pure cultures for dye decolorization, there is very limited research to unravel the application of bacterial-yeast/fungi consortium for the rehabilitation of our natural resources. In the present study, a bacterial-yeast consortium has been developed using a yeast-*Galactomyces geotrichum* with another potential dye degrading microbial species-*Brevibacillus laterosporus* to decolorize a sulphonated azo dye- Reactive red 198 (RR 198). *Brevibacillus laterosporus* is an aerobic bacterium and is a potent dye degrading species. Our previous reports provide strong evidence on abilities of *Brevibacillus laterosporus* in decolorization of several dyes, such as, Remazol red, Rubine GFL, Disperse brown 118, Golden Yellow HER and mixture of dyes.[27-30] In the yeast species, *Galactomyces geotrichum* can be a strong highlight, as it has been used to eliminate different variety of textile dyes which include Reactive yellow 84A, Rubine GFL and Methyl red.[14,23] Presence of wide range of biocatalytic enzyme system make these species strong candidates for enhanced dye degradation. High efficiency and biocatalytic performance was expected using the combination of these microbial species. RR 198 is a sulphonated monoazo dye which is toxic and recalcitrant in nature due to the presence of azo and sulphonic groups. It gives intense

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coloration to water at very low concentration, hence effluent containing this dye cause deterioration of water bodies and lead to harmful effects on aquatic flora and fauna. It is one of the most consumable azo dye in the textile industries in Ichalkaranji, India; hence this dye was used as a model azo dye for the decolorization experiments in the present study. This study put forward the detailed mechanism of how this bacterial-yeast consortium is superior over an individual microbial culture in eliminating the xenobiotic and toxic azo dye RR 198 with the evidence of enzymatic status, characterization of products extracted after dye decolorization using Fourier transform infrared spectroscopy (FTIR) and High performance thin layer chromatography (HPTLC) along with metabolic fate of RR 198 using Gas chromatography–Mass spectroscopy (GC-MS) data.

2.0. Experimental

2.1. Microorganisms and culture conditions

Brevibacillus laterosporus MTCC 2298 was obtained from Microbial Type Culture Collection, Chandigarh, India. The pure culture was maintained at 4 $^{\circ}$C on the Nutrient agar slant containing (g L^{-1}): NaCl, 5.0; bacteriological peptone, 5.0; yeast extract, 2.0; beef extract, 1.0 and agar powder 15.0 at 4 $^{\circ}$C. *Galactomyces geotrichum* MTCC 1360 was obtained from Microbial Type Culture Collection, Chandigarh, India. Pure culture was maintained on malt yeast agar slants at 4 $^{\circ}$C, the composition of malt extract medium was (g L^{-1}): malt extract 3, yeast extract 3, peptone 5, and glucose 10.

2.2. Dyes and Chemicals

All required chemicals were obtained from Sigma Aldrich, USA, Hi-media Laboratories Pvt. Ltd., Mumbai, India and Sisco Research Laboratory (SRLs), India. All chemicals used were of the highest purity available and of the analytical grade. Textile dye

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RR 198 was a munificent gift from Manpasand textile processing industry, Ichalkaranji, India.

2.3. Development of bacterial-yeast consortium

The consortium was prepared by the earlier reported protocol.[31] The surface culture biomass of *G. geotrichum* (1 cm pieces of mycelium cut from agar slants were used to inoculate 100 mL malt extract medium in 250 mL Erlenmeyer flasks and grown at 30 °C for 24 h) was aseptically transferred to the 24 h old culture of *B. laterosporus* (grown at 30 °C in 250 mL Erlenmeyer flask containing 100 mL of nutrient medium) to obtain the bacterial-yeast consortium.

2.4. Decolorization experiment

All decolorization experiments were performed in the 250 mL Erlenmeyer flasks. Textile dye RR 198 was added at a concentration of 50 mg L^{-1} in the flask containing developed consortium with 100 mL nutrient broth. Aliquot was centrifuged (4000 rpm for 20 min) to separate cell mass and clear supernatant was used to determine the decolorization. Decolorization was determined by measuring absorbance maxima of the dye (λ_{max} of RR 198-530 nm) using UV visible spectrophotometer (Hitachi U-2800) by using following formula.

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

To investigate the effect of different dye concentration on decolorization, the concentrations of the RR 198 was varied from 50 to 400 mg L^{-1} concentration (50, 100, 200, 300 and 400 mg L^{-1}) to determine the amount of dye that could be decolorized by consortium and decolorization was determined by the formula mentioned above.

View Article Online
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All the media used in this study were pre-sterilized before experiments by autoclaving it at 121 °C for 20 min. All decolorization experiments were carried out in triplicate. Abiotic controls (without microorganisms) were always included.

2.5. Preparation of cell free extract

The consortium was prepared as mentioned above and individual organisms were grown in their respective medium for 24 h at 30 °C and centrifuged at 10,000 rpm for 20 min. This biomass of consortium and individual organisms were separately suspended in 50 mM potassium phosphate buffer (pH 7.4), gently homogenized and used for sonication (sonics-vibracell ultrasonic processor) keeping sonifier output at 60 amplitude maintaining temperature below 4 °C and giving 12 strokes each of 30 sec with one min interval. These resulting extracts were then used as enzyme source. The culture supernatant obtained after centrifugation during the harvesting of cell biomass was directly used as a source of extracellular enzymes. Same procedure was carried out for samples obtained after degradation of the dye.

2.6. Determination of enzyme activities

All enzyme activities were assayed spectrophotometrically (Hitachi U-2800) in the cell free extract as well as culture media at room temperature (25 °C). Veratryl alcohol oxidase activity was determined by using the procedure reported earlier by Jadhav et al.[32] Laccase activity was monitored according to the procedure reported earlier.[33] 2 mL reaction mixture contained 5 mM 3', 3'-diaminobenzidine tetrahydrate (DAB) in 0.1M acetate buffer (pH 4.8) and increase in optical density was measured at 410 nm. Tyrosinase activity was determined by the procedure as follows; 3 mL reaction mixture contained 50 mM of catechol and 2.1 mM of ascorbic acid in 50 mM potassium phosphate buffer (pH 6.5) equilibrated at 25°C. ΔA_{265} nm was monitored until constant, and then 0.1 mL of the supernatant from the

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DOI: 10.1039/C4RA15834B

reaction mixture was added. The formation of *o*-benzoquinone and dehydro-ascorbic acid and decrease in optical density was measured at 265 nm. ΔA_{265} nm was obtained using the maximum linear rate for both the test and control. One unit of tyrosinase activity was equal to a ΔA_{265} nm of 0.001 per min at pH 6.5 at 25^{o}C in a 3.0 mL reaction mixture containing L-catechol and L-ascorbic acid.

The NADH-DCIP reductase and Riboflavin reductase activity were assayed by modifying earlier reported methods by Waghmode et al.[33] DCIP reduction was monitored at 590 nm and calculated using an extinction coefficient 0.019 μM^{-1} cm^{-1}. The reaction mixture (5.0 mL) prepared contained 25 μM substrate (DCIP) in the 50 mM potassium phosphate buffer (pH 7.4) and 0.1 mL enzyme. From this, 2.0 mL reaction mixture was assayed at 590 nm by addition of 250 μM NADH. Riboflavin reductase NAD(P)H:flavin oxidoreductase was measured by monitoring the decrease in absorbance at 340 nm. Cell free extract was added to a solution (final volume 2 mL) containing 100 mM of Tris-HCl (pH 7.5), 25 μM of NADPH and 10 μM of riboflavin. Reaction rates were calculated by using a molar extinction coefficient of 0.0063 μM^{-1} cm^{-1}. Azo reductase activity was assayed by earlier reported method.[33] The assay mixture (2 mL) contains 4.45 μM of methyl red (MR), 50 μM NADH, 1.2 mL of potassium phosphate buffer (20 mM, pH 7.5). The reaction mixture was pre-incubated for 4 min followed by the addition of NADH and monitored for the decrease in color absorbance (430 nm) at room temperature. The reaction was initiated by addition of 0.2 mL of the enzyme solution. Methyl red reduction was calculated by using its molar extinction coefficient of 0.023 μM^{-1} cm^{-1}. One unit of enzyme activity was defined as amount of enzyme required to reduce 1 μM of substrate min^{-1} mg of $protein^{-1}$. All the enzyme assays were run in triplicates.

2.7. Extraction and analysis of products obtained after dye decolorization

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Biomass was removed after decolorization by centrifugation (10, 000 rpm at 4 °C for 20 min) and supernatant was processed for extraction of degradation metabolites with equal volume of ethyl acetate. The extracted residue were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The crystals obtained were dissolved in small volume of High Pressure Liquid Chromatography (HPLC) grade methanol and used for High Performance Thin Layer Chromatography (HPTLC), Fourier Transform Infrared spectroscopy (FTIR) and Gas Chromatography - Mass Spectroscopy (GC-MS) analysis. The FTIR analysis of extracted metabolites was carried out using protocol reported earlier.[31] This FTIR analysis of metabolites was carried out on Perkin–Elmer, spectrum one instrument and compared with control dye in the mid IR region of 400-4000 cm^{-1} with 8 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio 5:95, pellets were fixed in sample holder, and the analysis was carried out. The GC–MS analysis of metabolites were carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm id, 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 and increased up to 200 °C with 10 °C min^{-1} – raised up to 280 °C with 20 °C min^{-1} rate. The interphase temperature of MS engine was maintained at 280 °C and ionization voltage to 70 eV. The minimum and maximum limit of m/z was kept to 40 and 950 respectively. The compounds were identified on the basis of mass spectra using the NIST library. To confirm the biodegradation of dyes, the same metabolites were analyzed by HPTLC using silica gel plates (HPTLC Lichrospher Silica gel 60 $F_{254}S$, Merck). 15 µL of sample was applied on the plate by micro syringe using sample applicator (Linomat V, Camag, Switzerland). The solvent system used for RR 198 was propanol: ethyl acetate: methanol: toluene (3: 6: 0.5: 0.5). The chromatogram was analyzed using scanner (Camag, Switzerland).

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DOI: 10.1039/C4RA15834B

2.8. Phytotoxicity study

This test was performed to assess the toxicity of the untreated and treated effluent with respect to two kinds of seeds commonly used in Indian agriculture; *Sorghum vulgare* and *Phaseolus mungo* using earlier reported method.[33] Ten seeds of each plant were sowed into a plastic sand pot. The sand pot was prepared by adding 15 g of washed sand into the plastic pot. Metabolites were extracted using procedure described in section 2.7 and these crystals obtained after ethyl acetate extraction were dissolved in distilled water. Toxicity study was carried out by watering the seeds of each plant with untreated textile effluent and extracted metabolites of effluent (5 mL per day). Control was kept by watering the seeds in distilled water (5 mL per day). Germination (%), lengths of the shoots and roots were recorded after 7 days. The experiment was carried out at room temperature.

2.9. Statistical Analysis

Data were analyzed by one-way analysis of variance using Tukey–Kramer multiple comparison test. Values were the mean of three independent experiments. Values were considered significant when $P < 0.05$.

3.0. Results and discussion

3.1. Decolorization experiment and effect of physico-chemical parameters

The decolorization of dyes is directly dependent upon the catalytic potential of a microorganism. Decolorization efficiencies of individual microorganism and the developed consortium are presented in Fig 1 in terms of percentage decolorization and time required for decolorization. This developed consortium is nothing but a combination of a bacterium (*B. laterosporus*) which is known to be a proficient decolorizer[27-29] with another yeast species (*G. geotrichum*). Consortium exhibited 92% decolorization of RR 198 within 18 h with dye concentration of 50 mg L^{-1} when optimized conditions (pH-7, 40 $^{\circ}$C and static condition) for

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decolorization were provided to the consortial culture. In contrast, *B. laterosporus* and *G. geotrichum* alone exhibited 58 and 42% decolorization respectively in same experimental conditions. Recently, we have reported the higher decolorization of textile dye Scarlet RR and textile industry effluent using the same consortium of yeast and bacteria.[31,33] Decolorization of most of the dyes by microbial cultures is generally carried out in neutral to slightly alkaline pH range. Consortium of *Bacillus* sp. and *G. geotrichum* also showed effective decolorization of Brilliant blue G at neutral pH[34] while natural microbial consortium required the same temperature range (40 $^{\circ}$C) for maximum decolorization of mixture of dyes and also with faster rate.[35] The loss of cell viability or the negligible oxido-reductive enzyme activities might be the cause of reduced decolorization in culture broth which was incubated beyond the optimum temperature range.

3.2. Effect of dye concentration on decolorization by consortium

An investigation of effect of dye concentration on decolorization ability of microbial system is very important as it explores the limits of a biological system. To assess this, culture of consortium was provided with higher doses of dye from 50 to 400 mg L^{-1}. It has been found that, higher concentration of dye decreased the decolorization performance of consortium. However, the difference in decolorization was insignificant ($P > 0.05$) as decolorization in flask with dye concentration of 400 mg L^{-1} was 59% as compared to 78% decolorization in flask containing the dye concentration of 50 mg L^{-1} within first 12 h (Fig 2A). Interestingly, the decolorization of RR 198 using consortium was found to be much higher with all dye concentration used with minor cessation in decolorization with increase in dye concentrations as compared to the decolorization by individual microorganisms. The decolorization of RR 198 by *B. laterosporus* was marginally reduced from 40% (at dye concentration of 50 mg L^{-1}) to 7% (at dye concentration of 400 mg L^{-1}) within first 12h;

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while at the same time, 29 and 12% decolorization was achieved using *G. geotrichum* at dye concentration of 50 and 400 mg L^{-1} respectively (Fig 2B and 2C). These results suggest that consortial culture can tolerate higher dye concentration with effective decolorization of dyes as compared to individual strains. It has been found that the higher dye concentration do not alter the decolorization when bacterial co-culture was used instead of pure culture, and this might be due to the synergistic effect of both microorganisms.[31,36] The gradual decrease in the decolorization rate at higher dye concentration in case of individual microbial cultures was probably due to the toxic effect of dyes with regard to the individual bacteria and/or inadequate biomass concentration or improper cell to dye ratio.[34] Based on previous reports,[37] the concentration of dye in the textile industry effluent is generally around 16-32 mg L^{-1} and as consortium is unaffected with higher dye concentration, this can be used as an effective tool in continuous wastewater treatment systems.

3.3. Enzyme analysis

A major mechanism of biodegradation by the microbial cells is controlled by the biotransformation enzymes produced by the cell itself. To prove the role of enzymes involved in biodegradation reaction, various biotransformation enzymes were monitored during the decolorization of RR 198 (18 h) in consortium and also in the individual microorganism i.e. *B. laterosporus* and *G. geotrichum* which are summarized in Table 1. In this set of experiment, significant induction of laccase (115%) in consortium was observed as compared to the activity in the cells of *G. geotrichum*. NADH-DCIP reductase activity was induced by 43 and 14% in consortium cells when compared to activity in the cells of *G. geotrichum* and *B. laterosporus* respectively. In addition to that, 22% induction in azo reductase activity was detected in consortium culture as compared to the activity detected in the cells of *B. laterosporus*. It should be noted that, there were absence of few biotransformation enzymes

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in the individual microbial species during decolorization. In contrast, the same enzymes were in positive action in consortium system during decolorization of RR 198. This collective action of all the oxido-reductive enzymes in consortium might be the key factor of rapid decolorization. The communal enzyme activities of mixed microbial cultures are far better for dye decolorization as compared to individual strains.[31,36,37] These enzymes have been found in an active role in the biodegradation of various textile dyes such as Acid Violet 19, Amaranth,[13,32] Green HE4B,[38] Malachite Green[39] and mixture of dyes.[35]

3.4. Product characterization

Chromatographic technique can be used to detect the biotransformation process of a substrate molecule. In the present study, the biotransformation of RR 198 was monitored by FTIR, HPTLC, and GC-MS. Fig 3A elucidated the FTIR spectrum of RR 198. The FTIR spectrum of the RR 198 dye showed the peaks at 2889 cm^{-1} for C-H stretching vibrations in alkanes, 1646 and 1490 cm^{-1} for C=N stretching vibrations in cyclic α, β unsaturated compounds, 1553 cm^{-1} C=N stretching vibrations in pyridines and N-H deformation in acyclic compounds, 1400 cm^{-1} for O-H deformation in phenols, 1209 cm^{-1} for C-O stretching in phenols and also for N-H deformation in bonded N-H, 1137 cm^{-1} for S=O asymmetric stretching in sulphones and C-H deformation in substituted benzenes, 1055 cm^{-1} for C-O stretching in phenols, 740 cm^{-1} for C-Cl stretching in halogenated compounds and benzene ring with five adjacent free H atoms, 621 cm^{-1} for C-N stretching and N-H deformation in acyclic compounds. On the other side, significant disappearance of major peaks and formation of new peaks in the FTIR spectrum of metabolites obtained after dye decolorization suggested the biotransformation and effective mineralization of RR 198 by consortium than individual microorganisms (Fig 3B-D).

View Article Online
DOI: 10.1039/C4RA15834B

HPTLC analysis of control dye and metabolites obtained after decolorization clearly suggest the biotransformation of RR 198 into simple compounds as multiple bands were obtained in the metabolites lane of consortium and also in metabolites produced by individual microorganisms (Fig 4). However, the number of separated compounds and their Rf values were different than control dye (data not shown). In the lane of metabolites obtained via consortial degradation, eleven bands were detected while nine and six bands were obtained in the lane of metabolites extracted after degradation of RR 198 by *B. laterosporus* and *G. geotrichum* respectively. These observations suggested the catalytic superiority of the consortium in dye degradation. The change of Rf value of RR 198 (0.90) and presence of multiple products in metabolites lane provided the supplementary substantiation for biodegradation of dyes as recorded by FTIR.

In order to get an insight of difference between the metabolic fate of RR 198 by consortium and pure cultures during degradation of dye, the degradative pathways was proposed using GC-MS data of metabolites extracted after the degradation of RR 198, which would help us to get the answer, how actually the consortium works? Fig 5 illustrates the proposed pathway of RR 198, degraded by consortium and individual organisms on the basis of GC-MS analysis of products formed within 18 h and Table 3 represents mass spectra of the metabolites formed. In all three possibilities, biodegradation of RR 198 was initially followed by reduction of azo bond. Azo dyes can be cleaved symmetrically and asymmetrically, with an active site available for an enzyme to excite the molecule.[27] Accordingly, the first step in degradation of RR 198 by consortium was asymmetric cleavage of azo bond via azo reductase which lead to formation of intermediate [A] 2-(phenylsulfonyl)ethanesulfonate detected at retention time in GC, Rt-24.325, having molecular weight of MW-249 based on the obtained mass to charge ratio of m/z-247(+2) Fig 5(c). Further desulphonation of this intermediate lead to the product [B] (ethylsulfonyl)benzene detected at retention time in GC,

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DOI: 10.1039/C4RA15834B

Rt-22.417 having molecular weight of MW-170 and the mass to charge ratio of m/z-170 by the action of veratryl alcohol oxidase. On the other side, laccase was responsible for dechlorination, desulphonation and deamination of postulated intermediate [1] which yielded product [C] naphthalen-1-ol [(Rt-20.167, MW-144) based on the obtained mass to charge ratio of m/z-146(-2)], and a reactive intermediate [D] 1,3,5-triazin-2-amine [(Rt-21.508, MW-96) based on the mass to charge ratio of m/z-97(-1)] and an unidentified product [2]. The formation of final product [E] 1,3,5-triazin [(Rt-19.500, MW-81) according to the mass to charge ratio obtained at m/z-83(-2)] indicates deamination of reactive intermediate [D] by veratryl alcohol oxidase.

In case of degradation of RR 198 by individual organism, different metabolites were obtained. In case of biodegradation of dye by *B. laterosporus*; formation of products [A] 2-(phenylsulfonyl)ethanesulfonate [detected at retention time of Rt-21.467 having molecular weight of MW-249 according to the mass to charge ratio of m/z-248(+1)], [B] (ethylsulfonyl)benzene [Rt-20.625, MW-170 based on the mass to charge ratio achieved at m/z-170] and [C] 6-chloro-*N*-phenyl-1,3,5-triazine-2,4-diamine [detected at Rt-17.350, MW-221 based on the mass to charge ratio of m/z-223(-2)] occurred due to the positive role of azoreductase and veratryl alcohol oxidase [Fig 5(a)]. Whereas, only one product i.e. [A] (ethylsulfonyl)benzene [Rt-19.892, MW-170, according to the mass to charge ratio of m/z-170] was obtained in a single step via azo bond cleavage and desulphonation by azoreductase and veratryl alcohol oxidase respectively from *G. geotrichum* [Fig 5(b)]. These results suggested individual organisms partially mineralized the parent dye molecule; whereas, consortium totally mineralized the RR 198 into simple forms. Recently, in a different approach, 96% decolorization of RR 198 was attained within 60h using a plant system,[40] but totally different metabolites were obtained which were not completely mineralized as

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DOI: 10.1039/C4RA15834B

consortium did in the present study. These observations suggest that, each species has its own way to act upon a substrate molecule with different fate of metabolism.

3.5. Phytotoxicity

The untreated textile effluent released to the environment influence their toxicity, when used directly for the agriculture purpose. Hence it is of prime intention to study the toxic effect of treated as well as untreated dye samples on commonly used agricultural plants. Table 2 represents the toxicity analysis of the RR 198 and its metabolites obtained after decolorization. In the dye samples, seed germination was inhibited by 50 and 30 % in *Sorghum vulgare* and *Phaseolus mungo* respectively. But after the treatment with consortium, germination of *Sorghum vulgare* and *Phaseolus mungo* was enhanced by 100 and 30% respectively when compared to untreated dye. In addition, the metabolites produced after degradation of RR 198 might have played a nutritive role in plant growth regulation as both the plants shown better growth in metabolites solution as compared to plants grown in plain distilled water. Reduction in shoot and root length of both the plants clearly suggested the toxicity of untreated azo dye. While, reduction in toxicity of dye after treatment proved the effectiveness of consortium.

Conclusions

This study clearly suggests that the bacterial-yeast consortium has an enormous strength to catalyze the azo dye-RR 198 within short period rather than individual microbial species. FTIR, HPTLC and GC-MS analysis indicated better mineralization of dye by consortium with significant reduction in its toxicity. The tolerance capacity of this developed consortium with high dye concentrations also recommends application of this consortium into effluent treatment plant.

View Article Online
DOI: 10.1039/C4RA15834B

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View Article Online
DOI: 10.1039/C4RA15834B

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View Article Online
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Figure captions

Figure 1 Decolorization of RR 198 by consortium (—◆—), *B. laterosporus* (—■—) and *G. geotrichum* (—▲—) at pH 7, 40 °C and in static condition with dye concentration of 50 mg l^{-1}.

Figure 2 Effect of different concentration of dye concentration [50 mg l^{-1} (—◆—), 100 mg l^{-1} (—■—), 200 mg l^{-1} (—▲—), 300 mg l^{-1} (—●—), 400 mg l^{-1} (—×—) on decolorization by consortium (A), *B. latersporus* (B) and *G. geotrichum* (C) at pH 7, 40 °C and in static condition.

Figure 3 FTIR spectrum of control dye RR 198 (a) and its metabolites extracted after decolorization (18 h) at pH 7, 40 °C in static condition by consortium (b), *B. laterosporus* (c) and *G. geotrichum* (d).

Figure 4 HPTLC chromatogram (I) and HPTLC plate (II) of control dye RR 198 (a) and metabolites produced after its degradation (18 h) at pH 7, 40 °C in static condition by consortium (b), *G. geotrichum* (c) and *B. laterosporus* (d).

Figure 5 Proposed metabolic pathway of RR 198 by *B. laterosporus* (a), *G. geotrichum* (b) and consortium (c).

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DOI: 10.1039/C4RA15834B

Table 1 Enzyme activities of cells obtained after decolorization (18 h) of RR 198.

		G. geotrichum	*B. laterosporus*	**Consortium**
Veratryl alcohol oxidase[a]		NA	1.846 ± 0.154	1.051 ± 0.106*[$]
Laccase[a]		0.942 ± 0.046	NA	1.084 ± 0.087*[$]
Tyrosinase[b]	Intracellular	1408 ± 151	1115 ± 18.57	NA
	Extracellular	1217 ± 111	253.4 ± 14.72	NA
NADH-DCIP reductase[c]		183.96 ±11.22	230.94 ± 34.36	263.03 ± 23.79*[$]
Riboflavin reductase[d]		14.39 ± 0.296	NA	4.80 ± 0.126*[$]
Azoreductase[e]		5.546 ± 0.481	3.890 ± 0.157	4.738 ± 0.227*[$]

[a] Activity in units/min/mg protein.
[b] Activity in IU.
[c] µg of DCIP reduced/min/mg protein.
[d] µg of Riboflavin reduced /min/mg protein.
[e] µM of MR reduced /min/mg protein.
NA- No activity.

Values are mean of three experiments ± SEM. Enzyme activities of consortium are significantly different from the *G. geotrichum* at *P < 0.001 and significantly different from the *B. laterosporus* at $P < 0.001 and by one-way ANOVA with Tukey-Kramer Multiple Comparisons Test.

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DOI: 10.1039/C4RA15834B

Table 2 Phytotoxicity study of RR 198 and the metabolites obtained after its decolorization (18 h) by consortium.

Observations	*Sorghum vulgare*			*Phaseolus mungo*		
	I	**II**	**III**	**I**	**II**	**III**
Germination (%)	100	50	100	100	70	100
Plumule length (cm)	3.47 ± 0.180	2.32 ± 0.182*	4.83 ± 0.217$^{\$}$	12.52 ± 0.271	10.31 ± 0.469*	14.28 ± 0.196$^{\$}$
Radicle length (cm)	5.11 ± 0.133	1.38 ± 0.22*	4.91 ± 0.271$^{\$}$	5.72 ± 0.116	2.35 ± 0.120*	4.76 ± 0.211$^{\$}$

I: Seeds germinated in distilled water.
II: Seeds germinated in RR 198.
III: Seeds germinated in metabolites obtained after decolorization of RR 198.

Data was analyzed by one way analysis of variance (ANOVA) with Turkey-Kramer multiple comparison test using mean values of germinated seeds of three experiments. Seeds germinated in RR 198 are significantly different from the seeds germinated in plain Distilled water at *$P<0.001$ and the seeds germinated in degradation products are significantly different from the seeds germinated in RR 198 at $^{\$}P<0.001$.

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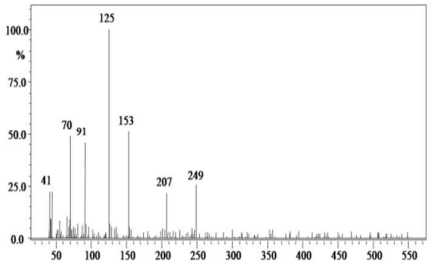
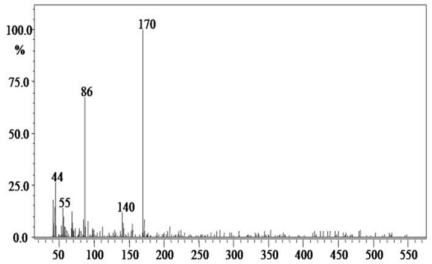
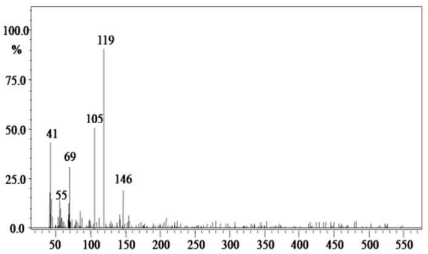
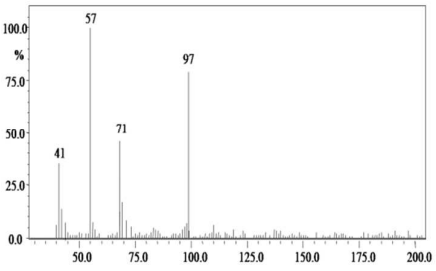
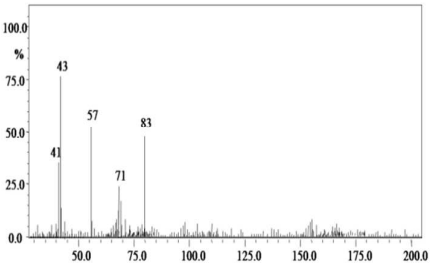
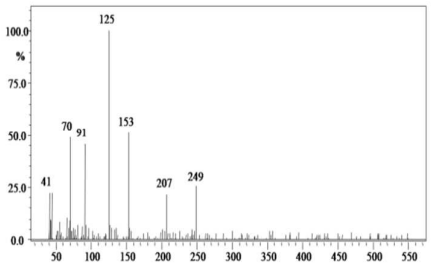
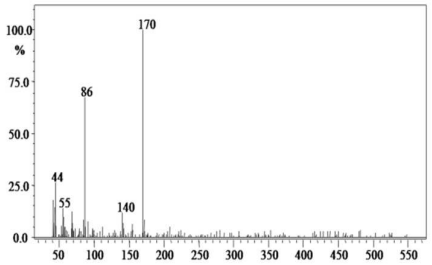
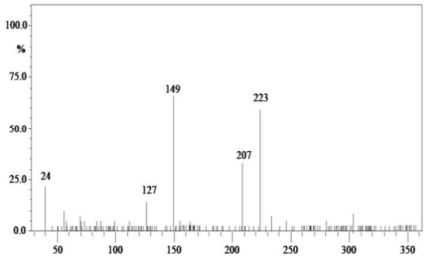
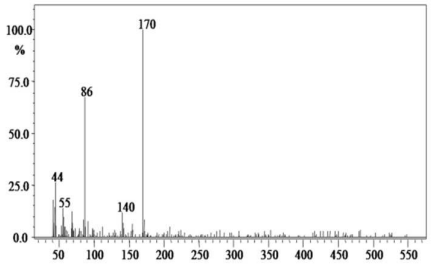
DOI: 10.1039/C4RA15834B

Table 3 The GC-MS analysis of metabolites obtained at 18 h decolorization of RR 198.

	Metabolite	Retention time (min)	m/z	Mass spectra
	1. Consortium			
[A]	2-(phenylsulfonyl)ethanesulfonate	24.325	249	
[B]	(ethylsulfonyl)benzene	22.417	170	
[C]	naphthalen-1-ol	20.167	146	
[D]	1,3,5-triazin-2-amine	21.508	97	
[E]	1,3,5-triazin	19.500	83	
	2. *B. laterosporus*			

View Article Online
DOI: 10.1039/C4RA15834B

[A]	2-(phenylsulfonyl)ethanesulfonate	21.467	249	
[B]	(ethylsulfonyl)benzene	20.625	170	
[C]	6-chloro-*N*-phenyl-1,3,5-triazine-2,4-diamine	17.350	223	
	3. *G. geotrichum*			
[A]	(ethylsulfonyl)benzene	19.892	170	

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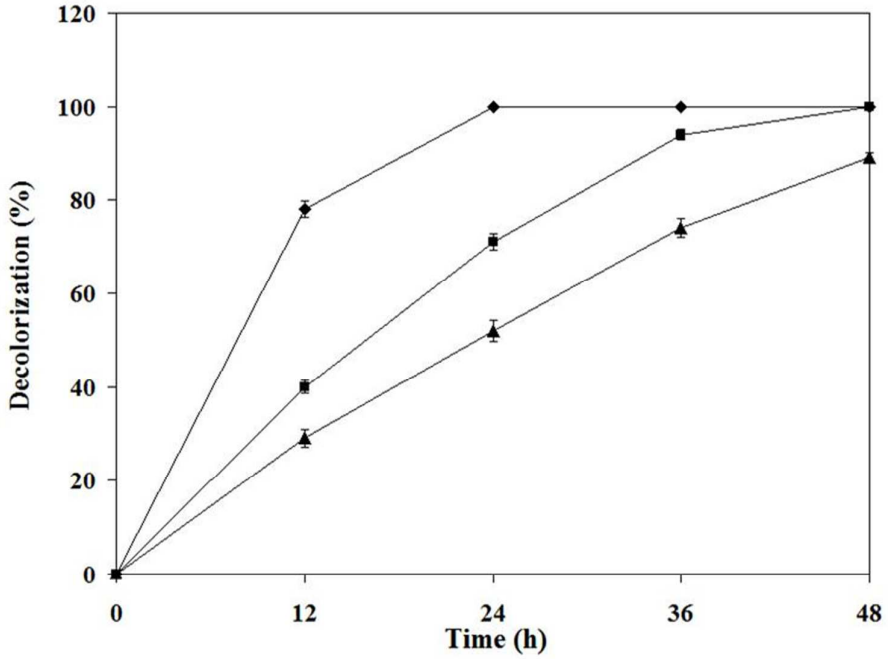

Fig. 1

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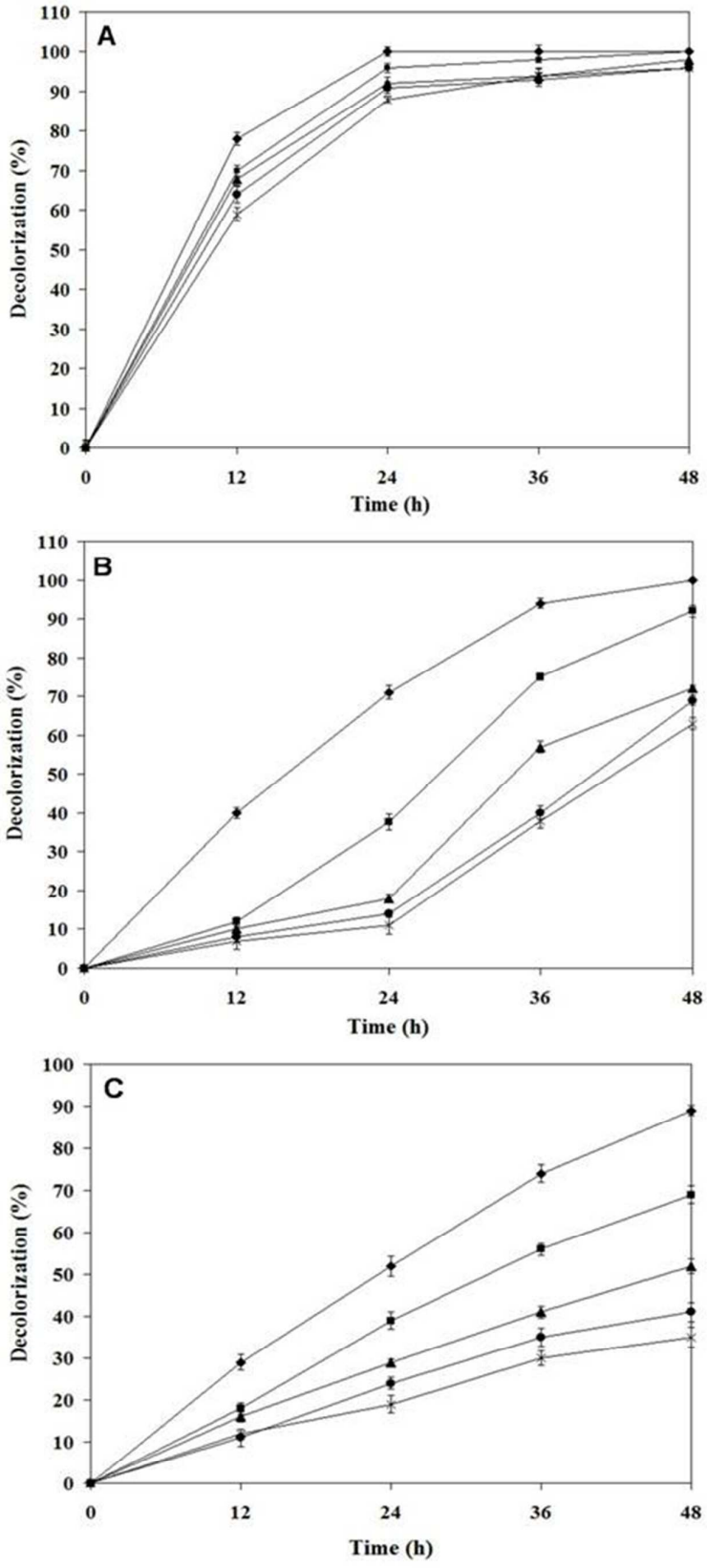

Fig. 2

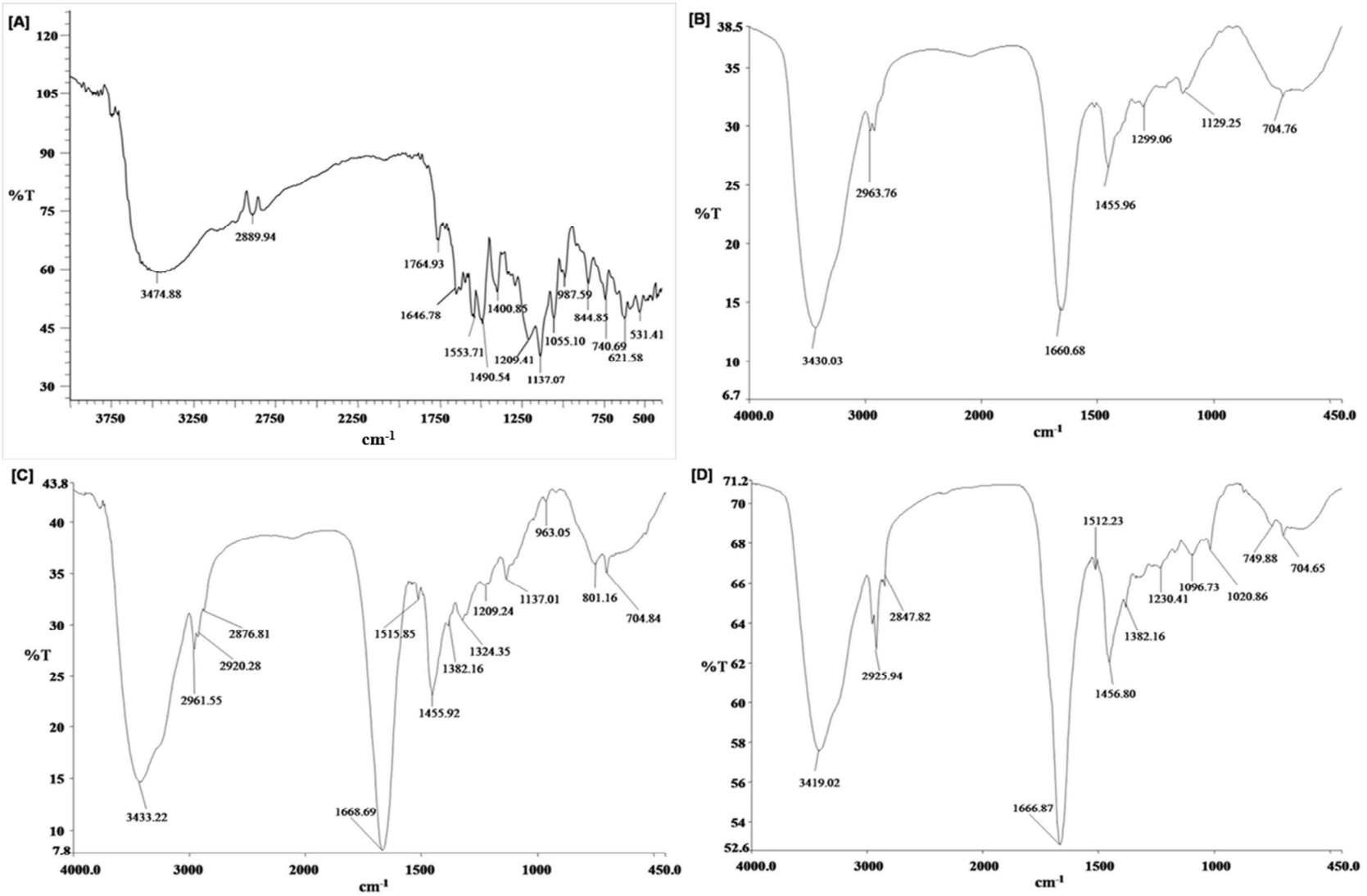

Fig. 3

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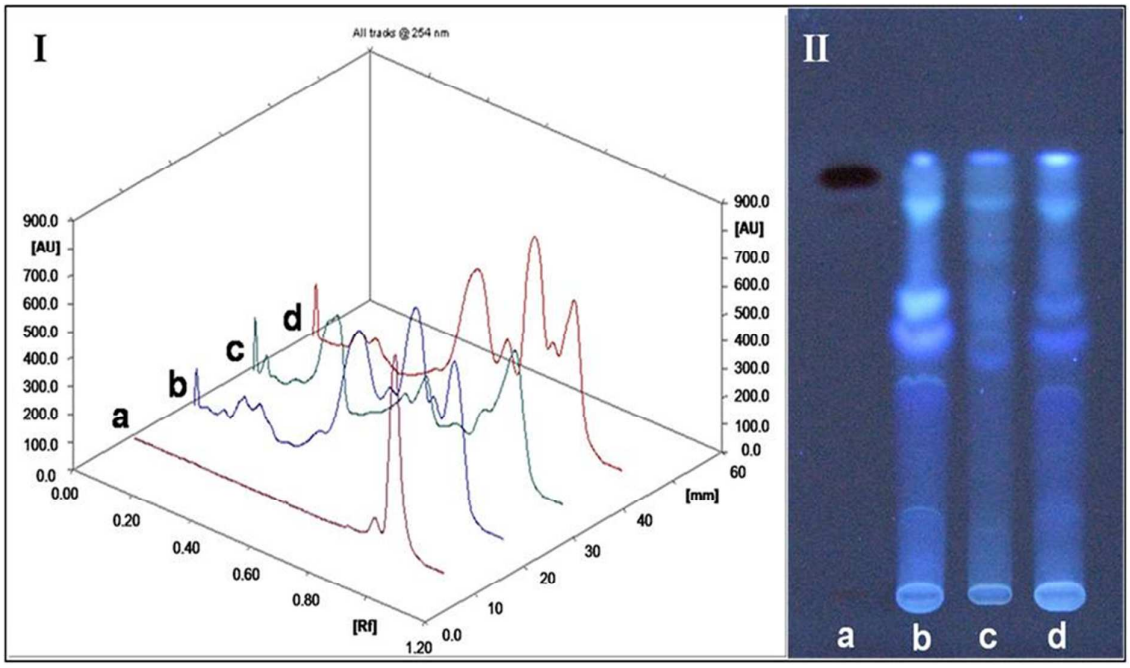

Fig. 4

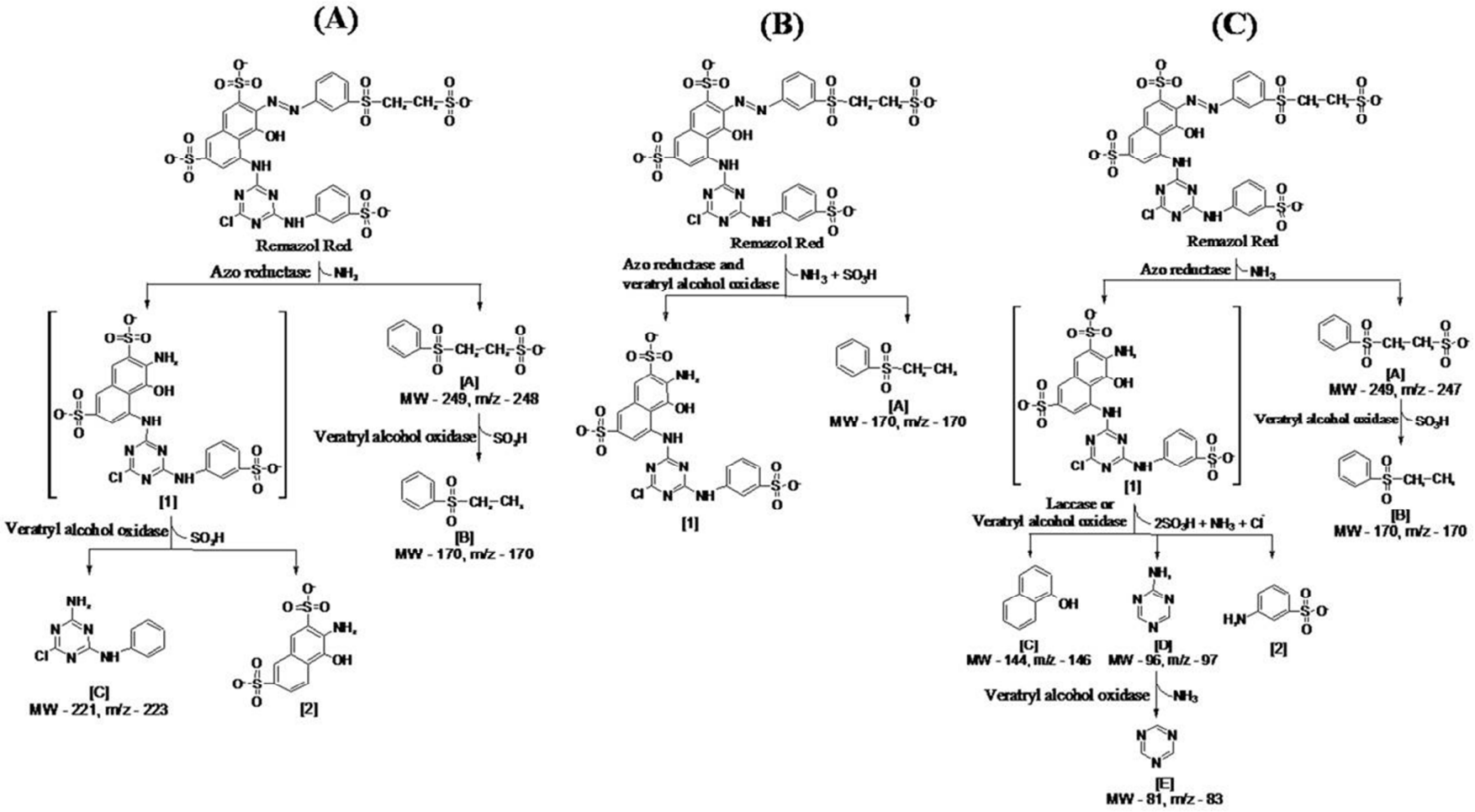

Fig. 5