Journal of Hazardous Materials 186 (2011) 713–723

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity, genotoxicity and oxidative stress studies

Swapnil S. Phugare [1], Dayanand C. Kalyani [1], Asmita V. Patil, Jyoti P. Jadhav *

Department of Biochemistry, Shivaji University, Kolhapur 416004, India

ARTICLE INFO

Article history:
Received 31 August 2010
Received in revised form 9 November 2010
Accepted 12 November 2010
Available online 19 November 2010

Keywords:
Consortium
Laccase
Oxidative stress
Protein oxidation
Lipid peroxidation

ABSTRACT

The present study aims to evaluate Red HE3B degrading potential of developed microbial consortium SDS using two bacterial cultures viz. *Providencia* sp. SDS (PS) and *Pseudomonas aeuroginosa* strain BCH (PA) originally isolated from dye contaminated soil. Consortium was found to be much faster for decolorization and degradation of Red HE3B compared to the individual bacterial strain. The intensive metabolic activity of these strains led to 100% decolorization of Red HE3B (50 mg l^{-1}) with in 1 h. Significant induction of various dye decolorizing enzymes viz. veratryl alcohol oxidase, laccase, azoreductase and DCIP reductase compared to control, point out towards their involvement in overall decolorization and degradation process. Analytical studies like HPLC, FTIR and GC–MS were used to scrutinize the biodegradation process. Toxicological studies before and after microbial treatment was studied with respect to cytotoxicity, genotoxicity, oxidative stress, antioxidant enzyme status, protein oxidation and lipid peroxidation analysis using root cells of *Allium cepa*. Toxicity analysis with *A. cepa* signifies that dye Red HE3B exerts oxidative stress and subsequently toxic effect on the root cells where as biodegradation metabolites of the dye are relatively less toxic in nature. Phytotoxicity studies also indicated that microbial treatment favors detoxification of Red HE3B.

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1. Introduction

There has been an alarming increase in the pollution of various water bodies due to industrialization during the past few decades. Textile industry is a leading contributor to the pollution of water bodies. The color produced by minute amount of organic dyes in water is considered very important because, besides having possible harmful effects, the color in water is aesthetically unpleasant [1]. Colored water eventually affects entire ecosystem. Sometimes dyes used in the coloration process are also toxic and even carcinogenic in nature, ultimately affecting the living system badly, including plant, animals and humans. Overall conditions dictate the necessity of dye containing water to undergo treatment before disposal to the environment.

During the past two decades, several physicochemical decolorization techniques have been reported, few, however, have been accepted by the textile industries [2,3]. The continuous application of these methods put forth their disadvantages as, expensive nature, formation of sludge and the emission of toxic substances [4]. Sometimes, physico-chemical treatments are unable to remove the recalcitrant azo dyes and/or their organic metabolites completely, eventually generating a significant amount of sludge that may cause secondary pollution problems [5].

Microbial decolorization of dyes has recently received much attention as it is a cost-effective method for dye removal [6,7]. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms [5]. Several microbial sources have been reported for dye decolorization including, fungal and bacterial cultures. Recently, trend is shifting towards use of mixed microbial culture compared to individual strain. Several microbial consortia have been reported for efficient dye removal [8,5]. In microbial consortium, the individual strains may attack the dye molecule at different positions or may utilize metabolites produced by the co-existing strains for further decomposition [9].

It is well established that pollution lowers the quality of life in various aspects, and affects health and life span; therefore bioremediation of pollutants for reduction of their toxic effects is of prime importance. The resurgence of genotoxicity studies has led to the development of more than 200 short term assays to evaluate the genotoxic effect of unknown environmental agents [10]. However, most animal assays are costly and thus the enforcement agencies are not using them for their routine monitoring. Keeping

* Corresponding author. Tel.: +91 231 2609153; fax: +91 231 2691533.
E-mail address: jpjbiochem@gmail.com (J.P. Jadhav).
[1] These authors have equal contributions.

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doi:10.1016/j.jhazmat.2010.11.049

this in view, plant bioassays, which are considerably less expensive, have been proposed from time to time for pollution monitoring [11]. Some of the plant bioassays, viz. *Allium cepa*, *Vicia faba* and *Tradescantia paludosa*, are used over few decades for genotoxicity assessment. Genotoxic, cytotoxic effects of various chemicals, industrial effluents on the root cells of *A. cepa* have been demonstrated previously [8,11–13].

Present study evaluates the potential of developed consortium SDS to degrade and detoxify Red HE3B, a commonly occurring dye in the textile effluent. To the best of our knowledge, demonstration of dye induced oxidative stress, lipid peroxidation and protein oxidation in the root cells of *A. cepa* is for very first time.

2. Materials and methods

2.1. Chemicals and dye stuff used

The dye Red HE3B (Reactive red 120) and other textile dyes used in the present study were courteously supplied by local textile industry Ichalkaranji, India. ABTS (2,2′-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid)) and NADH (Nicotinamide Adenine Dinucleotide reduced form), were purchased from Sigma Chemical Company (USA). Lower melting point agarose (LMPA) and normal melting point agarose (NMPA) were purchased from Sigma–Aldrich, USA. Thiobarbituric acid, trichloro acetic acid and veratryl alcohol were purchased from Hi-media, Mumbai, India.

2.2. Isolation, screening and identification of the dye degrading microorganisms

The isolation, screening and identification of efficient dye degrading microorganisms were carried out as per our earlier report [14]. The 16S rRNA analysis was performed at geneOmbio Technologies, Pune, India. The ∼1 kb rDNA fragment was amplified using high-fidelity PCR polymerase. The gene amplification by polymerase chain reaction was achieved using forward primer: 5′-AGAGTRTGATCMTYGCTWAC-3′ and reverse primer 5′-CGYTAMCTTWTTACGRCT-3′. The partial 16S rRNA gene sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences were downloaded. Phylogenetic tree was constructed by Neighbour-Joining method [15] using the MEGA4 package [16].

2.3. Medium used for culture maintenance and decolorization study

Both the bacterial strains (stock cultures) were maintained routinely on the yeast extract agar slants containing ($g\,l^{-1}$): NaCl 5.0, yeast extract 5.0 and agar 25.0 and stored at 4 °C until used. The organisms from stock culture were used for the decolorization studies after pre-culturing in yeast extract broth ($g\,l^{-1}$): NaCl 5 and yeast extract 5. A loopful of each microbial culture was inoculated separately in 10 ml culture tubes containing 5 ml yeast extract broth and incubated for initial 6 h, followed by aseptic mixing of each broth together in 90 ml of sterilized yeast extract medium and further incubation up to 24 h to develop the consortium. The consortium thus prepared was used during all of our studies and designated as consortium SDS.

2.4. Decolorization of studies using bacterial consortium SDS

The Red HE3B was added at the concentration of $50\,mg\,l^{-1}$ to 250 ml Erlenmeyer flask containing 100 ml consortium SDS (24 h grown). Aliquots (3 ml) of the culture media were withdrawn at different time intervals (15, 30, 45 and 60 min) and centrifuged at 7000 × *g* for 15 min to separate the bacterial cell mass. Decolorization of the dye Red HE3B was analyzed using UV–vis spectrophotometer (Hitachi U 2800, Tokyo, Japan) at 530 nm. All decolorization experiments were performed in triplicate and the decolorization activity was expressed in terms of the percentage decolorization using following formula

$$\text{Decolorization}(\%) = \frac{A_i - A_t}{A_i} \times 100$$

where A_i was the initial absorbance and A_t is the absorbance at incubation time *t*. The above mentioned protocol was followed while studying the effect of static and shaking conditions, physicochemical parameters, increasing dye concentration and repeated dye addition on decolorization.

2.5. Preparation of cell free extracts

The cells of consortium SDS developed in the yeast extract medium for 24 h were harvested by the centrifugation at 7000 × *g* for 20 min and considered as a control. The cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.4) and sonicated (Sonics-vibracell ultrasonic processor, 30 s, 7 strokes of the 60 amplitude) at 4 °C. The sonicated cells were centrifuged in cold condition (at 4 °C; 7000 × *g* for 20 min) and used as the source of intracellular enzymes. Similar procedure was used to quantify the enzyme activities after dye decolorization.

2.6. Enzymatic assays

Activities of azoreductase, laccase, veratryl alcohol oxidase and DCIP reductase were assayed spectrophotometrically. The laccase and azoreductase assays were performed as reported earlier [17,18]. Enzyme activities were calculated using extinction coefficient of oxidized ABTS ($3.6 \times 10^4\,M^{-1}\,cm^{-1}$) at 420 nm and of methyl red ($23{,}360\,M^{-1}\,cm^{-1}$) at 430 nm. Activity of veratryl alcohol oxidase and DCIP reductase was calculated by previously quoted methods [19,20]. The reduction of DCIP was calculated using extinction coefficient of $19\,mM\,cm^{-1}$.

2.7. Biodegradation analysis

The culture broth after Red HE3B decolorization was extracted with equal volume of ethyl acetate. The extracts were then dried by evaporation; small portion of remaining residue was redissolved in HPLC grade methanol and used for, HPLC and GC–MS analysis whereas remaining residue was mixed with stereoscopically pure KBr and used for FTIR analysis. FTIR analysis was carried out using Simadzu 8400S spectrophotometer in the mid-infrared region of 400–4000 cm^{-1} with 16-scan speed. HPLC analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector, using C_{18} column (4.6 × 250 mm) and HPLC grade methanol as mobile phase with flow rate $1\,ml\,min^{-1}$. The identification of metabolites formed after degradation of Red HE3B was carried using a QP2010 gas chromatography coupled with mass spectroscopy (Shimadzu).

2.8. Toxicological studies of dye and dye metabolites

Toxicological studies of dye and its metabolites were carried out using *A. cepa* test and phytotoxicity assay. *A. cepa* test was conducted with respect to parameters such as cytotoxicity, genotoxicity, Comet assay, antioxidant enzyme status, lipid peroxidation and protein oxidation.

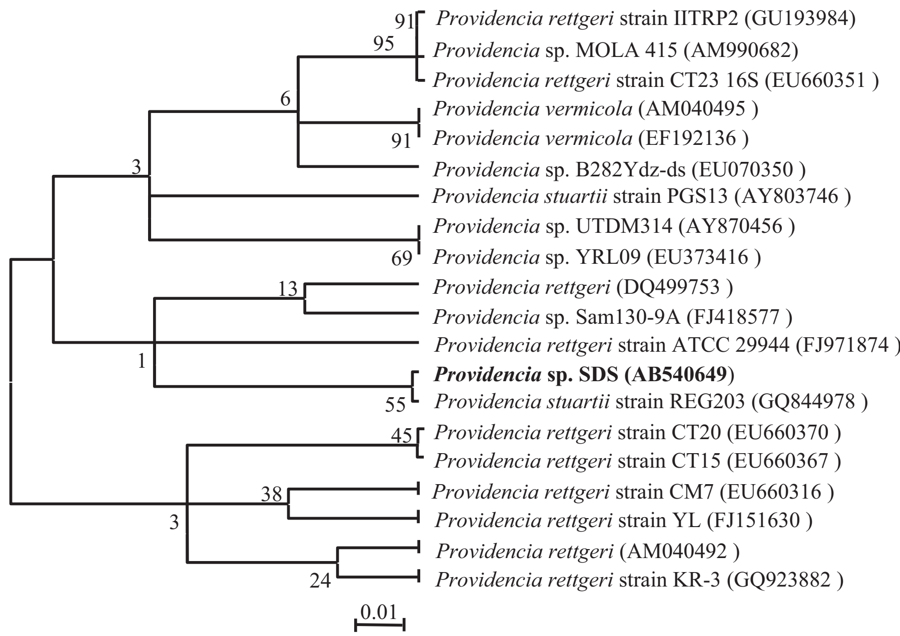


Fig. 1. Phylogenetic analysis of 16S rRNA gene sequence of *Providencia* sp. SDS. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 replicates. The scale bar (0.01) indicates the genetic distance. Brackets represent sequence accession numbers.

2.8.1. A. cepa test

2.8.1.1. Cytotoxicity and genotoxicity analysis. Small bulbs of *A. cepa* with uniform size and shape were exposed to water initially for development of roots. The bulbs thus prepared were grouped in to three sets, followed by 48 h exposure to dye sample (500 mg l^{-1} of Red HE3B, Set I), biodegradation metabolites of dye (500 mg l^{-1}, Set II) and water (control, Set III). After exposure, the bulbs were removed and thoroughly washed in running tap water and used for further cytotoxicity and genotoxicity studies as reported previously [8].

2.8.1.2. Comet assay (single cell gel electrophoresis). Comet assay was carried using previously quoted protocol [12,13]. For each slide, 25 randomly chosen nuclei were analyzed using an inverted microscope. A computerized image analysis system (Comet version 1.5) was employed to measure % DNA damage (%*T*) and tail length (TL).

2.8.1.3. Antioxidant enzyme status, protein oxidation and lipid peroxidation. Antioxidant enzymes namely catalase (CAT, E.C. 1.11.1.6), superoxide dismutase (SOD, E.C. 1.15.1.1), guaiacol peroxidase (GPX, E.C. 1.11.1.7) and ascorbate peroxidase (APX, E.C. 1.11.1.11) were analyzed by Spectrophotometric as well as In-gel assays using the procedure reported earlier [13]. Spectrophotometric assays were initiated by adding aliquots of enzyme extracts containing 50 μg of protein to 3 ml of reaction mixture. In case of In-gel enzyme assays, protein extracts containing equal amounts of soluble protein (100 μg) were subjected to native discontinuous polyacrylamide gel electrophoresis under non-reducing and non-denaturing conditions.

Protein oxidation and lipid peroxidation was assayed using previously quoted protocol [13]. The reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) was used to determine the amount of protein oxidation. Carbonyl concentration was calculated from the difference in absorbance of the test and the blank using extinction coefficient of DNPH (ε = 22 mM^{-1} cm^{-1}) and expressed in nmol of DNPH incorporated mg^{-1} of protein. Lipid peroxidation was measured as the amount of MDA produced by the TBA reaction. The concentration of MDA was calculated using an extinction coefficient (ε = 155 mM^{-1} cm^{-1}) and expressed in nmol g^{-1} FW.

2.8.2. Phytotoxicity assay

The phytotoxicity study was carried out at room temperature using *Phaseolus mungo* and *Triticum aestivum* seeds by irrigating separately 5 ml samples of Red HE3B (500 mg l^{-1}) and its degradation metabolites (500 mg l^{-1}) per day. Control set was carried out using plain water at the same time. Length of plumule (shoot), radical (root) and germination (%) was recorded after 7 days.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with the Tukey–Kramer multiple comparisons test.

3. Results and discussion

3.1. Identification and phylogenetic position of the isolates

The *Pseudomonas aeuroginosa* strain BCH, one of the strains used in present study was isolated previously [14]. The second strain used in the consortium development was isolated by enrichment culture technique and identified as *Providencia* sp. SDS using 16S rRNA gene sequence analysis. The 16S rRNA gene sequence was blasted at NCBI server followed by downloading of the homologues sequences and construction of phylogeny using MEGA4 software tool. Fig. 1 showed phylogenetic relationship between the *Providencia* sp. SDS and other related microorganisms found in the GenBank database. The digits adjacent to nodes are the statistical frequency of the indicated species. The numbers shown in parentheses are accession numbers of different species. The nucleotide sequence (954 bp) of strain *Providencia* sp. SDS is deposited in DDBJ under the accession number AB540649. *P. aeuroginosa* strain BCH was previously quoted for decolorization of Direct orange 39 [14]. Similarly, other *Pseudomonas* species are also well known for dye decolorization [8]. However, very few reports are available regarding the role of *Providencia* species in bioremediation.

Table 1
Various dye decolorization by *Providencia* sp. SDS (PS), *Pseudomonas aeuroginosa* strain BCH (PA) and consortium SDS.

Dyes	*Providencia* sp. SDS (PS)		*Pseudomonas aeuroginosa* strain BCH (PA)		Consortium SDS	
	% Decolorization	Time (h)	% Decolorization	Time (h)	% Decolorization	Time (h)
Red HE3B[a]	90	12	94	5	100	1
Red HE7B[a]	78	48	91	6	97	1
Scarlet GDR[a]	89	14	93	9	98	3
Brown 3 REL[a]	85	12	84	14	99	7
Green HE4BD[a]	94	48	96	3	100	2
Direct red 5B[a]	89	20	92	2	98	1.5
Ramzol black 5B[a]	88	20	92	5	100	1

[a] 50 mg l^{-1}.

3.2. Decolorization analysis

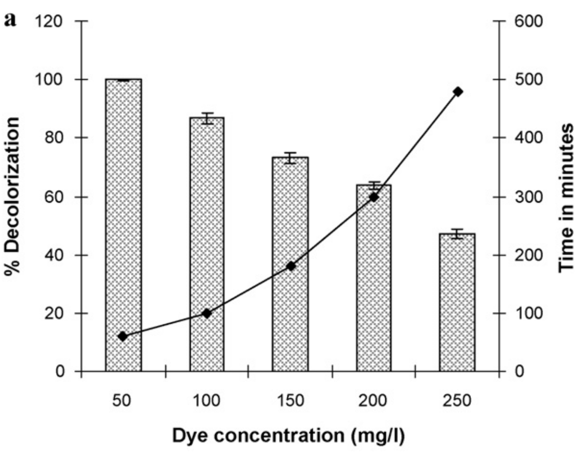
3.2.1. Comparative study of the decolorization of various industrial dyes by using individual strain (PA and PS) and developed consortium-SDS

The decolorization efficacy of a microorganism depends up on its potential to degrade various dyes [21]. The ability of a pure culture as well as the developed consortium SDS to decolorize various industrial dyes viz. Red HE3B, Red HE7B, Scarlet GDR, Brown 3REL, Green HE4BD, Direct red 5B and Ramazol black 5B (50 mg l^{-1}) were tested in the yeast extract medium under static conditions. Results presented in Table 1 clearly shows that developed consortium stand efficient compared to the pure cultures in terms of percentage decolorization and time required for decolorization. Enhanced rate of decolorization for various direct and reactive dyes by using bacterial consortium has been reported earlier [5].

3.2.2. Decolorization under various physico-chemical conditions

The effect of various physicochemical conditions (agitation, temperature, pH, dye concentration and repeated dye additions) on the decolorization of Red HE3B by using the consortium SDS was studied in detail. Monitoring of Red HE3B decolorization under static condition showed 100% decolorization within 1 h, however percentage decolorization activity was found to be retarded in case of shaking conditions, showing only 20% decolorization even after 24 h incubation. The inhibition of Red HE3B percentage decolorization under shaking conditions might be due to the azo nature of the dye. Under aerobic conditions azo dyes are generally resistant to attack by bacteria [22]. Azo dye decolorization by bacterial species if often initiated by enzymatic reduction of azo bonds, the presence of oxygen normally inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds [23]. Temperature studies revealed that consortium was operative over a wide rage of temperature, i.e. from 20 to 50 °C. The variation in temperature dose not seemed to hamper Red HE3B decolorization. On the other hand, pH was found to be the crucial parameter during the decolorization process. The pH studies showed that, neutral pH (7) was favorable for optimum decolorization, whereas extreme acidic (3 and 5) or alkaline pH (9 and 11) retards the decolorization. The pH is directly associated to the overall biochemical processes and eventually with the growth of microbial systems. Although, growth was observed at acidic and alkaline pH, the cell density might not be sufficient to favor the optimum decolorization.

To assess the maximum decolorization ability of consortium SDS on Red HE3B, consortium was tested against different concentrations of the dye (Fig. 2a). The dye concentration from 50 to 150 mg l^{-1} showed 100% decolorization. Higher dye concentrations such as 200 and 250 mg l^{-1} showed 85% and 70% decolorization respectively with variation in the time required for decolorization. This decrease in decolorization efficiency at high dye concentrations may be due to the toxicity of the dye to bacteria and/or inadequate biomass concentration for the uptake of higher concentrations of dye [5]. Repeated use of cell biomass, stand as an economical parameter in decolorization process and subsequently for industrial purpose. In the present study we have analyzed the decolorization efficacy of the consortium SDS by repeated dye addition. The consortium was able to retain its efficiency up to 6 repeated dye addition cycles with little variation in percentage decolorization as well as time requirement (Fig. 2b). The decreasing percentage decolorization was observed from 6th to 9th cycle with increase in the time requirement. Furthermore addition of dye in the 10th cycle dose not showed any decolorization. The



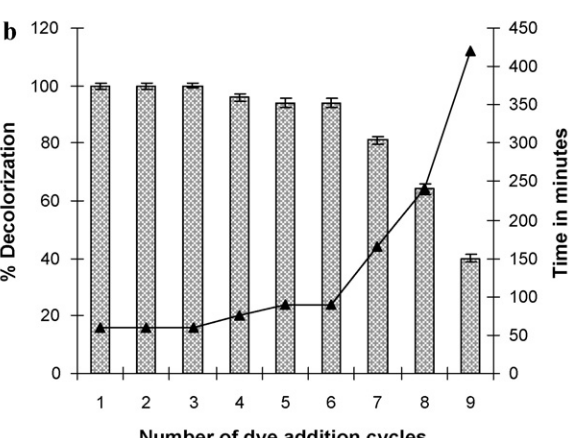


Fig. 2. Effect of initial dye concentration on decolorization (a) and effect of repeated addition of dye on decolorization (b).

Table 2
Enzyme activities in case of individual bacterium and consortium SDS.

Enzyme	Control cells			Cells obtained after decolorization of Red HE3B		
	PA	PS	Consortium SDS	PA	PS	Consortium SDS
Veratryl alcohol oxidase[a]	0.123 ± 0.23	N.D.	0.101 ± 0.43	0.298 ± 0.47*	N.D.	0.365 ± 0.34*
Laccase[a]	0.345 ± 0.032	0.232 ± 0.05	0.378 ± 0.23	0.473 ± 0.37*	0.398 ± 0.13*	0.645 ± 0.54*
DCIP reductase[b]	29.34 ± 0.034	23.54 ± 0.43	32.45 ± 0.76	36.47 ± 0.14*	35.39 ± 0.03*	51.43 ± 0.54*
Azo reductase[a]	N.D.	0.134 ± 0.07	0.125 ± 0.05	N.D.	0.324 ± 0.17*	0.312 ± 0.24*

PA: *Pseudomonas aeuroginosa* strain BCH and PS: *Providencia* sp. SDS (PS).
[a] Enzyme activity, Unit mg of protein^{-1} min^{-1}.
[b] μg DCIP reduced min^{-1} mg protein^{-1}.
Values are mean of three experiments ± SD. Significantly different from control cells at * < 0.001 by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

observed gradual decrease in decolorization might be due to the culture entering into the stationary phase and subsequently into the death phase, resulting in the inhibition of enzyme systems gradually. Similar studies were reported previously in case of individual bacterial strains [14,24].

3.3. Enzymatic analysis

The tracking of the various enzyme activities showed the involvement of various oxidative as well as reductive enzymes during the decolorization process. Induction of various enzymes during decolorization gives additional insight of decolorization mechanism and also supports the active role of microorganisms in the biodegradation process. Enzyme activities during decolorization of Red HE3B incase of individual bacterial strain as well as consortium SDS are as summarized in Table 2. Individually, strain PA shows presence of veratryl alcohol oxidase, laccase and DCIP reductase whereas PS shows presence of laccase, DCIP reductase and azo reductase. On the other hand consortium SDS bears all of these enzymes. The collective action of all of these enzymes might be the key of efficacy of consortium SDS. The role of enzymes like laccase, veratryl alcohol oxidase, DCIP reductase, azo reductase in dye decolorization is well documented [14,17–19]. The collective enzyme activities of mixed microbial cultures are far better for dye decolorization as compared to individual strains [5,8].

3.4. Biodegradation analysis

HPLC, FTIR and GC–MS analysis helped us to investigate the Red HE3B dye degrading mechanism of consortium SDS. The HPLC analysis of dye sample collected at the beginning showed a single peak at retention time 1.7 min (Fig. 3a). As, the decolorization progressed the emergence of additional peaks were observed due to degradation of parent dye (after 1 h) at retention time 1.7, 2.4 and 2.7 min (Fig. 3b). FTIR spectrum of control dye and its biodegradation metabolites is as shown in Fig. 4. The FTIR spectrum of Red HE3B showed presence of different peaks at 3435 cm^{-1} for –C–H stretching of single bridge alcoholic or phenolic compound, 2926 cm^{-1} for –CH_2 stretching of alkanes, 2097 cm^{-1} for displayed –NH_3 stretching of amino acid, 1629 cm^{-1} for –N=N– stretching of azo bonds, 1404 cm^{-1} for –OH deformation of alcohols, 1228 cm^{-1} for –S=O stretching of sulfites, 1047 cm^{-1} for –S=O stretching of sulfonic acids. The FTIR spectrum of Red HE3B metabolites showed major peaks at 2866 cm^{-1} for –C–H stretching of alkanes, 1635 cm^{-1} for stretching of secondary amides,

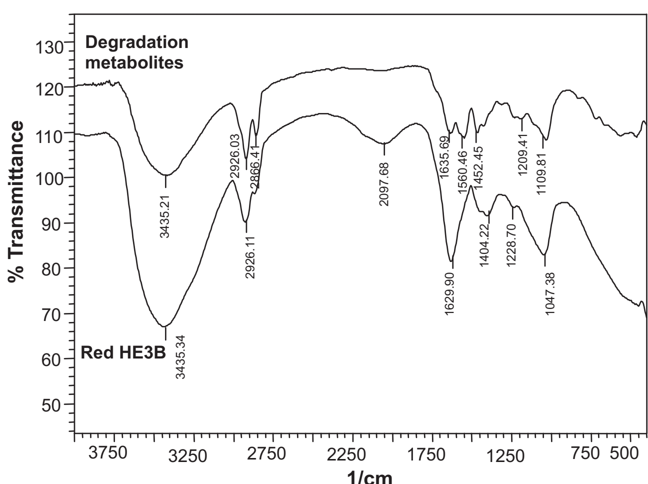


Fig. 4. FTIR spectra of Red HE3B and its biodegradation metabolites obtained after treatment with consortium SDS.

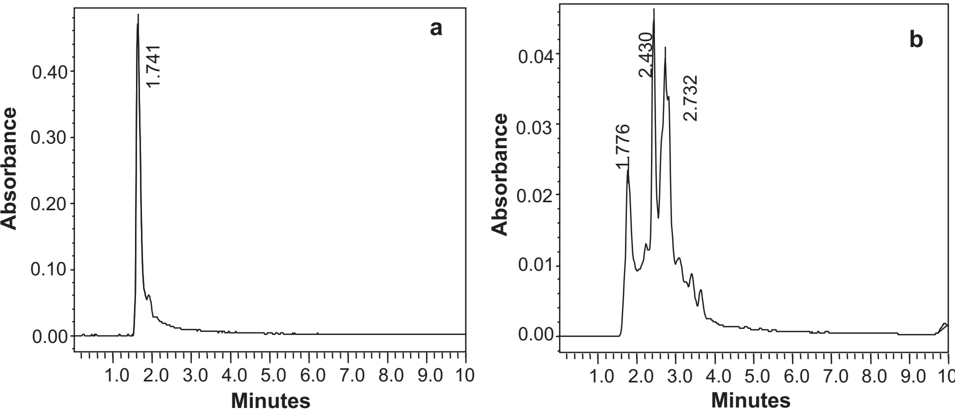


Fig. 3. HPLC elution profile of the Red HE3B (a) and its degradation metabolites obtained after treatment with consortium SDS (b).

Table 3
GC–MS spectral datasheet of metabolites formed after degradation of Red HE3B.

Sr. no.	Molecular weight of metabolite (M.W.)	Retention time (min)	Name of metabolites	Mass peaks
1	173	22.78	2-Aminobenzenesulfonic acid	
2	463	22.94	3-Amino-5[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino]-4-hydroxynaphathalene-2-sulfonic acid methanethiol (1:1)	
3	319	24.45	3-Amino-4-hydroxynaphthalene-2-7-disulfonic acid	
4	146	28.07	6-Chloro-1,3,5-triazine-2,4-diamine	
5	159	26.66	2-Aminonaphthalene-1-ol	
6	144	21.86	Naphthalene-1-ol	

1560 cm^{-1} for stretching of amino acid salts, 1452 cm^{-1} for –C–H deformation of alkanes, 1209 cm^{-1} for –S=O stretching of sulfonic acids, 1109 cm^{-1} for –C–OH stretching of secondary alcohols. The disappearance of peak 1629 cm^{-1} in case of dye metabolites gives the evidence of azo bond cleavage by the azoreductase.

The GC–MS analysis showed the probable metabolites produced during the Red HE3B biotransformation process. The structures of the detected compounds were assigned from the fragmentation pattern and m/z values. Table 3 shows molecular ion chromatograms for metabolites extracted from the culture super-

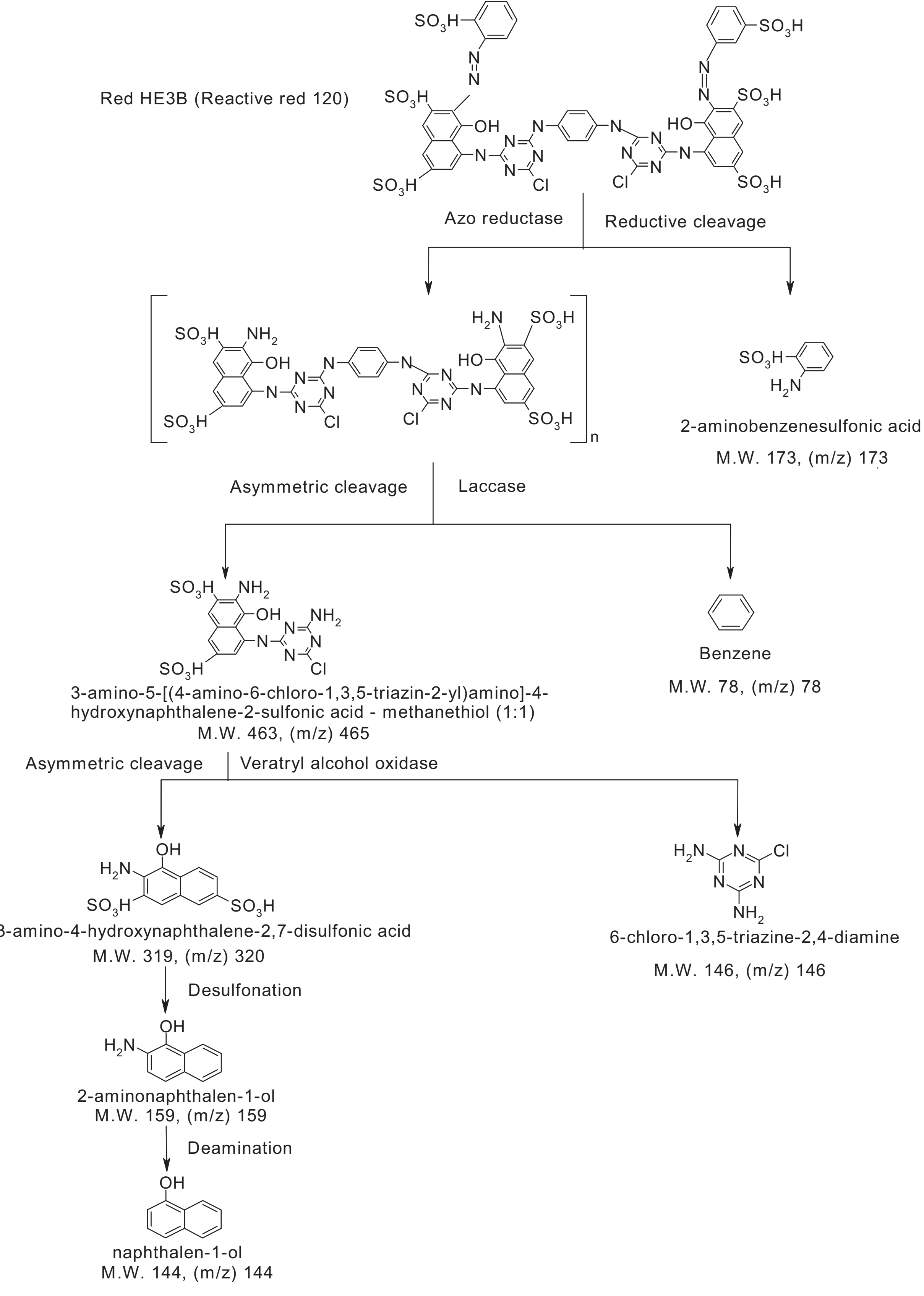


Fig. 5. Proposed pathway of Red HE3B biodegradation.

Table 4
Effect of untreated and treated (biodegraded) dye Red HE3B on root length, mitotic index (MI), number and frequency of micronuclei and chromosome breaks obtained for the *Allium cepa* tests.

Analysis	Samples		
	Control	Red HE3B3[a]	Red HE3B metabolites[a]
RL (cm)Mean ± SD	3.91 ± 0.12	3.32 ± 0.68	3.87 ± 0.34
MI Mean ± SD	11.10 ± 0.21	13.36 ± 1.16	10.98 ± 0.43
MN	0	5	1
CB	1	3	2
TA	1	8	2
TCA	2512	2589	2674
Frequency of TA	0.04 ± 0.006	0.30 ± 0.23*	0.07 ± 0.01

[a] 500 mg l^{-1}; RL: root length; MI: mitotic index; MN: micronuclei; CB: chromosome breaks; TCA: total number of cells analyzed; and TA: total number of alterations. Values are mean of three experiments, SD (±), significantly different from the control (roots germinated in water) *$P < 0.01$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

natants. On the basis various enzyme induction and mass spectrum analysis, the possible Red HE3B biodegradation pathway adapted by the consortium SDS is as illustrated in Fig. 5. Initial cleavage might start off with reduction of azo bond with action of azoreductase followed by subsequent oxidative and reductive cleavage with the help of various oxidative and reductive enzymes viz. veratryl alcohol oxidase, laccase and DCIP reductase. According to our proposal, the azoreductase catalyzed initially reductive cleavage of azo bond leads to the formation of 2-aminobenzenesulfonic acid having mass peak (m/z) 173 and postulated reactive intermediate. Asymmetric cleavage of this intermediate by laccase resulted in to formation of 3-amino-5[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino]-4-hydroxynapathalene-2-sulfonic acid-methanethiol (1:1) having mass peak (m/z) 465 accompanied by release of benzene (m/z) 78. The oxidative attack on this phenyl moiety by veratryl alcohol oxidase produce 3-amino-4-hydroxynaphthalene-2-7-disulfonic acid with mass peak (m/z) 320 and 6-chloro-1,3,5-triazine-2,4-diamine (m/z) 146. Further, 3-amino-4-hydroxynaphthalene-2-7-disulfonic acid undergoes desulfonation reaction to produce 2-aminonaphthalene-1-ol having mass peak (m/z) 159 followed by deamination reactions resulted to naphthalene-1-ol with mass peak (m/z) of 144. The conversion of complex dye molecules to simpler metabolites by oxidative and reductive mechanism with the aid of various oxidoreductive enzymes is well documented [8].

3.5. Toxicity analysis

Assessment of the ecological and genetic impact of dyes and their metabolites produced after biodegradation on plant populations is of great importance as plants are important commercial products and are consumed by people. In the present study we have used *Allium* test for toxicological analysis of dye and dye metabolites. Cytogenetic analysis showed strong genotoxic effect of dye Red HE3B on the root cells of *A. cepa* (Table 4). Cytotoxicity analysis is made depending up on increase or decrease in the mitotic index (MI). MI serves as an important parameter of cytotoxicity studies in environmental biomonitoring [25]. Insight to Table 4 gives clear idea regarding the strong genotoxic effect of Red HE3B dye molecules as compared to its biodegradation metabolites. Mitotic index of the samples exposed to 500 mg l^{-1} of the Red HE3B was 13.36%. On the other hand, mitotic index of the samples exposed to 500 mg l^{-1} of dye metabolites was found to be 10.98%, which is almost equal to that of control (11.10%). Decreased mitotic index compared to the control can serve as an indicator of presence of cytotoxic compounds in the environment [8,12]. However, increased mitotic index might be associated with

Table 5
Effect of untreated and treated (biodegraded) dye Red HE3B on number and frequency of chromosome aberrations obtained for the *Allium cepa* tests.

Analysis	Samples		
	Control	Red HE3B[a]	Red HE3B metabolites[a]
AB	3	16	3
DA	0	14	0
MC	0	3	1
LC	1	4	3
BC	0	2	0
AL	0	3	0
ML	1	3	0
TCA	5	45	7
TMO	279	346	294
Frequency of TCA	1.79 ± 0.23	13.00 ± 1.04*	2.38 ± 0.44

[a] 500 mg l^{-1}; AB: anaphase with bridge; DA: delayed anaphase; MC: multipolar chromosome; LC: laggard chromosome; BC: binucleated cell; AL: anaphase with loss; ML: metaphase with loss; TMO: total number of mitotic cells observed; and TCA: total number of cells with alterations. Values are mean of three experiments, SD (±), significantly different from the control (roots meristem germinated in water) *$P < 0.001$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

the tumor formation, uncontrolled cell division, ultimately exerting detrimental effects on the cells [8,25]. Besides mitotic index different kinds of chromosomal aberrations were observed, the most common chromosomal abnormalities in all treatments were c-mitosis, laggards, chromosome breaks, anaphase bridges, stickiness and micronuclei. Statistical analysis of the genotoxicity tests showed that the percentage of aberrant mitotic cells caused by the Red HE3B was significantly different from that of the control, as shown in Tables 4 and 5. Similar observations were previously noted with the textile effluents and dye, which concludes these molecules, exerts the strong chromosomal alterations as well as affects cell division process badly [8,25,26].

Comet assay (single-cell gel electrophoresis, SCGE) was performed to assess the genotoxic potential of the dye Red HE3B and its biodegradation metabolites. The sensitivity of the SCGE or Comet assay allows rapid prediction of genotoxic potential of compounds and has been shown to be useful for in vivo and in vitro biomonitoring of environmental pollutants [27,28]. In the present study *A. cepa* bulbs were exposed to dye Red HE3B and its biodegradation metabolites (500 mg l^{-1}). The isolated nuclei from the root meristem cells were used for electrophoretic analysis, due to naked condition and high DNA content the nuclei are highly sensitive to the electrical field. The percentage of tail DNA (% of DNA in comet tail) and tail length (μm) was measured and it was observed through ANOVA test that, a significant difference in the parameters tested as compared to control (Table 6), the comets observed are as shown in Fig. 6[A]. The results presented here collectively conclude that, this plant has great sensitivity towards the textile dye and can be used as a environmental monitoring agent. The samples treated with biodegradation metabolites showed significantly less DNA damage, the values almost matching to control. This concludes

Table 6
Detection of DNA damage in the root meristem cells of *Allium cepa* exposed to Red HE3B and its biodegradation metabolites using Comet assay.

Analysis	Samples		
	Control	Red HE3B[a]	Red HE3B metabolites[a]
TL (μm) Mean ± SD	26.64 ± 3.03	43.18 ± 3.02*	27.63 ± 2.03
%*T* Mean ± SD	40.24 ± 4.21	58.51 ± 4.12*	41.32 ± 3.43

[a] 500 mg l^{-1}; values are mean of three experiments, SD (±), significantly different from the control (roots meristem germinated in water), *$P < 0.001$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

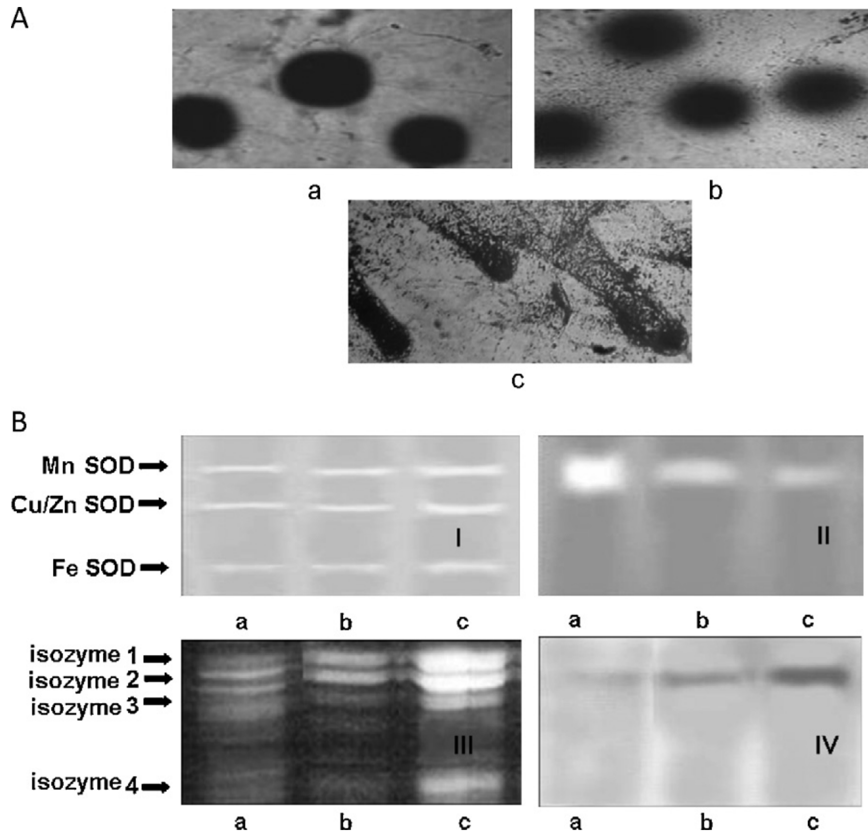


Fig. 6. Toxicity analysis using root cells of *Allium cepa* exposed to Red HE3B and its biodegraded metabolites. [A] Comets observed and [B] zymographs of various antioxidant enzymes as SOD – I; CAT – II; APX – III, GPX – IV. The samples tested are as-control (a); degradation metabolites of Red HE3B (500 mg l^{-1}) (b); and dye Red HE3B at concentration of 500 mg l^{-1} (c).

consortium SDS was found to be efficient not only for degradation of Red HE3B but also for its detoxification.

Reactive oxygen species (ROS) are chemically-reactive molecules containing oxygen and formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. Several studies have shown that environmental pollutants can induce the production of ROS in plant cells as well as in mammalian cells which induce oxidative stress and cause oxidative damage to lipid, protein and DNA [29,30]. Sometimes under environmental stress conditions ROS levels can increase dramatically, which results in significant damage to cell structures (leading to DNA damage, protein oxidation and lipid peroxidation) and this cumulates into a situation known as oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases, catalases, ascorbate peroxidase, lactoperoxidases, glutathione peroxidases and peroxiredoxins. In the present study, we have tried to evaluate the role of textile dye Red HE3B in the induction of oxidative stress using *Allium* test. Studies of various antioxidant enzymes viz. SOD, CAT, APX and GPX were assayed using the *A. cepa* root cells exposed to dye Red HE3B as well as its biodegradation metabolites. Results summarized in Table 7 shows that Red HE3B is indices activities of SOD, APX and GPX and suppressed activity of the CAT. Similar observations were noted previously in case of *A. cepa* exposed to aluminium [13]. The zymographs obtained after in gel enzyme assay are as shown in Fig. 6[B]. Such phenomenon of antioxidant enzyme stimulation or inhibition might be associated with gene induction, as it is well documented that genes encoding antioxidant enzymes are activated by Al-stress [31,32]. The studies with biodegradation metabolites showed that antioxidant enzyme activities were almost matching to the control, indicating reduction of the toxicity of dye molecules. Based on the over all experimentation and observations it might be possible that, the dye Red HE3B

Table 7
Analysis of antioxidant enzymes activities (SOD, CAT, APX, and GPX), protein oxidation, lipid peroxidation from the root cells of *A. cepa* exposed to dye Red HE3B and its biodegradation metabolites after treatment with consortium SDS.

Parameter analyzed	Control	Red HE3B[a]	Red HE3B metabolites[a]
SOD activity (inhibition of NBT reduction by 50%) mg^{-1} protein h^{-1}	3.92 ± 1.44	15.21 ± 1.54*	4.05 ± 1.79
CAT activity (nmol of H_2O_2 utilized) mg^{-1} protein	49.21 ± 3.43	24.16 ± 2.74*	39.65 ± 2.77*
APX activity (μmoles of oxidized ascorbate formed) min^{-1} mg^{-1} protein	0.67 ± 0.08	1.48 ± 0.32*	0.73 ± 0.21
GPX activity (μmoles of tetraguaiacol formed) min^{-1} mg^{-1} protein	18.32 ± 3.54	64.87 ± 3.72*	40.12 ± 2.88*
Protein oxidation (Carbonyl content in nmol mg^{-1} protein)	3.7 ± 1.65	6.47 ± 1.48*	4.07 ± 1.31
Lipid peroxidation (MDA nmol g^{-1} FW)	0.34 ± 0.18	0.96 ± 0.30*	0.38 ± 0.67

[a] 500 mg l^{-1}; values are mean of three experiments and SD (±) is significantly different from the control at, *$P < 0.001$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

Table 8
Phytotoxicity studies of Red HE3B and its biodegradation metabolites.

Parameters	*Triticum aestivum*			*Phaseolus mungo*		
	Control	Red HE3B[a]	Red HE3B metabolites[a]	Control	Red HE3B[a]	Red HE3B metabolites[a]
Germination (%)	100	40	70	100	60	80
Plumule (cm)	10.10 ± 1.35	5.16 ± 1.46*	7.21 ± 1.87	9.14 ± 1.78	4.66 ± 1.45*	8.51 ± 1.37
Radical (cm)	8.65 ± 1.23	3.35 ± 1.01*	6.01 ± 1.09	7.76 ± 1.53	3.87 ± 1.32*	7.18 ± 1.89

[a] 500 $mg\,l^{-1}$; values are mean of three experiments, SD (±), significantly different from the control (seeds germinated in water) at, *$P < 0.001$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

induces the antioxidant enzyme machinery in the root cells of *A. cepa*.

The subsequent part of oxidative stress development is oxidation of proteins and lipid peroxidation. Dose dependant increase was observed in both, protein oxidation as well as in lipid peroxidation (Table 7). The formation of ROS can lead to the lipid peroxidation chain reaction and protein oxidation which are stronger indices of oxidative stress than are alterations in antioxidant enzyme levels [33]. The induced level of lipid peroxidation it might be due to ROS spices generation through dye stress, leads to the induction of lipid peroxidation chain reaction and oxidative modification of proteins. Increased levels of lipid peroxidation and protein oxidation due to the stress developed by Al have been documented previously [13]. Lipid peroxidation and protein oxidation rate in case of samples treated with biodegradation metabolites seems to like that of control, indicating less toxic nature of dye metabolites. On the basis of previous literature and observed results in our present study, it can be concluded that textile dyes possibly serves role in the oxidative stress induction.

The untreated dyeing effluents may be hazardous to the environment, when straightly used for agriculture. Thus, it was of concern to assess the toxicity of the effluent before and after decolorization. The phytotoxicity testing was done with two agriculturally important seedlings *Phaseolus mungo* and *Triticum asetivum*, which showed better germination in case of biodegraded metabolites compared to the dye Red HE3B (Table 8). This study reveals that the metabolites generated after the biodegradation of dye are less toxic than the original dye molecule.

4. Conclusion

A developed consortium SDS bears a versatile property of dye decolorization as well as detoxification. The consortium system was found to be better alternative as compared to the individual bacterial strains. Involvement of dye decolorizing enzymes systems suggests their role in biodegradation. The dye Red HE3B was found to be inducer of overall oxidative stress in the root cells of *A. cepa*, and this stress seemed to be released in case of dye metabolites. Studies presented here demonstrate the success of consortium SDS for decolorization of dye and simultaneous reduction of toxicity.

Acknowledgements

Swapnil S. Phugare would like to thank Jawaharlal Nehru Memorial Fund, New Delhi, India for awarding JNMF fellowship for doctoral studies. Authors are thankful to Department of Environmental Science and Department of Zoology, Shivaji University Kolhapur for providing technical assistance.

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