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Decolorization and detoxification of dye mixture and textile effluent by lichen *Dermatocarpon vellereceum* in fixed bed upflow bioreactor with subsequent oxidative stress study

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ABSTRACT

Navy Blue HE22 (NBHE22), dye mixture and real textile effluent were decolorized and degraded by lichen *Dermatocarpon vellereceum*. Up-flow bioreactor showed about 80%, 70%, 80% and 65% removal of American dye manufacturer index (ADMI), biological oxygen demand (BOD), total suspended solids (TSS) and total dissolved solids (TDS), respectively of dye mixture at flow rate of 25 ml h^{-1}. The removal of ADMI, BOD, TSS and TDS of real textile effluent were 75%, 65%, 82% and 70%, respectively at flow rate of 30 ml h^{-1}. Significant induction of extracellular enzymes such as manganese peroxidase and lignin peroxidase was observed up to 46% and 36% during decolorization of dye mixture, while 43% and 24% during effluent treatment, respectively. Exponential enhancement in the activities of stress enzymes such as catalase (CAT) and guaiacol peroxidase (GPX) was observed after exposure to NBHE22 (116% and 125%, respectively), dye mixture (150% and 300%, respectively) and effluent (400% and 350%, respectively) endorsing the stress tolerance ability of model lichen. Phytotoxicity and genotoxicity studies demonstrated less toxic nature of metabolites resulted from biodegradation.

1. Introduction

Textile dyes are the chemical compounds synthesized to impart color to the fabric. Textile industry is the second largest employment generating industry in India thus plays a socioeconomically important role. Textile sector is responsible to create more pollution as they discharge colored wastewater. These wastewaters create aesthetically undesirable pollution effect if released untreated into water bodies. These wastes are non-recyclable, unavoidable and in very large volumes (Dasgupta et al., 2015). These colored pollutants are having even more hazardous and serious effects on ecology. Synthesis about thousands of different types of textile dyes is a multibillion dollar industry worldwide. It is estimated that nearly 40% of entire amount of dyes applied for dying would discharge through effluent into the water bodies. An Indian textile mill working to produce approximately 6×10^5 meters of fabric per day is likely to discharge about 1.5 million liters per day (MLD) of effluent (Basak and Dey, 2016). Textile wastewater possess higher amount of biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS) and total dissolved solids (TDS) (Archna and Siva, 2012), remain unchanged into the environment and enter the food web through water, thus inducing very serious structural as well as genetic deformities (Wong and Yu, 1999; Lade et al., 2012). Different inorganic salts present in effluent, may enter the air by evaporation thus causing allergic reactions (Khandegar and Saroha, 2013).

Biological enzyme system has proved as very efficient in dye degradation and decolorization which is relatively cheaper and can be operated at natural conditions (Kalyani et al., 2008). Oxidative enzymes such as laccase, lignin peroxidase and polyphenol oxidase are known to be involved in dye degradation, which have recently been applied in immobilized form with matrix of multiple-shelled-Fe_2O_3 yolk-shell particles (Patel et al., 2016). Different types of dyes have been successfully degraded by various biological systems. However various biological sources have been enlisted as natural pools of bioremediation enzymes including fungal (Corso and Almeida, 2009; Telke et al., 2010), bacterial (Sharma et al., 2007; Koschorreck et al., 2008; Jadhav et al., 2009; Telke et al., 2009), and plant sources (Gramss and Rudeschko, 1998; Shafique et al., 2002; Kagalkar et al., 2010). Lichen,

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Permelia perlata was also reported for participation of laccase and manganese peroxidase in biodegradation of Solvent Red 24 (Kulkarni et al., 2014).

Oxidative stress is a metabolic phenomenon arising from the free radical mechanism which challenges the cell membrane integrity (Meriga et al., 2004). Various metabolic processes cause formation of free radicals which may be threatening to biological system if left unprocessed. Studies have been revealed the involvement of textile dyes in generation of reactive oxygen species (Acharya et al., 2008; Jadhav et al., 2010). Living organisms produce certain enzymes which can scavenge free radicals thereby protecting the cell from non-specific oxidoreductive reactions. Enzymes like catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) are well known as the oxidative stress managing enzymes. Lichens are natural biomonitors of pollution hence applied as a promising tool for study of stress induction by examining the stress enzyme status (Powlik-Skowronska and Backor, 2011; Sen et al., 2014).

Lichen is a natural symbiosis of algae and fungus; in which both symbionts get benefitted as phycobiont provides nutrients to mycobiont getting shelter and protection in return. *Dermatocarpon* is a genus of lichens belonging to family Verrucariaceae, commonly known as stipple back lichens. Heidmarsson (2001) depicted the umbilicate structure of *Dermatocarpon* sp. of lichens and their preferred growth on rock surfaces. This study demonstrates the ability of *Dermatocarpon vellereceum* in biodegradation of textile dye Navy blue HE22 (NBHE22), dye mixture and real textile effluent along with biodegradation, detoxification, characterization and oxidative stress response studies.

2. Materials and methods

2.1. Chemicals and dyestuff

Tartaric acid, n-Propanol, potassium iodide was purchased from Sisco Research Laboratory, Mumbai, India. Navy Blue HE22 and other dyes were purchased from Mahesh Dying Industry, Ichalkaranji, India. Real textile effluent was collected from disposal site. The normal melting point agarose and low melting point agarose were purchased from HiMedia, Mumbai, India. HPLC grade methanol, isopropanol, toluene and acetic acid were purchased from SD Fine Chemicals, Ltd.

2.2. Collection of lichen Dermatocarpon vellereceum

Botanical excursion was carried out for collection of lichen biomass in the hilly area near Panhala province situated at 18 km northwest of Kolhapur city of Maharashtra, India. Collection location was situated at 16°49′12″ north and 74°7′12″ east at elevation 753.7704 m above MSL. Lichen *Dermatocarpon vellereceum* was collected from rock surfaces by gently removing with forcep and scalpel without disturbing the structural parts. The collected biomass was brought to laboratory, washed to remove attached rock particles with soft brush, then air dried and stored at 4 °C temperature. Aliquots were removed and used for experiments.

2.3. Decolorization studies with process optimization

2.3.1. Screening of dyes

Dyes belonging to different structural groups, with different absorption maxima and with different colors were screened. Dyes namely Navy Blue HE22, Solo Blue GL, Orange 3R, Green HE4B, Red M5B, Golden Yellow HER, Orange RL and Rubin 3B (with 20 mg L^{-1} concentration) were used for screening. The solutions were treated with lichen *D. vellereceum* for 24 h and color was measured spectrophotometrically at respective λ_{max}. Percent decolorization was calculated with formula mentioned previously (Kadam et al., 2013).

2.3.2. Decolorization of NBHE22

The aliquots of collected and stored biomass of *D. vellereceum* were subjected for decolorization studies. The decolorization of NBHE22 was measured at λ_{max} 600 nm. The biomass was washed with surface sterilizing agent (0.5% hypochlorite solution) to reduce surface microbial flora. Optimizations regarding amount of biomass were done with a series of flask studies. Presoaked, partially dried lichen biomass of 0.4 gm per 10 ml dye solution (50 mg L^{-1}) was found be effective in decolorization after 28 h of exposure. Further, other decolorization parameters were standardized such as pH (2, 4, 6, 8 and 10), temperature (10, 20, 30, 40 and 50 °C), increasing dye concentration (50, 100, 150, 200 and 250 mg L^{-1}) and repeated exposures of initial dye concentration (50 mg L^{-1}) (Kulkarni et al., 2014).

2.4. Development of fixed bed up-flow column bioreactor for decolorization of dyes

The bioreactor was composed of a hollow glass column with inlet. Lower end of column was supplemented with inlet and upper opening was sealed with rubber cork bored at centre as outlet. Rubber tube was fitted into the cork with adapter for collecting the treated sample. Column was fitted to stand perpendicular to the table top. The inlet tube was fitted to peristaltic pump (Miclins, India) for flow rate adjustment. Lichen biomass was packed into the column aseptically. Flow was adjusted in upward direction against gravity to increase hydraulic retention time and accelerate decolorization process.

Developed up-flow bioreactor was applied for decolorization of dye mixture and diluted real textile effluent. Three structurally dissimilar dyes viz. triphenylmethane, Navy Blue HE22 (NBHE22); reactive, Brilliant Blue R (BBR) and solvent, Solo Blue GL (SBGL) were used to simulate dye mixture.

2.4.1. Study of color removal of dye mixture and effluent using bioreactor

ADMI Tristimulus method operated with three wavelengths (590, 540 and 438 nm) is well accepted for decolorization assessment of compositionally various and intensely colored dye mixtures and real textile effluents (Kang and Kuo, 1999). Aliquots were centrifuged ($4000 \times g$ for 10 min) and clear supernatant was used to estimate decolorization. ADMI values were calculated as per formula mentioned previously (Kurade et al., 2012).

Increasing dye mixture concentration (30, 60, 90, 120 and 150 mg L^{-1}) and different effluent concentrations (20%, 40%, 60% and 80%) were applied to determine the effective color removal. Various flow rates 55, 35, 25 ml h^{-1} (dye mixture) and 60, 40 and 30 ml h^{-1} (real effluent) were applied for investigation of optimum color removal.

2.4.2. Effect of semi continuous reactor feeding on decolorization efficiency

Operational efficiency of lichen bioreactor was determined for simulated dye mixture and real effluent separately, by feeding the reactor with effective concentration of dye mixture and effluent at their corresponding effective flow rate in semi continuous manner. Untreated dye mixture and effluent were pumped into the bioreactor using peristaltic pump at optimized respective flow rates separately. The treated samples were procured after every cycle of maximum decolorization without adding lichen biomass. Treated solutions were subjected to estimate removal of ADMI, BOD, TSS and TDS.

2.4.3. Monitoring the decolorization ability of treated biomass free solutions (extracellular enzymatic cocktail) for dye mixture and effluent

Biomass free treated solution of dye mixture and effluent obtained after biodegradation were again subjected for decolorization and stability testing of extracellular enzymes. One hundred ml of extracellular enzymatic cocktail was repeatedly exposed with dye mixture (15 mg L^{-1}) and effluent (30%) for 5 consecutive cycles. % ADMI removal was monitored in both cases after every 24 h of incubation.

2.5. Characterization of dye mixture and effluent before and after treatment

Dye mixture and effluent was characterized before and after treatment with respect to BOD, TSS, TDS, DO (dissolved oxygen), TS (toxicological studies) to check their environmental acceptability after biodegradation. All experimental protocols were performed as per procedure mentioned in APHA (1998).

2.6. Preparation of cell free extract from lichen biomass and enzymatic analysis

To study the enzymatic status, the lichen biomass was exposed to NBHE22, dye mixture and textile effluent separately. Cell free extracts for enzymatic analysis were prepared as reported previously (Kulkarni et al., 2014). Biotic control was kept assessing the presence of intracellular and extracellular oxidoreductive enzymes. Assessment of intracellular stress enzymes was performed with cytosolic extracts.

2.6.1. Assay procedures for analysis of oxidative enzymes in D. vellereceum

For assessment of laccase activity, the reaction mixture of 2 ml was containing 0.1 ml o-toluidine and 20 mM sodium acetate buffer of pH 4.0. The oxidation of substrate was measured at 366 nm (Telke et al., 2011). For assaying manganese peroxidase activity, reaction mixture containing 0.85 ml distilled water, 40 µl manganese sulphate substrate and 0.5 ml sodium tartarate with 0.2 ml enzyme was subjected for reaction by initiating with 1 mM hydrogen peroxide. Enzyme activity was measured at 238 nm (Murugesan et al., 2007). L-tyrosine was used for assessment of tyrosinase activity with reaction mixture containing 2.5 ml sodium acetate buffer (20 mM, pH 4.0) and 100 µM of L-tyrosine. The reaction was started by adding 0.2 ml of enzyme solution and increase in absorbance was measured at 280 nm (Kadam et al., 2013). The veratryl alcohol oxidase activity was assessed at 310 nm. Two ml reaction mixture contained 1.7 ml sodium citrate buffer (pH 3.0) and 100 mM veratrole 0.1 ml. The reaction was initiated with 0.2 ml enzyme solution (Jadhav et al., 2009). Lignin peroxidase assay was performed as per earlier report with n-propanol as substrate (Yadav and Yadav, 2006).

2.6.2. Assay procedures for intracellular stress enzyme analysis in D. vellereceum

Catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities were determined spectrophotometrically. CAT activity was determined with the procedure given earlier (Bedekar et al., 2014). APX activity was measured by monitoring decrease in absorbance at 290 nm. Reaction mixture containing 0.2 mM EDTA, 0.5 mM ascorbate, 0.1 ml H_2O_2 and 105 µM enzyme solution was assessed spectrophotometrically. Enzyme activity was expressed in terms of µmol of ascorbate oxidized per ml of enzyme solution per min (Kawakami et al., 2000). GPX activity was measured at 470 nm. Reaction mixture was prepared by adding 2.7 ml sodium phosphate buffer (pH 7), 0.1 ml of guaiacol, 0.1 ml H_2O_2 and 0.1 ml enzyme solution. Formation of tetraguaiacol was measured at 470 nm spectrophotometrically. Enzyme activity was measured as µmol of guaiacol oxidized per ml of enzyme solution per min (Acharya et al., 2008).

2.7. Extraction and analysis of biodegradation metabolites of NBHE22, dye mixture and effluent after treatment

Decolorized solutions of NBHE22, dye mixture and effluent were assessed to confirm the biodegradation ability of *D. vellereceum*. Extraction of metabolites from treated solutions was performed as per method reported earlier (Kulkarni et al., 2014). High performance thin layer chromatography technique was used to confirm the degradation of NBHE22 and dye mixture. Solvent system was standardized for both the solutions as n-propanol: methanol: acetone: acetic acid: toluene (4.2: 5.0: 1.3: 0.5: 0.3 v/v). The mixture was mounted on aluminium TLC plate coated with silica gel K60 with Linomat 5 applicator with the help of micro syringe and nitrogen as spray gas. After application, the plates were run with above mentioned standardized solvent system in trough chamber for development and later analyzed with tungsten lamp in Camag TLC scanner 3. FTIR analysis was performed for NBHE22, dye mixture and effluent with special assistance of Sophisticated Analytical Instrumentation Facility (SAIF) of IIT Bombay, Powai, Mumbai. Samples in the form of dry powder were mixed with spectroscopy grade potassium bromide and analyzed to develop spectrum on mid IR region (400–4000 cm^{-1}). Spectra for both control and extracted metabolites were developed and compared for structural deformation and biodegradation analysis.

2.8. Detoxification assessment of NBHE22, dye mixture, effluent and their respective extracted metabolites

Toxicity assessment of NBHE22, dye mixture, effluent and their corresponding extracted metabolite was performed using phytotoxicity and genotoxicity analyses. Phytotoxicity was studied on germinating seedlings of common crop plants *Phaseolus mungo* and *Sorghum vulgere* using the protocol mentioned earlier by Waghmode et al. (2012a, 2012b). Genotoxicity analysis was performed with the procedure reported by Acharya et al. (2008), with slight modifications. Single cell gel electrophoresis was performed and comet characters were compared with distilled water control for analysis of genotoxicity.

2.9. Statistical analysis

The statistical analysis of data was performed with one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test using the software Graph Pad InStat version 3.06.

3. Results and discussion

3.1. Study of decolorization potential of Dermatocarpon vellereceum

Tolerance along with efficiency is the demand of current biological wastewater treatment technologies. Lichens are well-known for their ability of adaptation to the changing environmental conditions as well as environmental stresses (Powlik-Skowronska and Backor, 2011). Direct application of lichen biomass minimized the synthetic media requirements and relatively simplified the operation process. Utilization of dye molecule as a carbon and nitrogen source by lichen *D. vellereceum* was confirmed by applying lichen biomass into dye solution without fortification by any synthetic media component.

Maximum decolorized triphenylmethane dye NBHE22 (λ_{max}-600 nm) was selected as a model dye for decolorization studies. NBHE22 (50 mg L^{-1}) was decolorized 98% within 28 h by *D. vellereceum* (Fig. 1a). Repeated exposure of biomass to NBHE22 (50 mg L^{-1}) showed the effective decolorization (90%) upto 5 consecutive cycles, however it was declined upto 75% after 8th cycle (Fig. 1b). *Lycinibacillus* sp. RGS demonstrated effective decolorization upto 94% at 21st cycle of repeated addition of dye Reactive Orange 16 (50 mg L^{-1} for each cycle) within 220 min (Bedekar et al., 2014). NBHE22 (10 mg L^{-1}) was also decolorized by *Rhizobium radiobacter* MTCC 8161 (Parshetti et al., 2009).

Percent decolorization observed at various pH 2, 4, 6, 8 and 10 were 47%, 67%, 80%, 95% and 67%, respectively, while those were 48%, 66%, 75%, 91% and 57% at temperature 10, 20, 30, 40 and 50 °C, respectively (Fig. 1c). Optimum pH was found to be 8 and temperature at 40 °C. Similar results have been observed in case of lichen *Permelia perlata* (Kulkarni et al., 2014) which remarks that the oxidative enzyme system in lichens might be operative at slightly alkaline pH and requires slight higher temperature than the mesophilic range. Effective pH and temperature may be having possible role in bioavailability of the

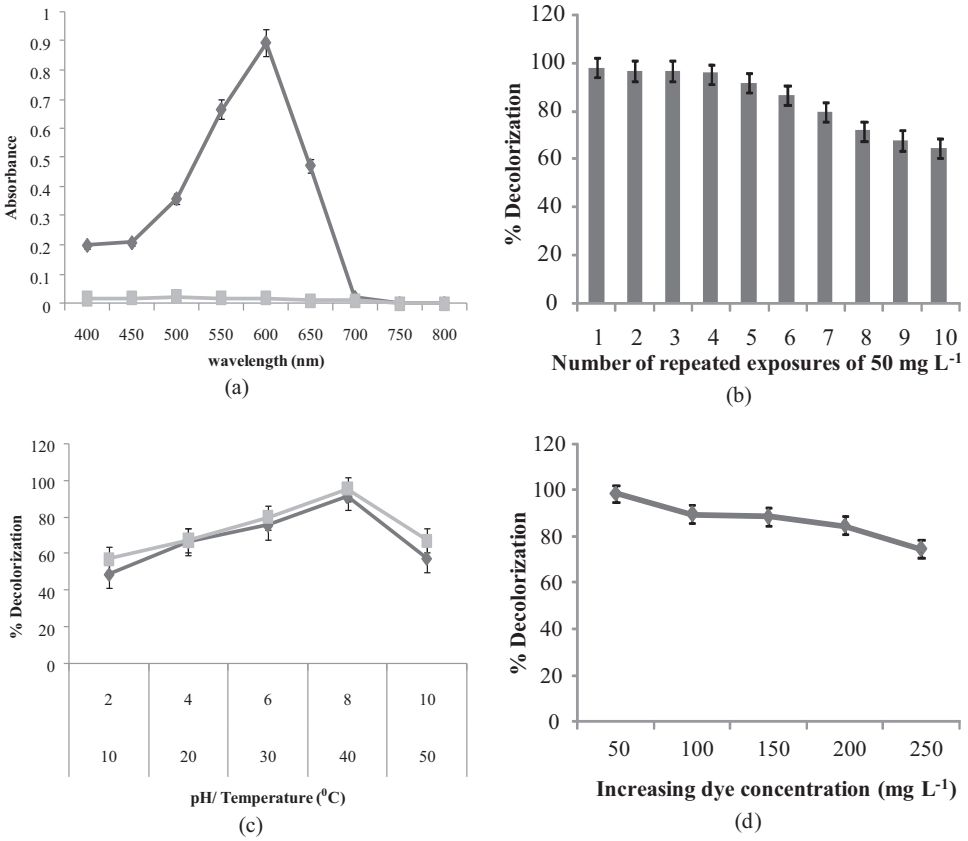

Fig. 1. Study of decolorization of NBHE22 by *D. vellereceum*, (a) Scanning spectra showing removal of NBHE22 after incubation, (b) Effect of repeated exposures of NBHE22 on decolorization, (c) Optimum pH and temperature for decolorization of NBHE22, (d) Effect of increasing dye concentration on efficiency of decolorization.

pollutant in case of lichens. Biosorption of Pb (II) and Cr (III) were observed maximum at 50 °C and pH 5 by lichen *Parmelina tiliaceae* (Uluozlu et al., 2008), however biosorption of cesium by lichen *Hypogymnia physodes* was observed at 20 °C and pH 4–5 (Pipiska et al., 2005). Hence there is possible role of effective pH and temperature in biosorption of dyes in lichens.

Percent decolorization for increasing dye concentrations such as 50, 100, 150, 200 and 250 mg L^{-1} were 98%, 90%, 89%, 85% and 75%, respectively (Fig. 1d). Decolorization percentage was found in inverse proportion with increasing dye concentration. Effect of increasing dye concentration demonstrates the ability of system to tolerate high dye concentration without losing the decolorization potential which makes it more applicable as remediator. Though the decolorization percentage had lowered at some extents, *D. vellereceum* showed its efficiency of dye removal up to 75%, hence showing significant dye tolerance. *Lycinibacillus* sp. RGS could tolerate upto 300 mg L^{-1} concentration of Reactive Orange 16 on addition of increasing concentrations (Bedekar et al., 2014).

3.2. Decolorization of dye mixture and textile effluent in the fixed bed upflow column bioreactor

3.2.1. Optimum effective flow rate

Percent reductions in ADMI, BOD, TSS and TDS were observed such as 75%, 65%, 82% and 70%, respectively for effluent at flow rate 30 ml h^{-1}, whereas those were 80%, 70%, 80% and 65%, respectively for dye mixture at optimized flow rate 25 ml h^{-1}(Fig. 2A & B). Effect of increasing concentration of dye mixture and effluent demonstrated that the system could retain its ability of decolorization with 60% ADMI removal upto 60 mg L^{-1} (20 mg each dye) for dye mixture and 65% ADMI removal for 50% effluent concentration (Fig. 2C & D). Upward flow of dye mixture and effluent in the column bioreactor enhanced its time of contact with the fixed bed lichen biomass. Regulated influent rate endorsed the desired concentration of dye molecules to come into contact with extracellular enzyme molecules favoring proper mineralization reaction.

3.2.2. Characterization of dye mixture and effluent after repeated feeding of reactor

ADMI removal of dye mixture was 80% after 1st batch, it declined to 60% by 8th batch. However, effluent exhibited 75% ADMI removal after 1st batch and further declined to 50% by 8th batch (Fig. 3 A). In case of dye mixture BOD, TSS and TDS were also reduced to 70%, 80% and 65%, respectively after 1st batch and 64%, 78% and 60%, respectively after 8th batch. Effluent solution showed decrease in BOD, TSS and TDS by 65%, 82% and 70%, respectively at 1st batch and 55%, 78% and 60%, respectively at 8th batch (Fig. 3C & D). Thus, selected rock lichen *D. vellereceum* can be endorsed for its dye decolorization efficiency.

3.2.3. Decolorization ability of cell free treated solutions of dye mixture and effluent

Treated solutions devoid of biomass resulted from passage of dye mixture and effluent through bioreactor column showed significant ADMI removal after repeated exposures of dyestuff (Fig. 3B). The batch wise experiment showed ADMI removal up to 65% after first cycle of 24 h and 57% at fifth cycle of dye mixture exposure. Similar experiments with effluent solution showed ADMI removal up to 60% at first cycle and 52% at fifth cycle of 24 h incubation. This study revealed that the treated cell free effluent and dye mixture solutions can serve as a source of extracellular enzymatic cocktail to achieve constant decolorization of untreated textile wastewater. Fig. 4

3.3. Analysis of enzymes in lichen D. vellereceum

3.3.1. Study of oxidative enzymes

Enzymatic analysis elucidated inhibition of intracellular oxidative enzymes whereas induction of extracellular enzyme (SM1-Table 1).

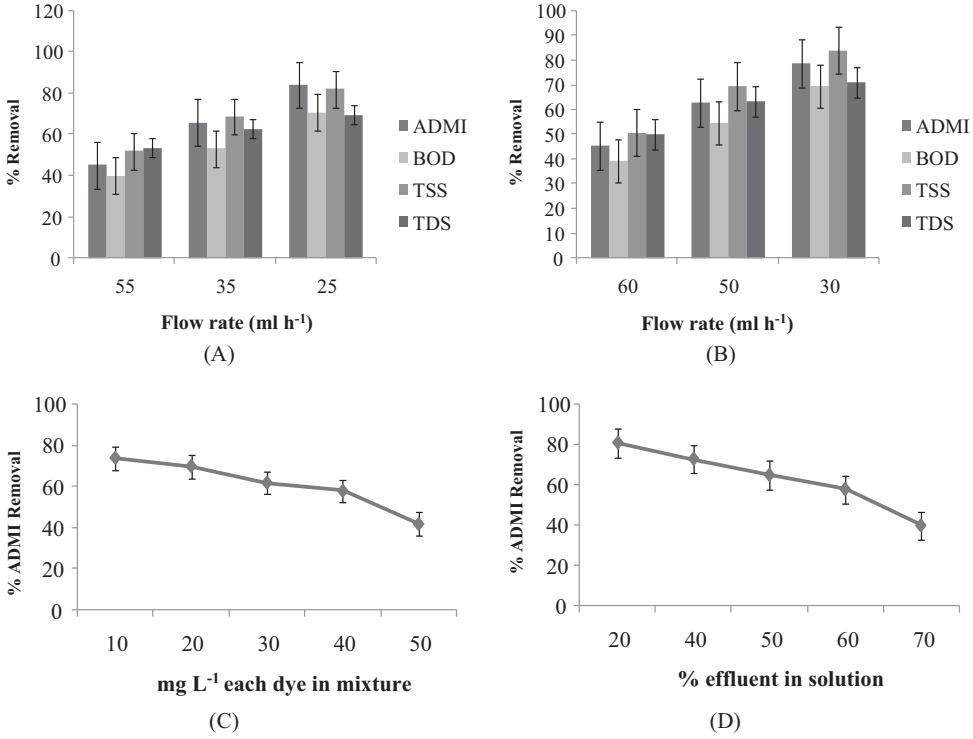

Fig. 2. % Removal in ADMI, BOD, TSS and TDS of (A) Dye mixture at flow rate 55, 35 and 25 ml h^{-1} and (B) Effluent at flow rate 60, 50 and 30 ml h^{-1}; effect of increasing concentrations of dye mixture (C) and effluent (D) to the bioreactor.

Significant induction of extracellular manganese peroxidase was estimated up to 46% and 43% upon exposure to dye mixture and effluent, respectively. Extracellular lignin peroxidase was also expressed considerably up to 34% and 24% when exposed to effluent and dye mixture, respectively. Extracellular tyrosinase (17%) was induced only after effluent exposure (SM1-Table 1). Tyrosinase was naturally occurring in non-Peltigeralean *Dermatocarpon miniatum* (L.) (Beckett et al., 2013). Enhanced activities of extracellular peroxidases elucidated the restricted entry of dye molecules into the cellular environment thus protecting the intracellular integrity and metabolism. Constituents of effluent and dye mixture, and their intermediate compounds during biotransformation act as substrate and redox mediator for enzymatic system of model lichen resulting enhanced color removal (Watharkar et al., 2015).

3.3.2. Study of antioxidant enzymes

Inhibition of intracellular oxidative enzyme activities was an indication of metabolic stress; hence estimation of stress enzyme

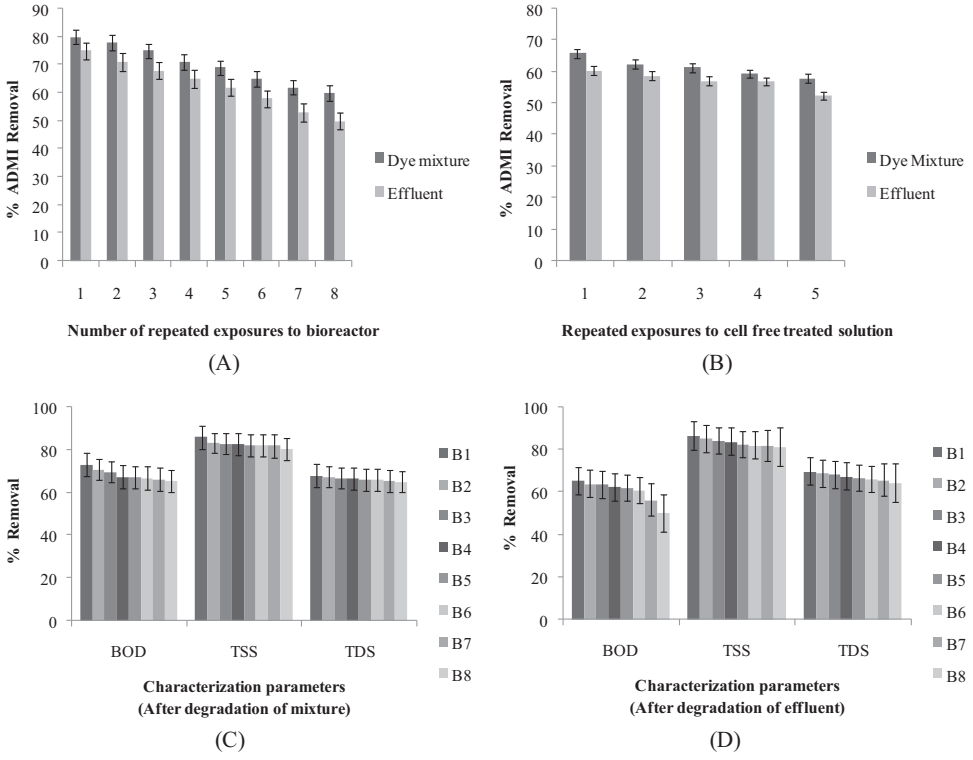

Fig. 3. (A) ADMI removal after repeated exposures of dye mixture and effluent to bioreactor, (B) Effect of repeated additions if dye mixture and effluent to biomass free treated solutions, Percent removal of BOD, TSS and TDS on batch wise reactor feeding, from batch 1–8, (C) From dye mixture and (D) From Effluent.

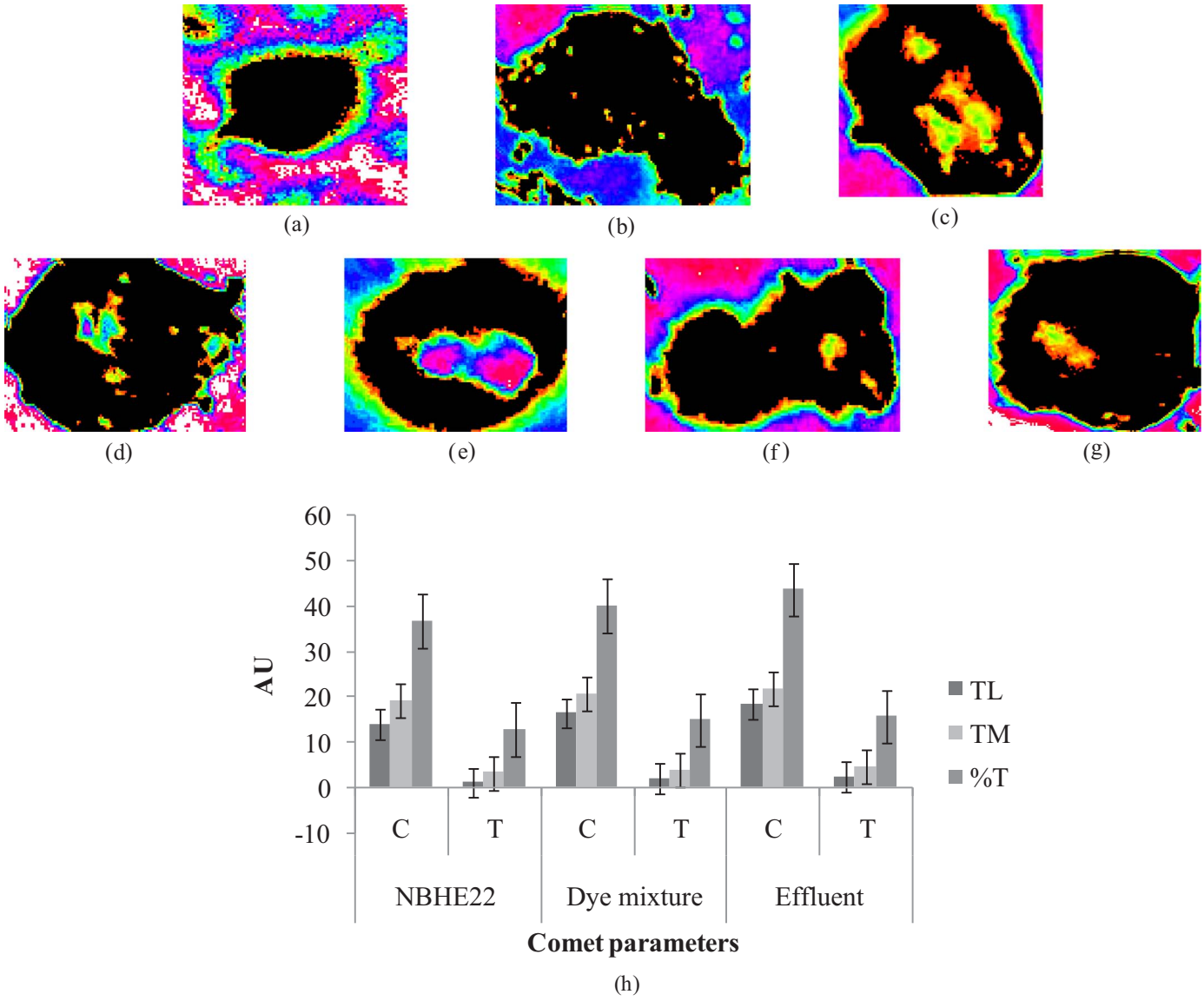

Fig. 4. Control sample exposed to distilled water (a), Comets developed on exposure to NBHE22 (b), dye mixture (d) and effluent (f); Integrated nuclei of cells exposed to metabolites of NBHE22 (c), dye mixture (e) and effluent (g); comparative account of comet parameters of NBHE22, dye mixture, effluent and their respective extracted metabolites (h).

Table 1
Analysis of oxidative stress enzymes in lichen *D. vellereceum* before and after exposure of NBHE22, dye mixture and effluent.

Name of enzyme		Activity of enzyme after exposure to		
	Control	NBHE22	Dye Mixture	Effluent
CAT[a]	$0.20 \pm 0.03 \times 10^{-8}$	$0.50 \pm 0.02 \times 10^{-8}$*	$0.52 \pm 0.17 \times 10^{-8}$*	$0.93 \pm 0.28 \times 10^{-8}$*
APX[b]	$0.15 \pm 0.07 \times 10^{-8}$	$0.39 \pm 0.05 \times 10^{-8}$*	$3.67 \pm 0.09 \times 10^{-8}$*	$5.79 \pm 0.27 \times 10^{-8}$*
GPX[c]	$0.16 \pm 0.07 \times 10^{-9}$	$0.36 \pm 0.09 \times 10^{-9}$*	$0.56 \pm 0.15 \times 10^{-9}$*	$0.66 \pm 0.09 \times 10^{-9}$*

Values are mean of three experiments ± SEM.
Significantly different from control at *P < 0.001 by one-way ANOVA with Tukey Kramer comparison test.
CAT[a]-catalase, APX[b]- ascorbate peroxidase, GPX[c]-guaiacol peroxidase.
NBHE22- Navy Blue HE22.
[a] Katal of H_2O_2 utilized ml of enzyme^{-1} min^{-1}.
[b] Katal of oxidized ascorbate formed ml of enzyme^{-1} min^{-1}.
[c] Katal of tetraguaiacol formed ml of enzyme^{-1} min^{-1}.

activities was performed. Exponential enhancement in activities of CAT was seen upto 400%, 150% and 116% and GPX activities up to 350%, 300% and 125% after exposure to effluent, dye mixture and NBHE22, respectively (Table 1). Similarly, activity of APX was induced upto 88%, 32% and 18% after exposure to effluent, dye mixture and NBHE22, respectively. Induction of oxidative stress enzymes in bacteria *Lysinibacillus* sp. RGS has been observed after exposure to dye Reactive Orange 16 (Bedekar et al., 2014). Elevated stress enzymes might be working for neutralizing the superoxides resulted by the metabolism, thus maintaining the viability and activity of the system. Overall study

Table 2
Estimation of occurance of oxidative enzymes in biomass free treated solutions of dye mixture and effluent.

Name of enzymes	Biomass free solutions exposed to		
	Distilled water	Dye mixture	Effluent
Lignin peroxidase[a]	$11.55 \pm 1.3 \times 10^{-8}$	$19.97 \pm 3.8 \times 10^{-8}$*	$25.40 \pm 2.7 \times 10^{-8}$**
Manganese peroxidase[b]	$0.44 \pm 0.05 \times 10^{-8}$	$0.63 \pm 0.14 \times 10^{-8}$*	$1.86 \pm 0.14 \times 10^{-8}$**

Values are mean of three experiments ± SEM.
Significantly different from control at *P < 0.01, ** P < 0.001 by one-way ANOVA with Tukey Kramer comparison test.
[a] Katal of $MnSO_4$ oxidized mg protein^{-1} min^{-1}.
[b] Katal of n-Propanol oxidized mg protein^{-1} min^{-1}.

demonstrated that lichen *D. vellereceum* possessed its own stress neutralizing enzyme machinery, thus showing effective stress tolerance potential.

3.3.3. Estimation of occurance of oxidative enzymes in biomass free treated solutions of dye mixture and effluent

Biomass free treated solutions obtained after passing the dye mixture and effluent were subjected to enzymatic analysis as they showed the efficient color removal after 24 h incubation. Activity of extracellular peroxidases from treated solutions in bioreactor showed significant induction up to 134% and 59% in case of 50% effluent and 15 mg L^{-1} dye mixture, respectively. However, manganese peroxidase also induced up to 314% and 40% after exposure to 50% effluent and 15 mg L^{-1} dye mixture, respectively (Table 2). Presence of active enzymes in treated sample even after first cycle of exposure to dye stuffs confronts enhanced application of extracellular enzymatic cocktails for dye degradation.

3.4. Analytical account of biodegradation of NBHE22, dye mixture, effluent and their respective extracted metabolites

Biodegradation of dye molecule is initiated with aromatic ring cleavage resultant of activity of oxidoreductases (Khandare et al., 2011b) which is successively followed with demethylation and deamination (Kabra et al., 2012). HPTLC is an analytical technique which interprets removal of dye from the solution. Absence of peaks of dye NBHE22 (Rf = 0.6) in test track elucidated their significant removal (SM2-Fig. 1). No peaks in the visible range scan of treated simulated dye mixture endorsing the lichen-mediated degradation to complete mineralization (SM2-Fig. 1, iii & iv). FTIR is effective analytical technique to elucidate biotransformation of a dye molecule into different metabolites as it shows altered fingerprinting pattern after biodegradation of dye than that before (Watharkar and Jadhav, 2014). Structural deformation of complex group is well studied with this technique (Khandare et al., 2011a, 2011b; Chakraborty et al., 2013). FTIR spectrum of control and test samples clearly indicated the altered structure of dyes after treatment thus confirming the biodegradation of NBHE22, dye mixture and effluent (SM3-Fig. 1). After FTIR analysis, disappearance of major peaks of Brown 3REL dye was evident after biodegradation by consortium GG-BL indicating its biodegradation (Waghmode et al., 2012b), however additional peaks were observed after biodegradation of dye mixture by ornamental plant *Petunia grandiflora* indicating formation of intermediate metabolites resultant of biotransformation which was demonstrated effectively with FTIR analysis. NBHE22 belongs to class of triphenylmethane dyes. These dyes contain three phenolic rings in general structure. Since structure of NBHE22 dye is not available, so it is difficult to establish biodegradation pathway. However, fingerprinting pattern obtained through FTIR analysis and induced enzyme status suggest enzymatic degradation of these dyes to complete mineralization. Detailed results of both HPTLC and FTIR analysis of NBHE22, dye mixture and effluent before and after biodegradation have been explained in SM2 and SM3, respectively.

3.5. Toxicity study of NBHE22, dye mixture, effluent and their respective extracted metabolites before and after biodegradation

3.5.1. Phytotoxicity studies

Polluted river water imposes direct impact at physical, physiological as well as genetic level of all living organisms. Color removal is not only the aim of textile dye biodegradation, but its transformation with reduced toxic nature is equally important for its safe discharge into the environment. In this study, germination inhibition was observed in case of seedlings of *Phaseolus mungo* and *Sorghum vulgere* exposed to NBHE22 up to 60% and 70%, respectively, while it was reduced up to 20% and 10%, respectively in case of extracted metabolites. Dye mixture and effluent were found to be more toxic than single dye as they showed germination of *Phaseolus mungo* by 30% and 20%, respectively and that of *Sorghum vulgere* by 30% each. However, extracted metabolites solutions of dye mixture and effluent showed germination up to 80% and 70%, respectively in case of *Sorghum vulgere* and 70% in case of *Phaseolus mungo* for both. Considerable reduction in toxic effect on root and shoot length was also observed after biodegradation for all cases (Table 3). Values of root lengths and shoot lengths of seedlings of both the crops exposed to treated solutions were found significantly different as compared to untreated solutions and nearly same to biotic control of distilled water when analyzed by ANOVA test.

3.5.2. Genotoxicity studies

The alkaline single cell electrophoresis of randomly selected nuclei from root apical meristem of *Allium cepa* bulbs was carried out for assessment of extent of DNA damage due to genotoxic effect of dyes and effluent. This technique is well accepted as naked isolated nuclei exhibit ability to demonstrate dye imposed toxicity in the form of affected nucleolar integrity (Watharkar and Jadhav, 2014). This study showed the comparative account of all comet parameters before and after biodegradation of test dye solutions. DNA percentage in tail of comet was 43%, 40% and 37% after exposure to effluent, dye mixture and NBHE22, respectively was reduced upto 15%, 14% and 12%, respectively after treatment (Table 4). Tail length of comets seen in *A. cepa* nuclei exposed to NBHE22, dye mixture and effluent were reduced from 14, 16 and 18 AU to 1.1, 2.1 and 2.5 AU, respectively after exposure to corresponding degraded samples. DNA disruption has already been demonstrated in *A. cepa* bulbs with fly ash of coal exposure (Chakraborty et al., 2008).

4. Conclusion

NBHE22, dye mixture and effluent was successfully degraded by lichen *Dermatocarpon vellereceum*. Developed semi continuous up-flow bioreactor with packed bed of *D. vellereceum* showed efficient decolorization of dye mixture and effluent with release of extracellular enzymes. Fed batch study demonstrated the efficacy of system even after repeated exposure. Significant reduction in BOD, TSS and TDS was observed after treatment thus suggesting safe for discharge. Presence and induction of oxidative enzymes suggested enzyme mediated bioremediation. Antioxidant enzymes showed their role in neutralizing the

Table 3
Phytotoxicity analyses of NBHE22, dye mixture, effluent and their respective extracted metabolites before and after biodegradation.

	Phaseolus mungo			*Sorghum vulgere*		
Sample	%Germination	Shoot length (cm)	Root length (cm)	%Germination	Shoot length (cm)	Root length (cm)
DW[a]	100	21.3 ± 0.7	9.7 ± 0.1	100	18.9 ± 0.5	6.3 ± 0.1
NBHE22	40	12.2 ± 0.4*	3.2 ± 0.1*	30	11.8 ± 0.2*	3.7 ± 0.1*
EM[b] of NBHE22 DM[c]	30	10.3 ± 0.8*	2.9 ± 0.1*	30	09.2 ± 0.7*	2.8 ± 0.1*
EM[b] of DM[c]	80	19.4 ± 0.7[$]	8.9 ± 0.1[$]	90	16.9 ± 0.6[$]	5.4 ± 0.5[$]
Effluent	20	09.9 ± 0.9*	2.6 ± 0.1*	30	08.7 ± 0.6*	2.5 ± 0.1*
EM[b] of effluent	70	16.9 ± 0.8[$]	7.2 ± 0.6[$]	70	14.8 ± 0.8[$]	4.9 ± 0.1[$]

Values are mean of three experiments ± SEM.
**Significantly different from DW control at * P < 0.001, significantly different from respective control at $P < 0.05, by one-way ANOVA with Tukey Kramer comparison test.
NBHE22- Navy Blue HE22.
[a] DW- Distilled water.
[b] EM- Extracted Metabolites.
[c] DM- Dye Mixture.

Table 4
Genotoxicity analyses of NBHE22, dye mixture, effluent and their respective extracted metabolites before and after biodegradation.

Sample	Comet characters		
	TL[a]	TM[b]	%DNA[c]
Distilled water	00.00	00.00	11.00 ± 0.3
NBHE22[d]	14.0 ± 0.2*	19.13 ± 0.4*	36.79 ± 0.6*
EM[e] of NBHE22	01.1 ± 0.1[$]	03.29 ± 0.4[$]	12.90 ± 0.5[$]
Dye mixture	16.5 ± 0.9*	20.80 ± 0.7*	40.21 ± 0.7*
EM[e] of dye mixture	02.1 ± 0.3[$]	03.87 ± 0.3[$]	14.90 ± 0.5[$]
Effluent	18.6 ± 0.7*	21.90 ± 0.6*	43.82 ± 0.6*
EM[eb] of effluent	02.5 ± 0.4[$]	04.68 ± 0.3[$]	15.70 ± 0.4[$]

Values are mean of three experiments ± SEM.
Significantly different from distilled water control at *P < 0.001, significantly different from respective control $P < 0.001, by one-way ANOVA with Tukey Kramer comparison test.
NBHE22
.
[a] Tail length.
[b] Tail moment.
[c] %DNA in tail.
[d] Navy Blue HE22.
[e] Extracted metabolites.

intracellular stress thus enabling the system to tolerate dye containing environment. HPTLC and FTIR analysis confirmed biodegradation. Toxicological analyses elucidated less toxic nature of extracted metabolites.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/xx.xxxx.xx.xxx.

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