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Differential fate of metabolism of a disperse dye by microorganisms *Galactomyces geotrichum* and *Brevibacillus laterosporus* and their consortium GG-BL

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Abstract

The present work aims to evaluate Brown 3 REL degrading potential of developed microbial consortium GG-BL using two microbial cultures, *Galactomyces geotrichum* MTCC 1360 (GG) and *Brevibacillus laterosporus* MTCC 2298 (BL). Microbial consortium GG-BL showed 100% decolorization of a dye Brown 3 REL, while individually *G. geotrichum* MTCC 1360 and *B. laterosporus* MTCC 2298 showed 26% and 86% decolorization under aerobic condition (shaking) respectively. Measurements of biochemical oxygen demand (BOD) (76%) and chemical oxygen demand (COD) (68%) were done after decolorization by consortium GG-BL. No induction in activities of oxidoreductive enzymes found in *G. geotrichum* while *B. laterosporus* showed induction of veratryl alcohol oxidase, Nicotineamide adenine dinucleotide-dichlorophenol indophenol (NADH-DCIP) reductase and riboflavin reductase indicating their role in dye metabolism. Consortium GG-BL showed induction in the activities of laccase, veratryl alcohol oxidase, tyrosinase, NADH-DCIP reductase and riboflavin reductase. Two different sets of induced enzymes from *G. geotrichum* and *B. laterosporus* work together in consortium GG-BL resulting in faster degradation of dye. The degradation of Brown 3 REL was analyzed using high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FT-IR) and gas chromatography mass spectroscopy (GC-MS). Phytotoxicity study revealed that metabolites formed after degradation was significantly less toxic in nature.

Key words: consortium GG-BL; decolorization; Brown 3 REL; biochemical oxygen demand; laccase; biodegradation
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Introduction

Rapid industrialization and urbanization have resulted in large amount of wastes into the environment causing major pollution problem. Among many pollutants, textile industry effluents are the major source of environmental pollution. Synthetic dyes are widely used in the textile, leather, pharmaceutical, cosmetic, and food industries. However, at least 10%–15% of the dyes used in textile processing are released into wastewater leading to alters pH, increases BOD, COD and reduction of sunlight penetration, which in turn decreases photosynthetic activity and deteriorates the water quality, lowering the gas solubility which causes acute toxic effects on aquatic flora and fauna (Saratale et al., 2009a). There is necessity to treat this effluent prior to discharge into the environment.

Existing physicochemical methods viz., chemical oxidation, reverse osmosis, coagulation, flocculation, filtration, adsorption, photo degradation and membrane processes are effective for color removal but these methods are not suitable due to high cost, low efficiency and inapplicability to a wide variety of dyes. Also they use more energy and chemicals than biological processes and may cause secondary pollution problems in the form of sludge (Tamboli et al., 2010; Kurade et al., 2011; Waghmode et al., 2011). Several emerging technologies such as electrochemical destruction, advanced oxidation and sorption have potential for decolorization but these approaches involve complicated procedures or are not feasible economically (Sandhya et al., 2005).

There is a need of novel mechanism for the removal of dyes from textile water. Bioremediation can be used as an alternative technology for treating the textile effluent. Bioremediation is the use of living organisms for the recovery or cleaning up of contaminated sites (soil, sediment, air, water). Compared with chemical/physical methods, biological processes have received much more attentions due to cost effectiveness, lower sludge production and environmental friendliness (Joe et al., 2008; Wang et al., 2008; Phugare et al., 2011a).

The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microor-

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ganisms and structure of dye itself. However, the presence of azo, nitro, and sulfo groups make dyes more resistant to microbial degradation and their residues accumulate in biota. Several numerous attempts have been done to develop biological process for treatment of textile dyes and effluents, including enzyme (Phugare et al., 2011b), fungi viz. *Aspergillus fum*igatus XC6 (Jin et al., 2007); *Kocuria rosea* MTCC 1532 (Parshetti et al., 2010); *Schizophyllum* sp. F17 (Tang et al., 2011) and bacteria viz. *Rhodopseudomonas palustris* (Liu et al., 2006); *Shewanella decolorationis S12* (Xu et al., 2007); *Comamonas* sp. UVS (Jadhav et al., 2008a); *Rhodopseudomonas palustris* W1 (Wang et al., 2008); *Citrobacter* sp. CK3 (Wang et al., 2009). Recent trend is shifting towards the use of mixed microbial culture as compared to individual strains. Thus the treatment systems composed of mixed microbial populations possess higher degree of biodegradation and mineralization due to synergistic metabolic activities of microbial community and offer considerable advantages over the use of pure cultures in the degradation of dyes and textile dyestuff (Khehra et al., 2005; Saratale et al., 2009b; Phugare et al., 2011a). In consortium, the individual strains may attack the dye molecule at different positions or may utilize metabolites produced by the co-existing strains for further mineralization of xenobiotic compound.

In the present study, a defined consortium of two organisms *Galactomyces geotrichum* MTCC 1360 and *Brevibacillus laterosporus* MTCC 2298, designated as consortium GG-BL was used for the decolorization of an industrial disperse dye Brown 3 REL under aerobic (shaking) condition. As both the microorganisms are found to be efficient in the dye degradation, the consortium composed of these two microorganisms achieved faster degradation by optimizing various physicochemical conditions. We have also studied the difference in the enzymatic status and the fate of metabolism of Brown 3 REL by individual organisms and with the consortium GG-BL. The various intermediates formed have been analyzed during the degradation of Brown 3 REL using HPTLC, HPLC, FT-IR and GC-MS techniques. In addition, phytotoxicity study was used to evaluate the toxicity of degradation products of Brown 3 REL by the consortium GG-BL.

1 Experimental

1.1 Chemicals

Brown 3 REL was obtained from the Yashwant Textile Processing Industry, Ichalkaranji, India. L-catechol, Methyl Red, veratryl alcohol, peptone, yeast extract and malt extract were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India. Remaining chemicals were purchased from Sisco Research Laboratory (SRL), India. All chemicals used were of the highest purity available and of analytical grade.

1.2 Microorganisms and culture conditions

G. geotrichum MTCC 1360 and *B. laterosporus* MTCC 2298 were obtained from Microbial Type Culture Collection, Chandigarh, India. The pure culture of *G. geotrichum* and *B. laterosporus* were maintained on malt extract agar and nutrient agar slants at 4°C respectively. The composition of malt extract medium used for decolorization studies was (g/L): malt extract (3.0), yeast extract (3.0), peptone (5.0) and glucose (10.0). The composition of nutrient medium used for decolorization studies was (g/L): NaCl (5.0), bacteriological peptone (10.0), yeast extract (2.0), beef extract (1.0).

1.3 Development of consortium GG-BL for decolorization of Brown 3 REL

Consortium GG-BL was prepared by aseptically transferring the mycelial biomass of 24 hr grown culture of *G. geotrichum* at 30°C in 250 mL Erlenmeyer flasks containing 100 mL malt extract medium to the flask containing 24 hr grown *B. laterosporus* (grown at 30°C in 250 mL Erlenmeyer flasks containing 100 mL of nutrient medium).

1.4 Decolorization experiment and physicochemical parameters

Biodegradation of Brown 3 REL (50 mg/L) by using consortium GG-BL was carried out in 250 mL Erlenmeyer flask containing 100 mL nutrient medium. Decolorization potential of individual organisms was also studied in their respective growth medium. Aliquots (4 mL) of the culture supernatant were withdrawn after regular time intervals during decolorization process. Suspended particles were removed from the culture medium by centrifugation at 4000 r/min for 20 min and decolorization was monitored by measuring absorbance by UV-Vis spectrophotometer (Hitachi U 2800, Japan) at 440 nm. All decolorization experiments were performed in triplicate and decolorization activity was expressed in terms of percentage decolorization (Phugare et al., 2011a). Abiotic controls (without microorganism) were always included. The above said protocol was followed while studying the potential of consortium GG-BL to decolorize Brown 3 REL at wide pH (3–11) and temperature (10–50°C) range. The potential of consortium GG-BL to tolerate higher concentration dye and repeated addition of Brown 3 REL (50 mg/L) in a fed batch manner was also checked. Reduction in COD and BOD were also measured. Reduction in COD was determined by using earlier reported method (APHA, 1995). The chloride interference was removed by using $HgSO_4$; the dye sample (before and after treatment) and $K_2Cr_2O_7$ was refluxed (acidic condition and Ag_2SO_4 as catalyst) in a reflux flask equipped with condenser on a COD digester apparatus (Spectra Lab, 2015D, India) for 2 hr. The refluxed sample was titrated against ferrous ammonium sulfate (FAS) as titrant and COD was calculated. BOD of the sample was determined by measuring the dissolved oxygen levels of the control samples (uninoculated solution of dyes) and test samples (the solution of dyes inoculated with consortium) before and after incubation for 3 days. Winkler's iodometric method was used for this estimation (APHA, 1995), and furthermore, BOD was calculated.

1.5 Effect of various carbon, nitrogen sources and agricultural waste on decolorization

Bushnell Haas medium comprised of (BHM) (g/L) ($MgSO_4$ (0.2), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $CaCl_2$ (0.02), $FeCl_3$ (0.05), NH_4NO_3 (1.0)) supplemented with yeast extract (0.5) was used to study the effect of carbon and nitrogen sources at the concentration of 5.0 g/L on the decolorization of Brown 3 REL (50 mg/L). In addition to different carbon and nitrogen sources, effect of different agricultural waste extract were seen on the decolorization of Brown 3 REL in BHM medium (5 mL extract of 10 g/L boiled agricultural residue).

1.6 Preparation of cell free extract

The consortium GG-BL was prepared as method described in Section 1.3 and individual organisms were grown in their respective medium for 24 hr at 30°C and centrifuged at 9000 r/min for 25 min. The biomass of consortium and individual organisms was separately suspended in 50 mmol/L potassium phosphate buffer (pH 7.4) and sonicated (sonics-vibracell ultrasonic processor, 12 strokes of 30 sec each for 1 min interval based on 60 amplitude output) at 4°C. The sonicated cells were centrifuged in cold condition (4°C, at 9000 r/min for 25 min) and supernatant used as the source of intracellular enzymes. Similar procedure was used to determine the enzyme activities after Brown 3 REL decolorization.

1.7 Enzymatic assay

1.7.1 Oxidative enzymes during decolorization

Activities of laccase, veratryl alcohol oxidase and tyrosinase were assayed spectrophotometrically in cell free extract and culture supernatant at room temperature (25°C) Laccase activity determined according to the procedure reported earlier (Tamboli et al., 2010). Two milliliter reaction mixture contained 5 mmol 3',3'-diaminobenzidine tetrahydrate (DAB) in 0.1 mol acetate buffer (pH 4.8) and increase in optical density was measured at 410 nm. Veratryl alcohol oxidase activity was determined by modifying earlier reported method using veratryl alcohol as a substrate (Phugare et al., 2011b). The reaction mixture contained 1 mmol veratryl alcohol, in 0.1 mol citrate phosphate buffer, pH 3.0, and 0.2 mL enzyme. Total volume of 2 mL was used for the determination of oxidase activity. Oxidation of the substrate at room temperature was monitored by an absorbance increase at 310 nm due to the formation of veratraldehyde. Tyrosinase activity was determined by modifying earlier reported method (Kandaswami and Vaidyanathan, 1973). The 3 mL reaction mixture contained 50 mmol of catechol and 2.1 mmol of ascorbic acid in 50 mmol potassium phosphate buffer (pH 6.5) equilibrated at 25°C. The $\Delta A_{265\,nm}$ was monitored until constant, and then 0.1 mL of the supernatant from the reaction mixture was added. The formation of *o*-benzoquinone and dehydro-ascorbic acid and decrease in optical density was measured at 265 nm. One unit of tyrosinase activity was equal to a $\Delta A_{265\,nm}$ of 0.001 per min at pH 6.5 at 25°C in a 3.0 mL reaction mixture containing L-catechol and L-ascorbic acid.

1.7.2 Reductive enzymes during decolorization

The nicotineamide adenine dinucleotide-dichlorophenol indophenol reductase and riboflavin reductase activities were assayed by modifying earlier reported methods (Waghmode et al., 2011). Dichlorophenol indophenol reduction was monitored at 590 nm and enzyme activity was calculated using molar extinction coefficient of 0.019 per μmol/cm. The 5.0 mL reaction mixture contained 25 μmol substrate (DCIP) in 50 mmol 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 μmol 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 mmol of Tris-HCl (pH 7.4), 25 μmol of NADPH and 10 μmol of riboflavin. Reaction rates were calculated by using molar extinction coefficient of 0.0063 per μmol/cm. Azo reductase activity was assayed by modifying earlier reported method (Telke et al., 2010). Two milliliter reaction mixture contained 25 μmol of Methyl Red (MR), 50 μmol NADH, 1.2 mL of potassium phosphate buffer (50 mmol, pH 7.4). 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. The reduction of Methyl Red was calculated using molar extinction coefficient of 0.023 μmol/cm. One unit of enzyme activity was defined as amount of enzyme required to reduce 1 μmol of substrate per min per mg protein. All the enzyme assays were run in triplicates.

1.8 Metabolite analysis

The decolorization of Brown 3 REL by *G. geotrichum*, *B. laterosporus* and consortium GG-BL were monitored by using UV-Vis spectrophotometer (Hitachi U 2800, Japan). The metabolites produced during degradation were extracted with equal volumes of ethyl acetate; dried over anhydrous Na_2SO_4 and dissolved in small volume of HPLC grade methanol and used for further analysis. HPTLC analysis was performed by using HPTLC system (CAMAG, Switzerland) (Kurade et al., 2011). The 10 μL of control and formed metabolites were loaded on precoated HPTLC plates (Lichrospher silica gel plate, Merck, Germany) by spray gas nitrogen and TLC sample loading instrument (CAMAG LINOMAT V). The composition of mobile phase was acetone:ethyl acetate (6:4, *V*/*V*). The control dye and formed metabolites were visualized in UV chamber and scanned at 280 nm with slit dimension 5 × 0.45 mm by using TLC scanner and the results were analyzed using HPTLC software WinCATS 1.4.4.6337. HPLC analysis was carried out (Waters model no. 2690, USA) on C_{18} column (symmetry, 4.6 mm × 250 mm) by isocratic method with 10 min run time (Phugare et al., 2011a). The mobile phase was methanol with flow rate of 1 mL/min and UV detector was kept at 440. The samples were filtered with a 0.2 μm membrane filter and about 10

μL of sample was manually injected into the injector port.

FT-IR (Shimadzu 8400S, Japan) was used for investigating the changes in surface functional groups of the samples before and after microbial decolorization. FT-IR analysis was done in the mid IR region of 400–4000/cm with 16 scan speed (Saratale et al., 2009a). The pellets were prepared using spectroscopic pure KBr (5:95, *W*/*W*) and fixed in the sample holder for the analysis. The identification of formed metabolites was carried using a QP2010 gas chromatography coupled with mass spectroscopy (Shimadzu, Japan) (Kalyani et al., 2009). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 80°C for 2 min, then increased linearly at 10°C to 280°C/min, and held for 7 min. The temperature of the injection port was 280°C and the GC-MS interface was maintained at 290°C. The helium carrier gas flow rate was 1.0 mL/min. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta) of the GC-MS.

1.9 Phytotoxicity analysis

Phytotoxicity of Brown 3REL was performed to assess the toxicity of textile industry effluent to common agricultural crop. The obtained product was dissolved in water to form a final concentration of 1000 mg/L. Ten seeds of *Phaseolus mungo* and *Sorghum vulgare* plants were sowed into a plastic sand pot with daily watering of (5 mL) Brown 3REL (1000 mg/L) and its degradation metabolites (1000 mg/L) obtained after degradation by consortium GG-BL. Control set was carried out using distilled water (daily 5 mL watering) at the same time. The length of shoot and root was recorded after 7 days. The study was carried out at room temperature. Germination (*G*, %) was calculated by the following equation:

$$G = \frac{N_g}{N_s} \times 100\%$$

where, N_g is the number of seed germinated; N_s is the number of seed sowed.

1.10 Statistical analysis

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

2 Results and discussion

2.1 Decolorization experiment and physicochemical parameters

Decolorization of Brown 3 REL using consortium GG-BL was carried out at aerobic (shaking) and microaerophilic (static) condition, but the decolorization was found to be more at aerobic condition. Therefore, the study continued at aerobic condition. It was observed that prepared consortium GG-BL had a greater potential of decolorization as compared to *G. geotrichum* and *B. laterosporus* under aerobic condition. Khehra et al. (2005) showed mutualism of individual strains in consortium for better decolorization of AR-88 by formed consortium HM-4, but the consortium HM-4 took 24 hr for the decolorization of AR-88 (20 mg/L). Whereas consortium GG-BL took only 10 hr for complete decolorization of Brown 3 REL (50 mg/L). Two different systems of *G. geotrichum* and *B. laterosporus* work together in the consortium GG-BL resulting in greater potential giving faster decolorization. Microbial consortium GG-BL showed 100% decolorization of a dye Brown 3 REL with significant reduction of BOD (76%) and COD (68%), while individually *G. geotrichum* and *B. laterosporus* showed 26% and 86% decolorization with 12% and 46% of BOD as well as 6% and 34% of COD reduction within 10 hr, 30°C, at aerobic condition (120 r/min) respectively. Also consortium showed 86% decolorization within 5 hr under aerobic condition, so the time require for decolorization of Brown 3 REL using consortium was less as compared to individual strains. Decreases in biochemical and chemical oxygen demand after decolorization of Brown 3 REL showed the low molecular weight metabolites could be completely mineralized, suggesting the potential use of consortium for the treatment of textile wasterwater. The optimum pH and temperature for Brown 3 REL decolorization were 7.0 and 30°C respectively (Fig. 1). No change in pH during the whole decolorization process. Consortium GG-BL also showed 99% decolorization at the concentration of 100 and 150 mg/L within 18 and 24 hr, respectively. However, at 300 mg/L dye concentration consortium showed 82% decolorization within 60 hr (Fig. 2a). This proved that prepared consortium showed great potential towards degradation of Brown 3 REL due to the mutualism of individual strains in consortium. Consecutive cycles of dye decolorization were studied by the repeated additions of Brown 3 REL (50 mg/L) in flask containing 100 mL growth of consortium at aerobic condition (Fig. 2b) and it showed effective dye decolorization up to 7 tested cycles. The result of dye decolorization at repeated cycles showed the decolorization efficiency of consortium GG-BL was more than 82% within 10–12 hr.

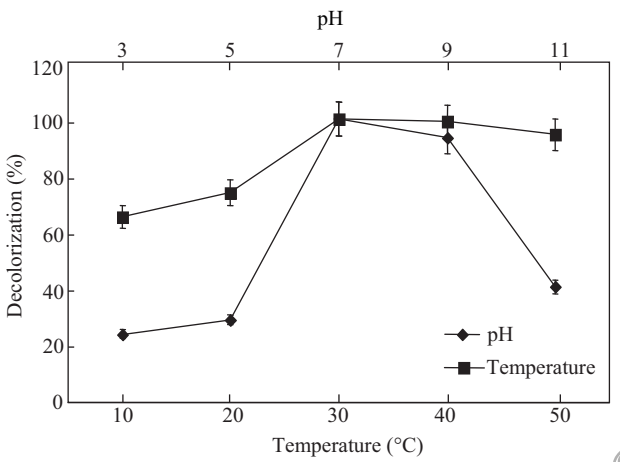

Fig. 1 Effect of pH and temperature on the decolorization of Brown 3 REL by using consortium GG-BL.

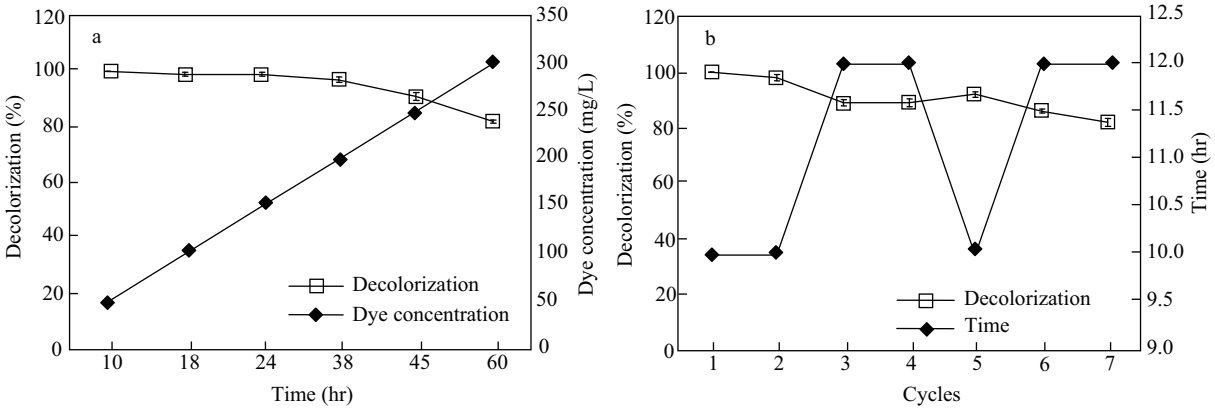

Fig. 2 Effect of initial dye concentration (a) and repeated addition of dye on decolorization (b) of consortium GG-BL.

2.2 Effect of various carbon, nitrogen source and agricultural waste on decolorization of Brown 3 REL

Biodegradation activity of consortium GG-BL greatly varied according to the type of carbon and nitrogen sources used in Bushnell Haas medium (BHM). In BHM medium (control), 30% decolorization of Brown 3 REL dye was observed in 24 hr. In an attempt to enhance decolorization in control medium it was supplemented with extra carbon and nitrogen sources and extracts of agricultural residues. It was found that bagasse and rice bran showed 84% and 76% decolorization, respectively. BHM containing extract of wheat bran, rice husk and wood shaving showed 68%, 56%, and 54% decolorization of Brown 3 REL within 24 hr respectively. There was supportive impact of agricultural residue components for the growth of microorganism as well as decolorization of the dye. The component present in agricultural extract could be act as mediators for oxidoreductive enzyme induction as well as enhances faster dye decolorization. Previous study reported that use of synthetic mediator enhances Crystal Violate degradation by laccase secreted by white rot fungi (Yan et al., 2009). Glucose showed 95% decolorization in alone and in combination with other nitrogen sources viz. urea, yeast extract and ammonium chloride showed 100%, 100% and 98% decolorization respectively. Waghmode et al. (2011) reported that glucose and yeast extract act as electron donors for faster decolorization of dyes. Tobajas et al. (2012) reported that use of glucose as cometabolic substrate responsible for induced degradation of 4-cholorophenol by *Comamonas testosteroni.* Similar results were obtained with the degradation of Reactive Black 5 using yeast isolate (Yang et al., 2008). Also urea and ammonium chloride showed 9% and 75% decolorization in alone but in combination with yeast extract and glucose showed 45% and 98% decolorization respectively. Similar results were found during degradation of Reactive Blue 220 by *Lentinus critinus* when glucose used in combination with urea in culture media (Niebisch et al., 2010). This showed concerted action of carbon and nitrogen sources in decolorization of Brown 3 REL. *Cunninghamella elegans* UCP 542 also showed similar results during decolorization of reactive azo dyes (Ambrósio and Campos-Takaki, 2004). Also starch and peptone showed 29% and 49% decolorization respectively.

2.3 Enzymatic assay

2.3.1 Oxidative enzymes during decolorization

Various oxidative enzymes were found to be effective in dye degradation. Induction of various enzymes during decolorization gives additional insights into decolorization mechanism and also supports the active role of microorganisms in the biodegradation process. Oxidative biodegradation takes place upon action of enzyme such as laccase and veratryl alcohol oxidase. In this study, induction in the oxidative enzymes (laccase and veratryl alcohol oxidase) was responsible for the decolorization of Brown 3REL within 10 hr. Laccase and veratryl alcohol oxidase were responsible for oxidative cleavage as well as demethylation during decolorization of Brown 3 REL in case of *G. geotrichum*, *B. laterosporus* and consortium GG-BL. Laccase was responsible for the demethylation of complex intermediate into simpler one during decolorization of Brown 3 REL by *Bacillus* sp. VUS (Dawkar et al., 2008). Phugare et al. (2011b) reported oxidative cleavage of Remazol Black B during decolorization by purified veratryl alcohol oxidase from *Pseudomonas aeruginosa* BCH. Significant induction in the activity of laccase by 700% and 863% in consortium (after decolorization) as compared to control consortium (before decolorization) and 83% and 120% induction as compared to *G. geotrichum*, while no laccase activity observed in case of *B. laterosporus*. Veratryl alcohol oxidase was induced by 32% and 36% in consortium GG-BL after decolorization as compared to control consortium GG-BL (before decolorization) but it was less as compared to *B. laterosporus* (after decolorization). Veratryl alcohol oxidase activity was absent in *G. geotrichum*. Intracellular and extracellular tyrosinase activity was tremendously induced in consortium GG-BL as compared to *B. laterosporus* and *G. geotrichum* after decolorization (Table 1). Jadhav et al. (2008b) reported similar induction pattern of tyrosinase in consortium-GB during the decolorization of Brown 3 REL. Previous study reported the involvement of laccase and tyrosinase for the degradation of textile dyes (Saratale et al., 2009a; Khandare et al., 2011).

2.3.2 Reductive enzymes during decolorization

The enzymatic analysis of consortium GG-BL and *B. laterosporus* (after decolorization) for reductive enzymes

Table 1 Enzyme activities in control cells (at 0 hr of dye addition) and cells obtained after 10 hr of Brown 3 REL decolorization by *G. geotrichum, B. laterosporus* and consortium GG-BL

Enzyme	*G. geotrichum*		*B. laterosporus*		Consortium GG-BL		
	0 hr	10 hr	0 hr	10 hr	0 hr	5 hr	10 hr
Laccase (unit/(min·mg protein))	0.070 ±0.05	0.046 ±0.00	NA	NA	0.016 ±0.00	0.128 ±0.00**	0.154 ±0.00**
Veratryl alcohol oxidase (unit/(min·mg protein))	NA	NA	0.276 ±0.03	7.83 ±0.05**	0.667 ±0.08	0.904 ±0.08*	0.883 ±0.01
Tyrosinase (unit/(min·mg protein))							
Intracellular	1411 ±451	NA	357 ±24.00	NA	217 ±40.6	2386 ±423**	1476 ±218**
Extracellular	1217 ±111	1165 ±267	565 ±72	NA	239 ±13.98	383 ±57.93	370 ±55.79
NADH-DCIP reductase (μg DCIP reduced/(min·mg protein))	183.9 ±11.35	19.36 ±1.88**	62.67 ±4.64	123.0 ±1.64**	53.62 ±0.81	49.39 ±5.48	76.65 ±4.83*
Azo reductase (μmol Methyl Red reduced/(min·mg protein))	5.54 ±0.48	NA	3.53 ±0.90	NA	2.95 ±0.55	NA	NA
Riboflavin reductase (μg riboflavin reduced/(min·mg protein))	14.39 ±0.29	NA	NA	21.82 ±6.37	3.70 ±0.71	11.25 ±1.93**	6.73 ±1.31*

Values are mean of three experiments ± standard error mean. Significantly different from respective control at $*P<0.01$ and $**P < 0.001$ by one-way ANOVA with Tukey Kramer comparison test.

showed induction in NADH-DCIP reductase and riboflavin reductase as compared to consortium GG-BL (before decolorization) (Table 1), while in case of *G. geotrichum* showed reduction in NADH-DCIP reductase and riboflavin reductase after decolorization as compared to control. Induction of riboflavin reductase was also observed in consortium-GB during the decolorization of Brown 3 REL (Jadhav et al., 2008b). Also Dawkar et al. (2008) reported that induction of NADH-DCIP reductase enzyme during decolorization of Brown 3 REL by *Bacillus* sp. VUS. Azo reductase was absent in individual strains as well as in consortium after decolorization. NADH-DCIP reductase and riboflavin reductase were induced 43%, 204% and 82% in consortium (after decolorization) as compared to control (before decolorization) while it was less as compared to induced in *B. laterosporus* (after decolorization). In case of *B. laterosporus* showed 96% and 2082% of induction in NADH-DCIP reductase and riboflavin reductase enzymes after the decolorization of Brown 3 REL. Khandare et al. (2011) reported the induction and involvement of riboflavin reductase enzyme during the decolorization of sulfonated azo dye Remazol Red by *Aster amellus* Linn.

2.4 Metabolite analysis

To explain the possible mechanism of the dye decolorization, we analyzed the products of biotransformation of Brown 3 REL by UV-Vis spectral analysis, HPTLC, HPLC, FT-IR and GC-MS. The spectrophotometric analysis of degraded culture supernatant by consortium GG-BL at 400–800 nm showed significant reduction in absorbance at 440 nm as compared to control Brown 3 REL (at 0 hr of dye addition), *G. geotrichum* (after decolorization) and *B. laterosporus* (after decolorization) (Fig. 3). The metabolites obtained after 10 hr of decolorization of Brown 3 REL were extracted with ethyl acetate, crystallized, dissolved in HPLC grade methanol and used for the analysis. The HPTLC analysis of degraded metabolites showed a different degradation pattern with different R_f values in consortium GG-BL as compared to control Brown 3 REL, *G. geotrichum* and *B. laterosporus* (Fig. 4a, b). The difference in R_f value of control dye Brown 3 REL (0.76) and formed metabolites by *G. geotrichum* (0.42, 0.57, 0.58, 0.77, 0.84, 0.93), *B. laterosporus* (0.44, 0.57, 0.78, 0.85, 0.94) and consortium GG-BL (0.34, 0.44, 0.57, 0.64, 0.74, 0.84, 0.94) indicate the biodegradation of Brown 3 REL (Fig. 4a). HPLC chromatogram of control Brown 3 REL showed two major peaks at the retention time of 2.997 and 3.217 min (Fig. 5a). Decolorization of Brown 3 REL by *G. geotrichum* showed two peaks at the retention time of 2.538 and 2.968 min (Fig. 5b), while decolorization by *B. laterosporus* showed two peaks at the retention time of 2.741 and 2.940 min (Fig. 5c), whereas the degradation of Brown 3 REL by consortium GG-BL showed three major peaks at the retention times of 2.522, 2.836 and 2.996 min (Fig. 5d), respectively, which suggested the further conversion of degradation products into various metabolites by consortium GG-BL as compared to individual organism.

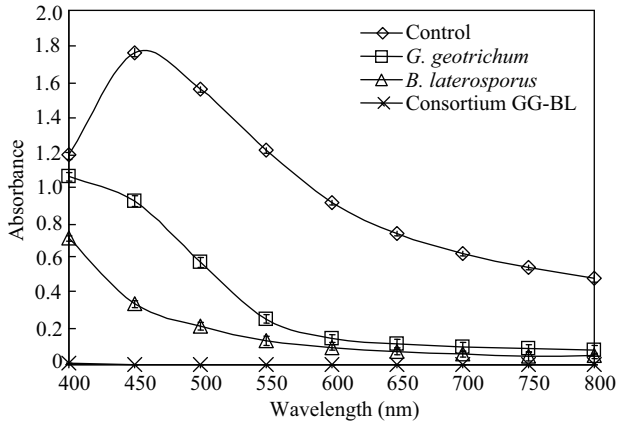

Fig. 3 UV-visible spectra of Brown 3 REL degradation by *G. geotrichum*, *B. laterosporus* and consortium GG-BL.

The comparison of FT-IR spectrum of control dye and the products formed after 10 hr of degradation revealed the biodegradation of the dye Brown 3 REL by *G. geotrichum*, *B. laterosporus* and consortium GG-BL (Fig. 6). The FT-IR spectrum of the Brown 3 REL showed the peaks at 3204.23 cm^{-1} for C=O stretching, 2360.47 cm^{-1} and 3095.37 cm^{-1} for N–H stretching, 2874.03 cm^{-1} for alkanes, 1593.74 cm^{-1} for C=N and C=C stretching, 1342.98 cm^{-1} and 1515.14 cm^{-1} for C–N vibrations, 1138.52 cm^{-1} for C–N vibrations and C–H deformation in substituted benzenes, 808.68 cm^{-1}

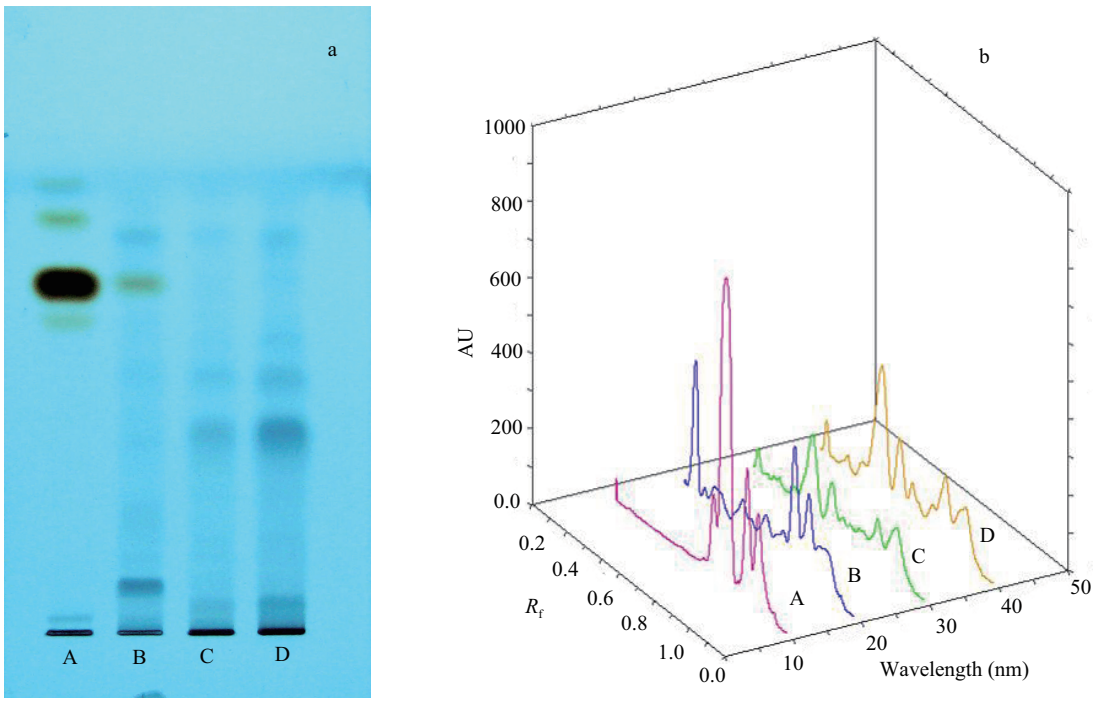

Fig. 4 HPTLC profile of TLC chromatogram (a) and 3D image profile (b) of control dye Brown 3 REL (A) and its metabolites obtained after 10 hr degradation by *G. geotrichum* (B), *B. laterosporus* (C) and consortium GG-BL (D).

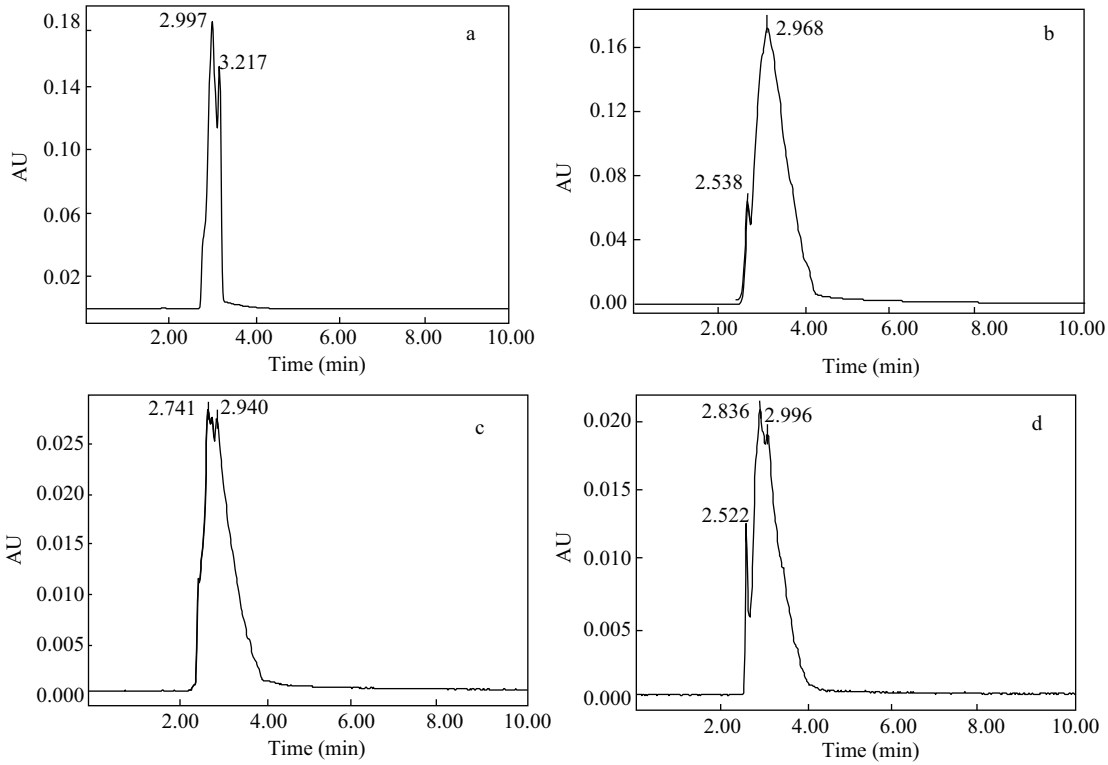

Fig. 5 HPLC elution profile recorded at 440 nm of control Brown 3 REL (a) and its metabolites obtained after 10 hr of degradation by *G. geotrichum* (b), *B. laterosporus* (c) and consortium GG-BL (d).

and 1043.04 cm^{-1} for ring C–H deformation and 678.00 cm^{-1} for C–Cl stretching in halides (Fig. 6a). Product formed by *G. geotrichum* showed peak at 3396.38 cm^{-1} for aromatic –OH stretch, 2927.19–2949.27 cm^{-1} for alkane, 1665.93 cm^{-1} for aryl carboxylic acid, 1512.23 cm^{-1} for the formation of pyridine and quinoline (C=C and C=N stretch), 1453.60 cm^{-1} for C–H deformation, stretch at 1031.70–1335.19 cm^{-1} for –OH deformation and peak at 1234 cm^{-1} showed presence of ether, and 670.39–749.88 cm^{-1} for halides (C–Cl) (Fig. 6b). Degradation by *B. laterosporus* showed NH_2 stretch at 3256.56 cm^{-1}, presence of alkanes ($–CH_3$ stretch) at 2862.31–2959.03 cm^{-1}, showed formation of quinones (C=O stretch) by a peak at 1665.43 cm^{-1}, presence of NH_2 group at 1515.60 cm^{-1}, C–N vibration at 1305.22–1338.81 cm^{-1}, peak at 1454 cm^{-1} showed C–H deformation, peak at 1110.09 cm^{-1}

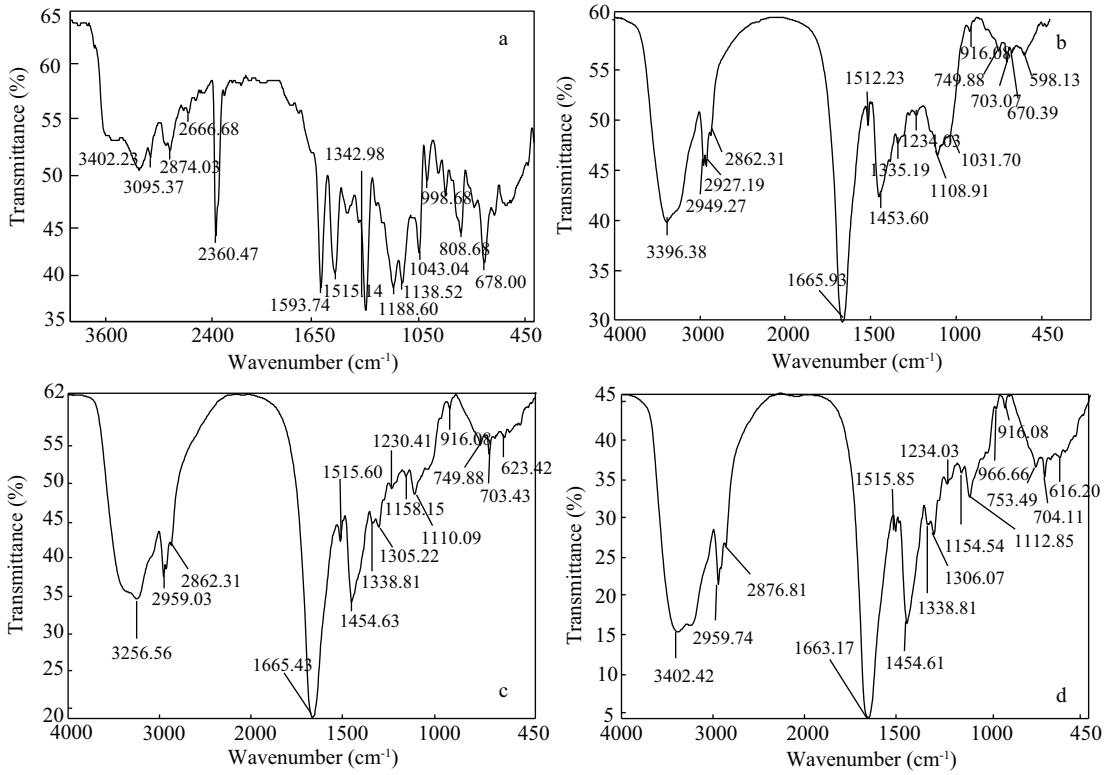

Fig. 6 FT-IR analysis of control dye Brown 3 REL (a) and its metabolites obtained after 10 hr of degradation by *G. geotrichum* (b), *B. laterosporus* (c) and consortium GG-BL (d).

indicate presence of aliphatic ethers (Fig. 6c). Whereas FT-IR analysis of consortium GG-BL showed differential peak patterns as compared to control dye, *G. geotrichum* and *B. laterosporus*. Degradation by consortium GG-BL showed N–H stretch at 3402.42 cm^{-1}, alkane ($–CH_3$) at 2876.81–2959.74 cm^{-1}, quinones (C=O) formation at 1663.17 cm^{-1}, peak at 1515.85 cm^{-1} indicate presence of NH_2 group, C–H deformation and vibration occurs at 1454.61 cm^{-1} and 1154.54–1338.81 cm^{-1} respectively, peak at 1112.85 cm^{-1} indicates presence of aliphatic ether, C–H deformation at 966.66 cm^{-1} (Fig. 6d). Disappearance of major peaks from FT-IR spectrum as compared to control obtained after degradation of Brown 3 REL by consortium GG-BL showed concerted action of oxidoreductive enzyme system of two microorganisms responsible for the complete mineralization of Brown 3 REL.

The GC-MS analysis showed the probable metabolites produced during Brown 3 REL biotransformation process. On the basis of induction of various enzymes and mass spectrum analysis, the possible biodegradation pathway adapted by *G. geotrichum*, *B. laterosporus* and consortium GG-BL is as illustrated in Fig. 7. In case of *G. geotrichum* asymmetric cleavage of Brown 3 REL mediated by oxidative enzyme laccase yields two reactive intermediates, one of which identified on the basis of GC-MS pattern as [(8-chloro-5-oxo-5,6-dihydroquinazolin-4-yl)oxy] acetic acid (m/z = 256). There was no further identified metabolite observed during the dye decolorization process. In case of *B. laterosporus* asymmetric cleavage mediated by veratryl alcohol oxidase enzyme yielded two reactive acetamide intermediates I and II. Intermediate I was identified as *N*-carbamyol-2-(quinazolin-4-yloxy) acetamide (m/z = 244) and intermediate II identified as *N*-(cyclopentyl carbamoyl) acetamide (m/z = 170). No further identified metabolite was observed in case of *B. laterosporus*. Whereas in case of consortium GG-BL, significant induction in laccase and veratryl alcohol oxidase enzymes showed asymmetric cleavage of Brown 3 REL yields two reactive acetamide intermediates I and II. The intermediate I was identified as *N*-carbamoyl-2-(quinazolin-4-yloxy) acetamide (m/z = 244) and intermediate II was identified as *N*-(cyclopentyl carbamoyl) acetamide (m/z = 170). Further hydroxylation and quinone formation in intermediate (I) by oxidative action of enzyme laccase yielded *N*-carbamoyl-2-[(5-oxo-5,6-dihydroquinazolin-4-yl) oxy] acetamide (m/z = 260) and demethylation of intermediate II by oxidative enzymes viz., laccase/veratryl alcohol oxidase yields urea compound identified as 1-cyclopentyl-3-formylurea (m/z = 154). Consortium GG-BL was found to be efficient in the decolorization as well as degradation of Brown 3 REL as compared to individual organisms, because enzyme system present in individual organisms showed mutualism in consortium or in consortium one organism could be able to utilize reactive intermediates produced by the action of another organism in consortium during degradation experiments towards complete mineralization of dye. It means that, in consortium concerted action of individual organisms and cumulative action of oxidoreductive enzymes present in both organisms were responsible for faster decolorization as well as complete mineralization of Brown 3 REL. Previous study reported the communal action of microorganism in consortia for the faster degradation of textile dyes (Moosvi et al., 2005; Junnarkar et al., 2006; Patil et al., 2010; Joshi et al., 2011).

Table 2 Phytotoxicity of Brown 3 REL and its degradation products extracted after 10 hr of degradation for *P. mungo* and *S. vulgare*

	Phaseolus mungo			*Sorghum vulgare*		
	I	II	III	I	II	III
Germination (%)	100	70	100	80	30	90
Shoot length (cm)	18.28 ± 0.12	8.36 ± 0.12*	18.10 ± 0.12**	1.87 ± 0.04	1.28 ± 0.04*	1.70 ± 0.01**
Root length (cm)	4.66 ± 0.07	1.40 ± 0.09*	5.42 ± 0.15**	2.37 ± 0.04	0.40 ± 0.06*	2.50 ± 0.09**

I: seeds treated with distilled water; II: seeds treated with formed metabolites (1000 mg/L); III: seeds treated with dye Brown 3 REL (1000 mg/L). Data was analyzed by one-way ANOVA test and mentioned values are the mean of ten germinated seeds of three sets SEM (±). Seeds germinated in Brown 3 REL are significantly different from the seeds germinated in plain water at $*P < 0.001$ and the seeds germinated in degradation products are significantly different from the seeds germinated in Brown 3 REL at $**P < 0.001$ when compared by Tukey Kramer Multiple comparison test.

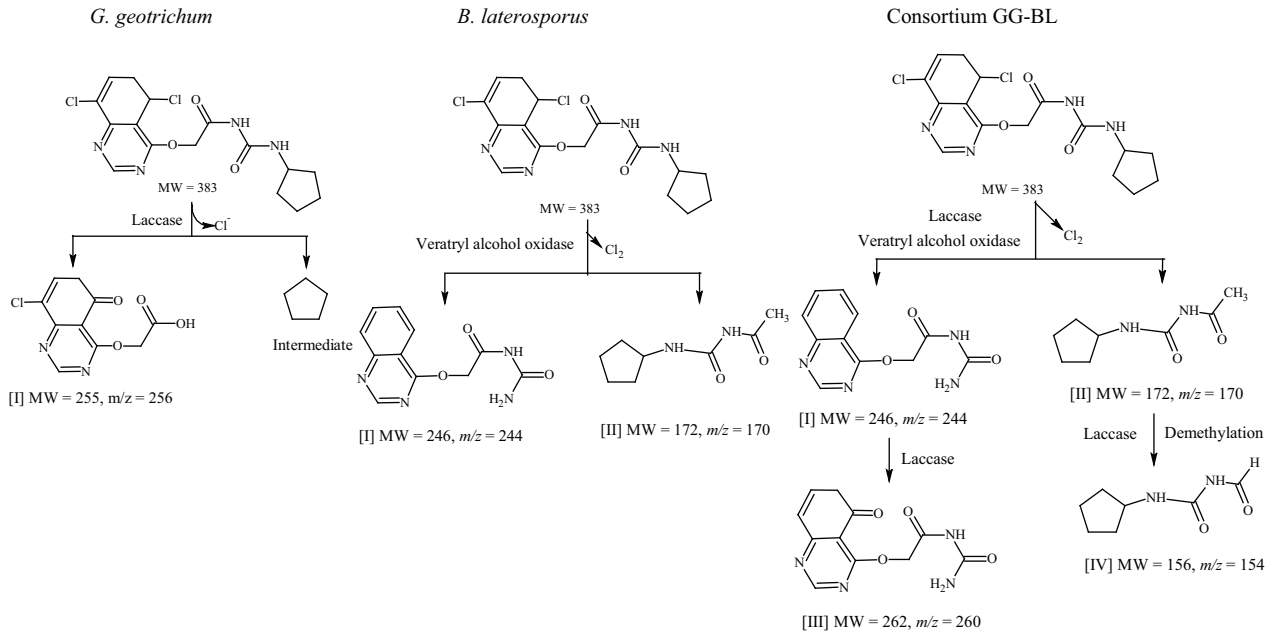

Fig. 7 Proposed pathways for the degradation of Brown 3 REL by *G. geotrichum*, *B. laterosporus* and consortium GG-BL.

2.5 Phytotoxicity analysis

The toxic nature of the dye and the textile effluent is mainly based on the chemical nature of the dye and number of dyes present in textile effluent. Phytotoxicity study revealed the toxic nature of Brown 3 REL to the *P. mungo* and *S. vulgare* plants. Germination of these plants was less with Brown 3 REL treatment as compared to the metabolites obtained after its decolorization and distilled water (Table 2). The Brown 3 REL significantly affected the shoot and root growth than the metabolites obtained after its decolorization. This study revealed the less toxic nature of degraded metabolites as compared to the control dye (Kabra et al., 2011).

3 Conclusions

In the present study, the combined activities of oxidoreductive enzymes in consortium GG-BL resulted in an increased decolorization of Brown 3 REL as compared to the individual strains. UV-Visible spectra of metabolites formed after the degradation of Brown 3 REL clearly showed the complete decolorization. HPTLC, HPLC, FT-IR and GC-MS analysis confirmed the biodegradation of Brown 3 REL. The consortium system was found to be better alternative as compared to the individual microbial strains. Phytotoxicity analysis indicated that the metabolites of dyes were nontoxic/less-toxic than that of Brown 3 REL. Results from this studies demonstrated that the consortium CG-BL could successfully applied in the bioremediation of textile effluent.

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