

Random UV Mutagenesis Approach for Enhanced Biodegradation of Sulfonated Azo Dye, Green HE4B

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Abstract The objective of the study was to execute mutant bacteria for efficient biodegradation of sulfonated azo dye, Green HE4B (GHE4B). UV irradiation was used to introduce random mutations in *Pseudomonas* sp. LBC1. Genetic alterations induced by UV irradiation in selected mutant bacteria were confirmed by random amplification of polymorphic DNA technique. The mutant bacteria named as *Pseudomonas* sp. 1 F reduced the time required for complete degradation of recalcitrant dye GHE4B by 25 % when compared with the wild one. The biodegradation was monitored by UV–Vis spectrophotometric analysis. Activities of enzymes like laccase, lignin peroxidase, veratryl alcohol oxidase, and NADH dichlorophenol indophenol reductase were found to be boosted in mutant bacteria as a consequence of UV-induced mutation. Matrix-assisted laser desorption/ionization-time of flight analysis of differentially expressed proteins of mutant bacteria suggested active role of antioxidant enzymes in the degradation of the dye. The degradation product was analyzed by Fourier transform infrared spectroscopy, high-performance thin-layer chromatography, and gas chromatography–mass spectrometry. Results revealed few variations in the degradation end products of wild-type and mutant bacteria. Phytotoxicity study underlined the safer biodegradation of GHE4B by mutant *Pseudomonas* sp. 1 F.

Keywords Degradation · Catalase · Azo dye · Mutation · UV irradiation · RAPD · Decolorization

Introduction

Water pollution has become an issue of concern in the last few years. Rapid industrialization is the root cause of the pollution. Textile industry effluent is the major source of water pollution. Highly colored effluent of textile industry comprises many recalcitrant azo dyes.

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Sulfonated azo dyes have great structural differences [1, 2] and consequently offer a great variety of color. Hence, these kinds of dyes are widely employed in textile industries. The stability and xenobiotic nature of azo dyes make them recalcitrant; hence, they are not readily degraded by conventional physical and chemical wastewater treatment processes [3]. However, microorganisms, being highly versatile, have developed enzyme systems for the decolorization and mineralization of azo dyes under certain environmental conditions [4].

Bacteria have enormous catabolic potential for remediating wastes; however, the interactions between bacteria and pollutants are complex, and suitable remediation does not always take place. Hence, molecular approaches are being applied to enhance bioremediation [5]. Random mutation is one of the attractive molecular strategies for developing more potent xenobiotic degrading strains. Mutagens such as UV irradiation, ethyl methyl sulfonate, and ethidium bromide (EtBr) are employed for inducing mutation [6]. Gopinath et al. [7] have employed random mutagenesis for improved bioremediation of congo red.

In the present work, we have used UV-induced mutations in order to enhance biodegradation prospective of *Pseudomonas* sp. LBC1. Green HE4B (GHE4B) is a sulfonated azo dye, and, due to its structural complexity, it is difficult to degrade higher concentrations of the dye in short time span. Hence, we have employed the mutant bacteria for faster and safer degradation of GHE4B. Wild *Pseudomonas* sp. LBC1 was also simultaneously used for the degradation studies in order to compare with the mutant one. Biodegradation of the dye was monitored with the help of UV–Vis spectrophotometry, high-performance thin-layer chromatography (HPTLC), and Fourier transform infrared spectroscopy (FTIR). The products formed after the degradation of the dye were identified with gas chromatography–mass spectrometry (GC–MS) analysis, and pathways for degradation of GHE4B with both wild-type and mutant bacteria were predicted. In order to find out possible rationale behind improved degradation capacity of mutant bacteria, protein analysis was done using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Differentially expressed prominent proteins were further subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis.

Material and Methods

Dyes, Chemicals, and Molecular Biology Material

Textile dyes were obtained from local textile industries in Ichalkarnji, India. NADH was purchased from Sigma Chemical Company (USA). *o*-tolidine and veratryl alcohol were obtained from SRL Chemicals, India. Nutrient broth with 1 % peptone and bovine catalase were procured from HiMedia Laboratory, India. All chemicals and reagents used in this study were of analytical grade.

All chemicals and reagents used for molecular analysis were of molecular biology grade. EDTA, Tris–HCL, sodium acetate, and SDS were purchased from HiMedia Laboratories, India. Agarose, TAE, PCR amplification reagents, nuclease-free water, and primers were obtained from Bangalore GeNei.

Microorganism and Culture Conditions

Pseudomonas sp. LBC1 used in this study was previously isolated in our laboratory from the textile dye-contaminated soil [8]. As the organism has potential of biodegradation of xenobiotics, it was selected for the present study.

Induction of Mutation and Screening of Mutants

The log-phase cultures of *Pseudomonas* sp. LBC1 were subjected for ultraviolet radiation of 15 W at 20-cm distance, receiving the radiation for 15, 30, 45, 60, 75, 120, 150, and 180 s, and then sheltered from the light immediately. The mutants were diluted with sterilized saline (0.8 % NaCl), then 150 μ l of diluted culture were outspreaded onto the nutrient agar plates containing GHE4B dye. The plates were sheltered from the light and incubated at 37 °C for 48 h.

After 48 h of incubation, the plates were observed for colonies showing zones of decolorization. All the colonies showing zones were again subjected for degradation of GHE4B (70 mg/l) in nutrient broth and the best degrader in terms of time required for complete decolorization was selected. The organism which was able to degrade the dye faster in all mutants screened was maintained as glycerol stock and used in further degradation studies. The best mutant thus obtained was named as *Pseudomonas* sp. 1 F.

Genetic Analysis of Mutation by RAPD

In the present study, genetic difference between wild-type and mutant organism was observed by random amplification of polymorphic DNA (RAPD). Extraction of DNA from wild-type and mutant organism was carried out as per our earlier report [9]. Amplifications were carried out in a 50- μ l reaction mixture. Each primer in Table 1 was used in this experiment. DNA amplification was performed as initial denaturation at 94 °C for 5 min and 45 cycles, as 1 min at 94 °C, 1 min at 36 °C, and 1 min at 72 °C, followed by a final extension cycle of 10 min at 72 °C. The RAPD products were resolved on agarose gel (1.5 %). To confirm reproducibility of the method, all amplifications were repeated for three times.

Degradation of GHE4B by Wild and Mutant Bacteria

For the decolorization experiment, sterile 100-ml nutrient broth was inoculated with 50 μ l of the glycerol stock of the wild-type bacteria and mutant *Pseudomonas* sp. 1 F. The flasks were incubated for 24 h at 37 °C at static conditions. Exactly after 24 h of incubation, 7 mg of dye GHE4B was added to each flask under aseptic conditions. The degradation was monitored. Aliquots (3 ml) of the culture media were drawn after regular time intervals for

Table 1 Primers used for RAPD analysis

Primer no.	Sequence
1	5'GTGACGTAGG3'
2	5'AGTCAGCCAC3'
3	5'GGGTAACGCC3'
4	5'AGGTGACCGT3'
5	5'GTTGGCGGCT3'
6	5'ACAACGCCTC3'
7	5'GGCGGTTGTC3'
8	5'TCAGTCCGGG3'
9	5'TCTGGCGCAC3'
10	5'CACCATCCGT3'

color measurement. Suspended particles from the culture medium were removed by centrifugation at 7,000 rpm for 20 min. Decolorization was monitored by measuring the absorbance of the supernatant at 630 nm using Hitachi U-2800 Spectrophotometer.

Preparation of Cell-Free Enzyme Extract

To assess enzyme profiles of *Pseudomonas* sp. LBC1 and *Pseudomonas* sp. 1 F, 50 μ l glycerol stock of each organism was inoculated. The bacterial cells grown in the nutrient broth were centrifuged at 8,000 rpm for 15 min. The resulting supernatant was used as a source of an extracellular enzyme, and the cells were uniformly suspended in sodium phosphate buffer (50 mM, pH7.0) by homogenization and sonicated (Vibracell ultrasonic processor, Sonics, New Town, CT), keeping sonifier output at 60 amp and giving seven strokes each of 15 s at 1.5-min intervals at 4 °C. This homogenate was centrifuged at 8,000 rpm for 20 min at 4 °C, and the supernatant was used as a source of crude intracellular enzyme. Similar procedure was used to extract and quantify induced enzyme activities after dye decolorization.

Enzyme Analysis

Activities of dye degrading enzymes were assayed spectrophotometrically in cell-free extract and culture supernatant. All enzyme assays were carried out at room temperature; reference blanks contained all components except the enzyme. NADH dichlorophenol indophenol (NADH-DCIP) reductase activity was determined using the procedure reported earlier by Salokhe and Govindwar [10]. DCIP reduction was monitored at 620 nm and calculated using the extinction coefficient of 19 mM/cm. The reaction mixture (5.0 ml) prepared contained 50-mM substrate (DCIP) in potassium phosphate buffer (50 mM, pH7.4) and 0.1-ml enzyme. From this, 2.0-ml reaction mixture was assayed at 620 nm by addition of 50 mM NADH. Lignin peroxidase activity was determined by monitoring the formation of propionaldehyde at 300 nm in 2.5-ml reaction mixture containing 100 mM *n*-propanol, 250 mM tartaric acid, and 10 mM H₂O₂.

For veratryl alcohol oxidase activity, the reaction mixture contained 4 mM veratryl alcohol, in citrate phosphate buffer (50 mM, pH3), and 0.2 ml of 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 [11]. Laccase activity was determined in a reaction mixture of 2 ml containing 5 mM *o*-tolidine in acetate buffer (20 mM, pH 4.0). The reaction was initiated by the addition of 0.2 ml of enzyme solution, and increase in optical density at 366 nm was measured [12]. For calculation of specific activity, protein concentration was determined by Lowry method [13] using bovine serum albumin as the standard.

Analysis of Degradation Products

The metabolites produced after decolorization of the dye were extracted by using equal volume of ethyl acetate. Extracted product metabolites were completely dried and then dissolved in small volume of HPLC-grade methanol. Same sample was then subjected for HPTLC and GC–MS analysis.

FTIR analysis was carried out using Perkin Elmer 783 spectrophotometer, and changes in % transmission at different wavelengths were observed. The FTIR analysis was done in the

mid-infrared region of 400–4,000 cm^{-1} . Dye powder and dried degradation products of GHE4B by both *Pseudomonas* sp. LBC1 and *Pseudomonas* sp. 1 F were analyzed by FTIR.

For HPTLC analysis, precoated TLC silica gel (60 F 254) plates supplied by Merck were used. Ten microliters of three samples (GHE4B, dye metabolites after degradation by *Pseudomonas* sp. LBC1, and metabolites after degradation by *Pseudomonas* sp. 1 F) was spotted on HPTLC plates by using a microsyringe (HPTLC Camag, Switzerland). The solvent system methanol/ethyl acetate/*n*-propanol/water/acetic acid (1:2:3:1:0.2, *v/v*) was used as mobile phase in HPTLC analysis. The dye chromatogram was scanned at 254 nm.

Metabolites obtained after degradation of GHE4B by *Pseudomonas* sp. LBC1 and mutant *Pseudomonas* sp. 1 F were analyzed by GC–MS. Along with the dye metabolites, regular metabolites of untreated bacterial cells of *Pseudomonas* sp. LBC1 and mutant *Pseudomonas* sp. 1 F grown in nutrient broth were subjected for GC–MS. The GC–MS analysis was carried out using a Shimadzu 2010 MS Engine, equipped with an integrated gas chromatograph and HP1 column (60 m long, 0.25 mm id, nonpolar). Helium was used as the carrier gas at a flow rate of 1 ml/min. The injector temperature was maintained at 280 °C, while the oven conditions were 80 °C for 2 min, followed by an increase to 210 °C based on a rate of 10 °C/min, and then followed by a further increase to 280 °C based on a rate of 20 °C/min.

Protein Analysis by MALDI-TOF

To study the differential expression of proteins in *Pseudomonas* sp. LBC1 and *Pseudomonas* sp. 1 F, bacterial cells were grown in nutrient broth for 48 h. Extracellular and intracellular proteins were extracted. The cellular response of induced (after exposure to dye) wild and mutant bacteria in terms of synthesized proteins was also observed. Extracellular and intracellular proteins of wild and mutant organism both before and after exposure to dye were subjected for SDS-PAGE. The discriminating protein bands were then analyzed by peptide mass fingerprinting (PMF) on MALDI-TOF. The bands were first subjected for in-gel trypsin digestion, and the resulting peptides were subjected to PMF [14]. MALDI-TOF spectra were recorded in reflector mode using the matrix α -cyano-4-hydroxy cinnamic acid. For identification of the proteins, MASCOT search tools were used. As the genome sequence of the organism is not known, the proteins were identified using relevant *Pseudomonas* sp. database and NCBI database.

Effect of Catalase on Degradation of GHE4B

To verify involvement of catalase in GHE4B degradation, we added pure bovine catalase (HiMedia) in dye decolorization study. For the decolorization experiment, sterile 100-ml nutrient broth was inoculated with 50 μl of the glycerol stock of the wild-type bacteria and mutant *Pseudomonas* sp. 1 F separately. The flasks were incubated for 24 h at 37 °C at static condition. Exactly after 24 h of incubation, 7 mg of dye GHE4B was added to the flasks under aseptic conditions. One flask of each organism was added with pure bovine catalase (HiMedia), and control without catalase was maintained. The degradation was monitored. In another experiment, the same concentration of dye solution (in 100 mM sodium phosphate buffer, pH 7.0) was incubated with catalase without bacterial cells at 37 °C.

Phytotoxicity Study

Phytotoxicity study was carried out in order to assess the toxicity of degradation metabolites in comparison with the original dye GHE4B. Two kinds of seeds, *Phaseolus mungo* and

Sorghum vulgare, were used in the study. The GHE4B and ethyl acetate extracted degradation product of wild as well as mutant bacteria were dissolved separately in distilled water to make the final concentration of 1,500 ppm. Toxicity study was done by growing ten seeds of each plant species separately into D/W as control, dye, and extracted degradation products of *Pseudomonas* sp. LBC1 and *Pseudomonas* sp. 1 F. Comparative data of germination (in percentage) and length of shoot and root were recorded after 7 days.

Statistical Analysis

All the experiments were performed in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) with the Tukey–Kramer multiple comparison test.

Results and Discussion

Screening of Mutants

After induction of mutation, all the plates were observed for zones of decolorization of dye. The colonies showing zones of higher diameter were selected and subjected for degradation of GHE4B in nutrient broth. GHE4B being sulfonated azo dye is recalcitrant to most biological degradation treatments. Hence, the same dye was selected to identify the most potent UV mutant in comparison with the wild-type bacteria. In initial studies, it was found that among the number of mutants screened, mutant *Pseudomonas* sp. 1 F was degrading GHE4B faster than the wild bacteria and other mutants. The best mutant was observed on the plate exposed for 75 s.

Genetic Analysis of Mutation by RAPD

UV radiation can produce several major types of DNA lesions such as cyclobutane-type pyrimidine dimers and the 6-4 photoproducts [15]. Other important types of DNA damages such as protein cross-links, DNA strand breaks, and deletion or insertion of base pairs can also be induced by UV irradiation. These different types of DNA damages could be detected by changes in RAPD profiles [16]. Genetic variation among different strains can be documented using various RAPD markers. Among the ten primers used in RAPD analysis, nine showed significant polymorphism. The key changes in the RAPD profiles were observed as an appearance or disappearance of different bands (Fig. 1) with variation in their intensity as well. UV-induced structural rearrangements in DNA must have resulted in polymorphism of RAPD bands.

Degradation of GHE4B by Wild and Mutant Bacteria

From spectrophotometric data (Fig. 2), it was observed that the wild-type bacteria achieved 100 % decolorization of the dye GHE4B in 48 h, while *Pseudomonas* sp. 1 F decolorized complete dye within 36 h.

Enzyme Analysis

The enzymes playing imperative role in the biodegradation of GHE4B were found to be laccase, DCIP reductase, veratryl alcohol oxidase, and lignin peroxidase. When enzyme

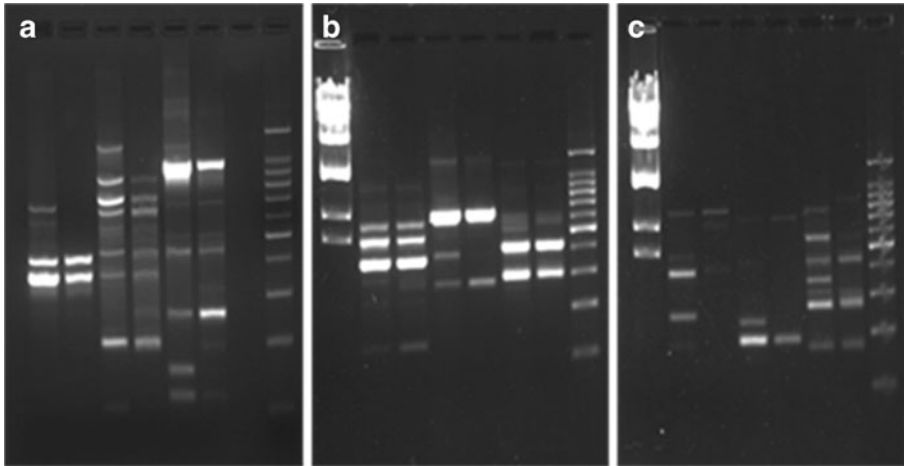


Fig. 1 Polymorphism in RAPD pattern using nine different primers. **a** Lanes 1, 3, 5 RAPD of wild DNA with primers 2, 3, and 4, respectively. Lanes 2, 4, 6 RAPD of genomic mutant DNA with primers 2, 3, and 4, respectively. Lane 8 A 200-bp ladder. **b** Lane 1 λ MluI marker. Lanes 2, 4, 6 RAPD of wild DNA with primers 5, 6, and 7, respectively. Lanes 3, 5, 7 RAPD of genomic mutant DNA with primers 5, 6, and 7, respectively. Lane 8 A 200-bp ladder. **c** Lane 1 λ MluI marker. Lanes 2, 4, 6 RAPD of wild DNA with primers 8, 9, and 10, respectively. Lanes 3, 5, 7 RAPD of mutant DNA with primers 8, 9, and 10, respectively. Lane 8 A 200-bp ladder

activities of the wild and the mutant bacteria were compared, interestingly, all the enzymes were found to be induced in the mutant organism (Table 2). As mutagens lead to mutation by inducing lesion or modification in base sequence of DNA that remains unrepaired, improvement in enzyme activity might be due to photolysis of pyrimidines to form dimers. UV irradiation might have caused error at replication and, hence, resulted in mutation. There might be an increase in copy number or expression of genes for dye degradation enzymes [17, 18]. Thus, induced oxidoreductases might have lead to faster degradation of GHE4B by mutant organism than wild type.

Analysis of Degradation Products

In FTIR analysis, the dye GHE4B (Fig. 3a) showed the presence of peaks like $3,444.42\text{ cm}^{-1}$ for symmetric N–H stretching as in the case of primary amines, peaks $1,633.98\text{ cm}^{-1}$, and $1,570.04\text{ cm}^{-1}$ correspond to the presence of N = N stretching as in azo bond. Peak at $1,288.22\text{ cm}^{-1}$ indicated the presence of C–N vibration as in the case of aromatic secondary

Fig. 2 Spectral analysis of bio-degradation of GHE4B. *Black circle*, degradation of GHE4B by *Pseudomonas* sp. LBC1; *black square*, degradation of GHE4B by *Pseudomonas* sp. 1 F

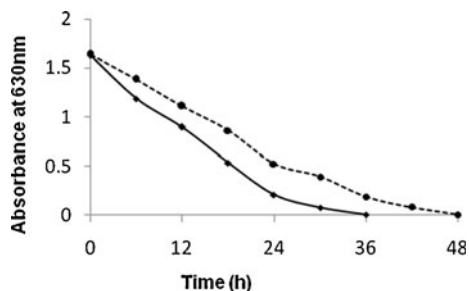


Table 2 Comparison of enzyme activities of *Pseudomonas* sp. LBC1 and mutant *Pseudomonas* sp. 1 F before and after degradation of dye

Enzyme	Before addition of dye		After degradation of dye	
	LBC1	1 F	LBC1	1 F
Laccase (extracellular)	0.544±0.009	0.610±0.006	0.920±0.023\$	1.114±0.022*\$
Laccase (intracellular)	1.220±0.007	1.413±0.009*	1.925±0.012\$	2.480±0.011*\$
Veratryl alcohol oxidase	0.851±0.006	0.970±0.001*	0.636±0.002\$	0.741±0.002*\$
Lignin peroxidase	0.559±0.006	0.742±0.003*	0.310±0.004\$	0.486±0.003*\$
NADH-DCIP reductase	240.96±1.54	269.17±1.69*	484.00±1.94\$	602.86±2.99*\$

Values are a mean of three experiments ± SE. Significantly different from wild-type *Pseudomonas* sp. LBC1 at * $P < 0.001$ and significantly different from uninduced cells at \$ $P < 0.001$ by one-way ANOVA with Tukey–Kramer comparison test

amines, while peak at $1,047.15 \text{ cm}^{-1}$ dictated the presence of S = O stretching in sulfonic acids. On the other hand, the FTIR spectra of degradation products of wild as well as mutant bacteria were found to be completely different from the control dye, clearly suggesting the biodegradation of the dye. Dye metabolites of *Pseudomonas* sp. LBC1 (Fig. 3b) showed peaks like $3,425.19 \text{ cm}^{-1}$ symmetric stretching as in the case of primary amines, $1,661.88 \text{ cm}^{-1}$ corresponds to C = C stretching of alkenes, peaks at $1,338.81 \text{ cm}^{-1}$ and $1,017.24 \text{ cm}^{-1}$ could be attributed to C–N vibrations as in the case of aromatic primary

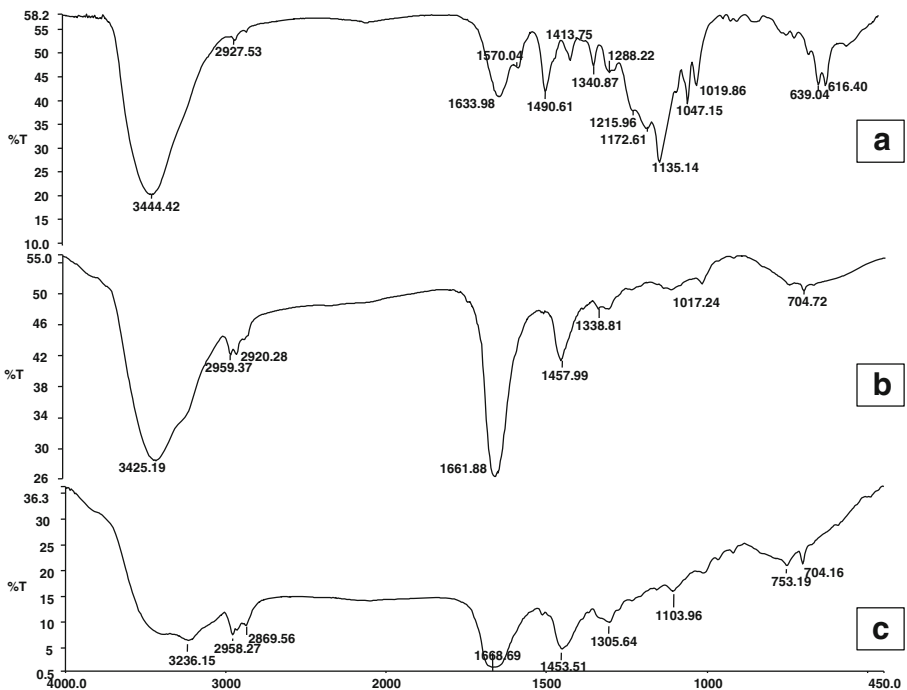


Fig. 3 FTIR analysis of GHE4B degradation. *a* FTIR spectrum of GHE4B. *b* FTIR spectrum of degradation metabolites of GHE4B by *Pseudomonas* sp. LBC1. *c* FTIR spectrum of degradation metabolites of GHE4B by *Pseudomonas* sp. 1 F

amines, and C–O stretching as in primary alcohols, respectively. Dye metabolites of *Pseudomonas* sp. 1 F (Fig. 3c) revealed the presence of $3,236.15\text{ cm}^{-1}$ for N–H stretching as in amines, $1,668.69\text{ cm}^{-1}$ corresponds to C = C stretching of alkenes, and $1,305.64\text{ cm}^{-1}$ represents C–N vibrations as in the case of aromatic primary amines.

The HPTLC analysis of control dye and degradation product showed different patterns of Rf values. GHE4B dye has shown spots at Rf 0.27, 0.61, 0.67, 0.76, and 0.97; while Rf values for degradation product of *Pseudomonas* sp. LBC1 were 0.05, 0.53, 0.59, 0.71, and 0.97, and product of mutant were 0.05, 0.24, 0.51, 0.57, 0.69, and 0.97. Three-dimensional graphs (Fig. 4) of GHE4B (a), degradation metabolites of *Pseudomonas* sp. LBC1 (b), and *Pseudomonas* sp. 1 F (c) clearly suggest degradation of the dye.

GC–MS analysis was used to determine the probable metabolites and predict pathways for degradation of the dye. GC–MS predicted compounds that were also present in uninduced cells were considered as routine cell metabolites, while the remaining metabolites were considered to be GHE4B degradation metabolites. Azo group N and aromatic ring C, substituted with hydroxyl/amino group in sulfonated dyes, is an acting site for lignolytic enzymes (laccases/peroxides) [19]. Cleavage of C–N bonds and desulfonation can be attributed to the action of laccase. Thus, correlating the enzyme activity results with the GC–MS analysis, in the proposed pathways for both wild-type and mutant bacteria, we can conclude that Green HE4B could have undergone first symmetric cleavage by lignin peroxidase to produce the unstable intermediate (A, hypothetical). In *Pseudomonas* sp. LBC1 pathway (Fig. 5), the intermediate “A” was then split into another unstable intermediate “B” and stable sodium 2,4-dihydroxy-3 methyl benzene sulfonate (I) (m/z 224).

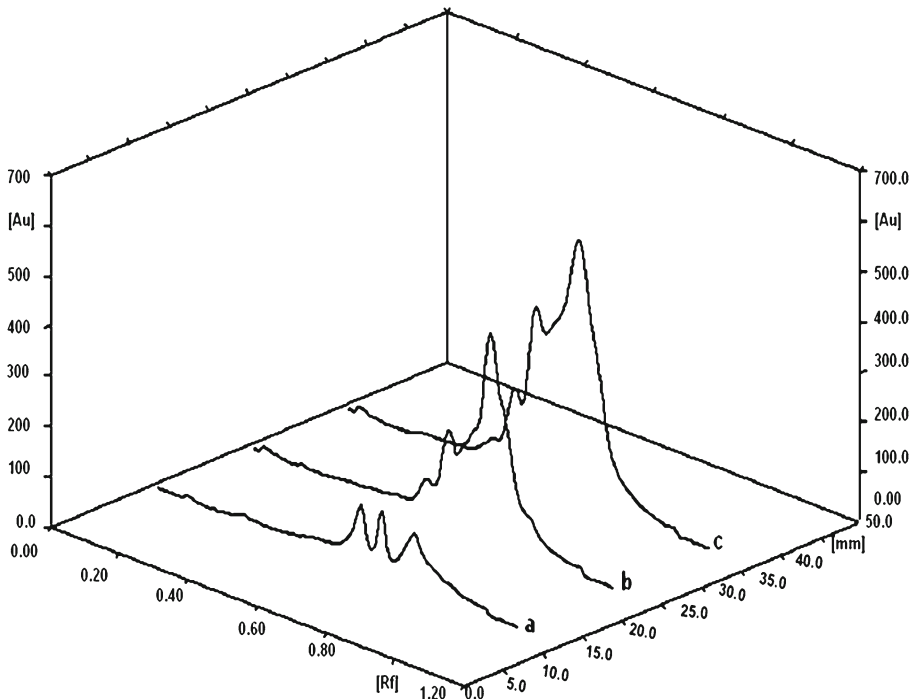


Fig. 4 HPTLC analysis of GHE4B degradation. *a* Three-dimensional graph of GHE4B. *b* Three-dimensional graph degradation metabolites of *Pseudomonas* sp. LBC1. *c* Three-dimensional graph degradation metabolites of *Pseudomonas* sp. 1 F

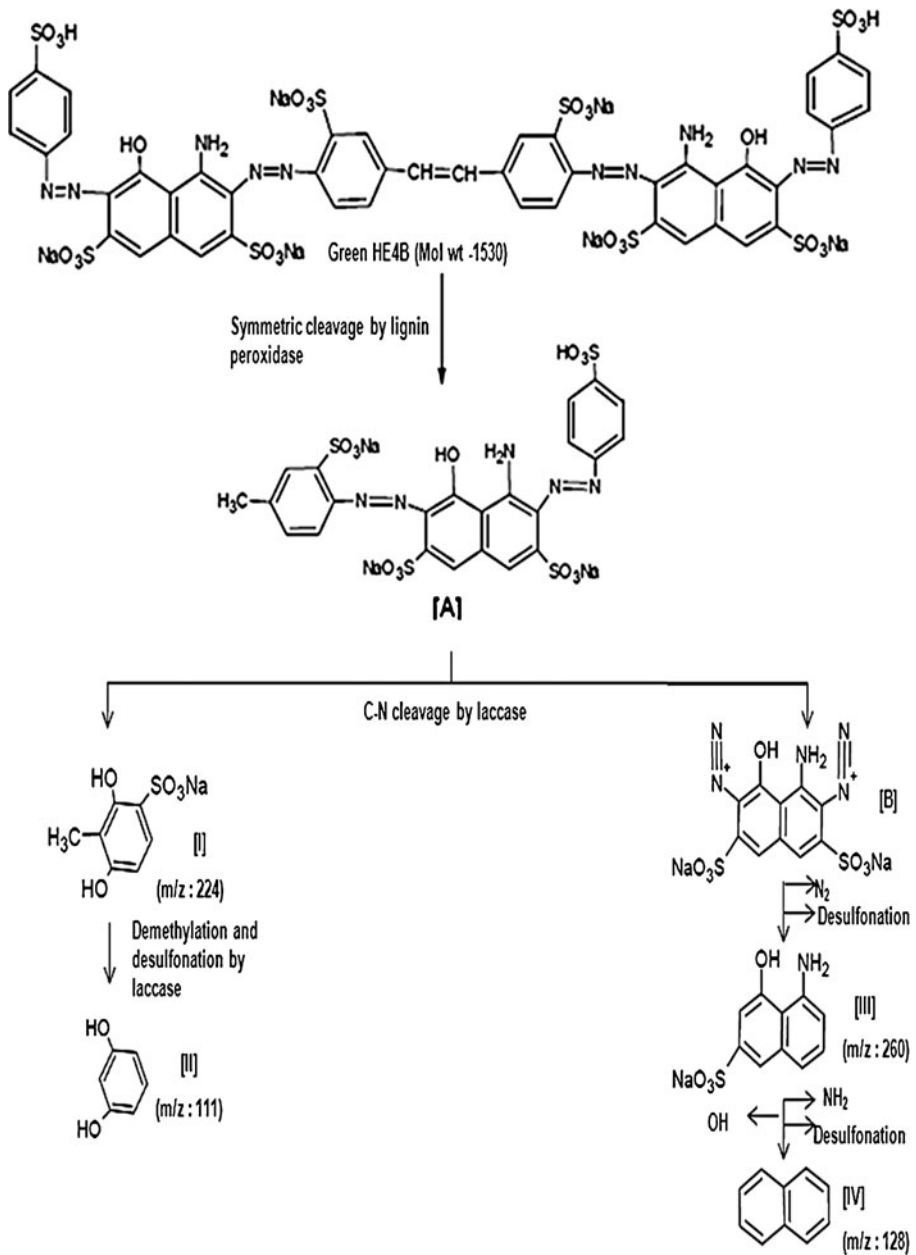


Fig. 5 GHE4B degradation pathway for *Pseudomonas* sp. LBC1

Product I was further metabolized to benzene 1,3-diol (II) (m/z 111). Unstable intermediate B underwent several further redox reactions to produce more stable intermediates identified as sodium 5-amino 4-hydroxy naphthalene-2-sulfonate (III) (m/z 260) and naphthalene (IV) (m/z 128). While mutant bacteria produced two distinguishing metabolites (Fig. 6) in comparison with wild-type bacteria, one was identified as 3-methyl phenol (I) (m/z 104),

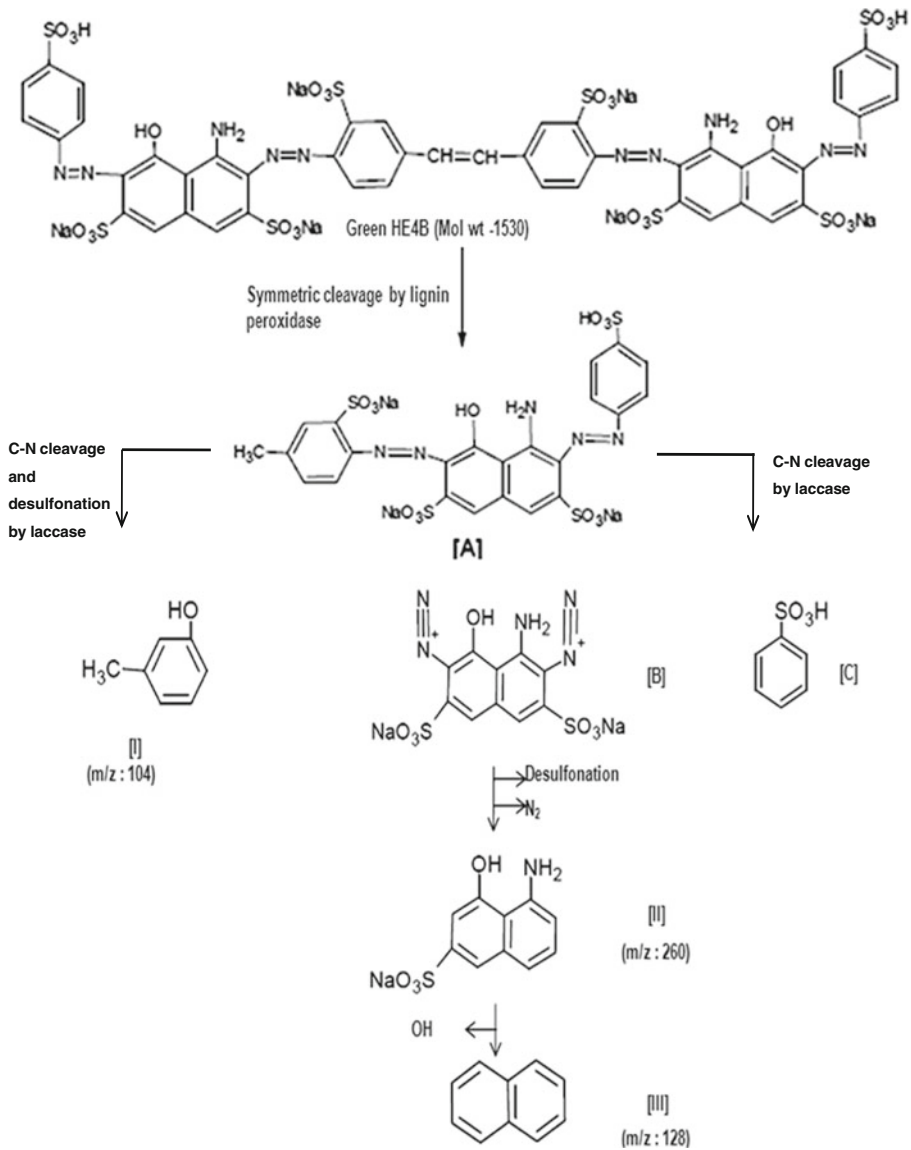


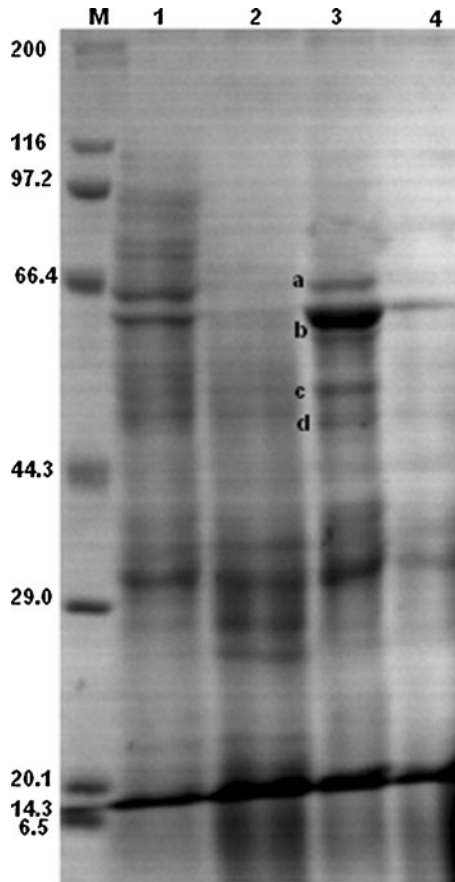
Fig. 6 GHE4B degradation pathway for *Pseudomonas* sp. 1 F

and the metabolite corresponding to m/z 238 could not be identified. Other two products of degradation [(m/z 260) and (m/z 128)] by GHE4B were found to be same as *Pseudomonas* sp. LBC1 products.

Protein Analysis by MALDI-TOF

SDS-PAGE analysis of extracellular proteins revealed three distinct bands in induced *Pseudomonas* sp. 1 F. Figure 7 shows overexpression of one extracellular protein in *Pseudomonas* sp. 1 F than *Pseudomonas* sp. LBC1. When these four bands were subjected for PMF, the overexpressed

Fig. 7 SDS-PAGE analysis. *M*: molecular weight marker (in kilodalton). *1*: Intracellular proteins of uninduced mutant *Pseudomonas* sp. 1 F. *2*: Intracellular proteins of uninduced *Pseudomonas* sp. LBC1. *3*: Intracellular proteins of green HE4B induced mutant *Pseudomonas* sp. 1 F. *4*: Intracellular proteins of induced *Pseudomonas* sp. LBC1



protein (Fig. 7, band b) was found to be catalase. The distinguishing proteins (Fig. 7, bands a, c, and d) were putative dehydrogenase, nitric oxide dioxygenase, and glutathione *s*-transferase (GST). Catalase and GST are major antioxidant enzymes. Expression of antioxidant enzymes is a result of exposure of mutant bacteria to UV irradiation as well as to the dye. Induction of catalase during biodegradation of sulfonated azo dyes has been previously reported in fungi by Laxminarayana et al. [20]. GST is a group of enzymes that is involved in the detoxification of many endobiotic and xenobiotics substances [21]. Though the exact role of antioxidant enzymes in degradation of azo dyes has not been thoroughly investigated, strong relationship between expression of antioxidant enzymes and degradation of xenobiotic compounds has been proposed in recent years [22–24]. Buckova et al. [22] have reported that the enzymes that participate in the protection of cells against oxidative stress are also involved in metabolic oxidation of ubiquitous phenolic contaminants of soil and water. Hence, in accordance with these interrelationships, we speculate that expression of catalase and glutathione *s*-transferase has a role in enhanced degradation of GHE4B.

Effect of Catalase on Degradation of GHE4B

According to MALDI results, induced antioxidant enzymes in mutants might have enhanced the degradation rate. As catalase was the most overexpressed protein, its involvement in dye

Table 3 Phytotoxicity study of GHE4B and its metabolites

	<i>Phaseolus mungo</i>				<i>Sorghum vulgare</i>			
	I	II	III	IV	I	II	III	IV
Germination (%)	100	60	90	100	100	50	100	100
Shoot length (cm)	18.1±0.78	9.7±0.67	11.7±1.12	15.6±0.41**\$	13.6±0.56	6.2±0.50	7.9±0.21	11.7±0.49***\$\$
Root length (cm)	6.5±0.41	2.7±0.43	4.2±0.28	5.9±0.64**	7.5±0.44	3.1±0.15	4.0±0.49*	6.0±0.41**

Values are a mean of three experiments ± SE. Significantly different from dye solution at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, and significantly different from dye metabolites of *Pseudomonas* sp. LBC1 at \$ $P < 0.05$ and \$\$ $P < 0.01$ by one-way ANOVA with Tukey–Kramer comparison test. I, plain D/W; II, GHE4B at the concentration 1,500 ppm; III, dye metabolites after degradation with *Pseudomonas* sp. LBC1; IV, dye metabolites after degradation with mutant *Pseudomonas* sp. I F

degradation was studied. It was found that the addition of external catalase reduced the time required for degradation up to 24 h. However, when only pure catalase without bacteria was used, no decolorization was observed even after 72 h. Thus, the study suggests indirect involvement of catalase in enhanced biodegradation of Green HE4B by mutant bacteria.

Phytotoxicity Study

Phytotoxicity study revealed the toxic nature of GHE4B to plants (Table 3). Germination as well as length of shoots and roots was drastically affected in the presence of dye. On the contrary, degradation metabolites were less toxic to the plants. Dye metabolites obtained after degradation with *Pseudomonas* sp. 1 F were least toxic to the plants.

Conclusions

UV mutation has yielded one potent azo dye-degrading strain. Faster degradation of the dye by mutant is because of the induction of dye-degrading oxidoreductases. UV mutation has not only induced dye degradation enzyme activities but it has also provoked antioxidant enzymes, which ultimately lead to quicker biodegradation of recalcitrant dye with mutant bacteria. In order to get profound insight into the precise involvement of antioxidant enzymes in the biodegradation of sulfonated azo dyes like GHE4B, future investigation is necessary.

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