Biodegradation of Green HE4B: Co-substrate effect, biotransformation enzymes and metabolite toxicity analysis

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Abstract A high exhaust reactive dye, Green HE4B (GHE4B) was 98% degraded in nutrient medium by *Pseudomonas desmolyticum* NCIM 2112 (pd2112) within 72 h at static condition. Decolorization time in synthetic effluent was reduced from 10 to 5 days in the presence of 10 g/l molasses. Addition of 5 g/l peptone to NaCl medium had reduced decolorization time from 108 to 72 h. Beef extract do not contribute more to the inducing effect of peptone, however it is a good co-substrate in sucrose or urea containing NaCl medium. Intracellular lignin peroxidase (Lip), laccase and tyrosinase activities were induced by 150, 355 and 212%, respectively till maximum dye removal took place. Aminopyrine N-demethylase (AND) and dichlorophenol indophenol reductase (DCIP-reductase) activities in pd2112 were induced by 130 and 20%, respectively at 72 h of incubation during GHE4B decolorization. By high performance liquid chromatography (HPLC) analysis, 4-hydroxybenzene sulfonic acid and 4-amino, 6-hydroxynaphthalene 2-sulfonic acids were identified as metabolites formed during 24–72 h incubation. Fourier transform infrared spectroscopy (FTIR) analysis supports the formation of these aromatic amines. pd2112, aerobically degraded GHE4B metabolites (formed at static condition) showing stationary phase of 6 days. There was no germination inhibition of *Sorghum*

bicolor and *Triticum aestivum* by GHE4B metabolites at 3,000 ppm concentration however untreated dye showed germination inhibition at the same concentration. GHE4B metabolites did not show any microbial toxicity at 10,000 ppm concentration.

Keywords Biodegradation · Co-substrate · Green HE4B · Reactive dye · Toxicity

Introduction

Reactive dyes are very soluble by design and, as a result, not all are used up by textile fibers during the dying process and therefore end up with the discharge from dye houses. They are hardly biodegraded under aerobic conditions [1]. These sulfonated and unsulfonated reactive dyes have a negative esthetic effect on the wastewater and some of these compounds and biodegradation products show carcinogenic and mutagenic characters [2]. As much as 90% of reactive dyes could remain unaffected after activated sludge treatment [1]. Fungi are reported to decolorize reactive azo dyes [3]. Some reactive dyes decolorization was mediated by mixed bacterial culture [4]. Many studies have reported only the effect of physicochemical parameters on decolorization of dyes. However, it is important to know the enzymes involved in decolorization process so that the structure-biodegradability relationship can be studied. A sulfonated dye, Green HE4B (GHE4B), is widely used in textile industries and its microbial decolorization has not studied. Here, we have reported GHE4B degrading enzymes in *Pseudomonas desmolyticum* NCIM 2112 (pd2112) at static anoxic condition. The phyto- and microbial-toxicity of the metabolites formed after complete dye decolorization have been assessed.

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Materials and methods

Chemicals

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and aminopyrine were purchased from Sigma-Aldrich, St. Louis, USA. Tartaric acid, n-propanol, catechol, 4-hydroxybenzene sulfonic (4-HBS) and 4-amino, 6-hydroxynaphthalene 2-sulfonic (4-A-6-NOH-2-S) acids were purchased from Sisco Research Laboratories, Mumbai, India. The textile dye GHE4B (C.I. Green 19A; $\lambda_{\max}$ 630 nm) was a generous gift from local textile industry, Solapur, India. All chemicals used were of the highest purity available and of the analytical grade.

Microorganisms and culture conditions

All bacterial cultures were obtained from National Center for Industrial Microorganisms, NCL, India. Pure culture was maintained on nutrient agar slants. For decolorization, the composition of nutrient medium was (g/l): NaCl 5, peptone 5, beef extract 3 containing GHE4B 0.1. The composition of synthetic effluent used for decolorization study was (g/l): GHE4B 0.1, soluble starch 1.0, acetic acid 0.15, $(\text{NH}_4)_2\text{SO}_4$ 0.28, NH_4Cl 0.23, KH_2PO_4 0.067, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.022, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.005, yeast extract 0.2, NaCl 0.15, NaHCO_3 1.0, and 1 ml/l of a trace element solution containing (g/l) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.1, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.392, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.248, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ 0.177 and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.02 [5]. Synthetic medium was used for degradation of aromatic amines produced after decolorization/degradation of GHE4B in nutrient medium and its composition was (g/l): NH_4Cl 2, KH_2PO_4 4, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.02, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.1, CaCO_3 0.08, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1. pd2112 was acclimatized [6] by gradually exposing to the increasing concentration of dye, i.e. 0.05–0.25 g/l and then used for all studies.

Dye decolorization

pd2112 was grown in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth for 24 h at 30°C. As A_{660} reached to 1.1, 100 mg/l dye was added in each flask and decolorization was studied at static as well as shaking condition (120 rpm) at 30°C. At certain time intervals, aliquot (3 ml) of the culture media was withdrawn, centrifuged at 5,000 rpm for 15 minutes and the A_{630} of culture supernatant was measured. The change in pH and reduction in chemical oxygen demand (COD) [7] were also studied in the same sample. Bacterial growth was followed by the gravimetric method after drying

at 80°C until constant weight. The percentage decolorization was calculated according to Saratale et al. [8].

For decolorization in synthetic effluent, cells were grown (A_{660} 1.1; 24 h) in nutrient medium and transferred aseptically to 100 ml synthetic broth. The effect of carbon (10 g/l glucose, sucrose and molasses) and nitrogen (5 g/l peptone, urea and malt extract) sources on decolorization performance of pd2112 in synthetic effluent was studied at 30°C at static condition.

The effect of individual nutrient medium components on decolorization was studied in 0.1 M NaCl medium. The concentration of glucose, sucrose, molasses, and peptone were 5 g/l, and urea concentration was 1 g/l in NaCl medium. For this 10% inoculum of pd2112 (nutrient broth; A_{660} 1.1; 24 h) was added to NaCl medium. Effect of beef extract addition (3 g/l) into NaCl medium was studied in the presence of all co-substrates. All decolorization experiments were performed in three individual sets. Uninoculated controls were used to compare color loss during the experiment.

Aerobic degradation [9] of aromatic amines formed after dye degradation in anoxic condition was studied. In brief, 5% inoculum of pd2112 was added (A_{530} 0.1) in 100 ml synthetic medium containing 0.1 g/l GHE4B metabolites and incubated at 30°C at 150 rpm. At different time intervals, culture supernatant was collected (5,000 rpm, 5 min), diluted in 0.10 M sodium phosphate buffer solution (pH 7.0) and absorbance was measured at 315 nm [10]. Growth of pd2112 on aromatic amines was measured at 660 nm periodically.

Enzyme assays

The cell-free extract was prepared as reported previously [6]. Lignin peroxidase (Lip) (EC 1.11.1.14) activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 ml containing 100 mM n-propanol, 250 mM tartaric acid and 10 mM H_2O_2 [5]. Laccase (EC 1.10.3.2) activity was determined in a reaction mixture of 2 ml containing 0.1% ABTS in 0.1 M acetate buffer (pH 4.9) and measured increase in optical density at 420 nm [11]. Tyrosinase activity (EC 1.14.18.1) was determined in a reaction mixture of 2 ml, containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) at 410 nm [12]. All enzyme assays were carried out at 30°C, where reference blanks contained all components except the enzyme. One unit of enzyme activity was defined as a change in absorbance unit/min/mg/protein. The assay mixture for nicotinamide adenine dinucleotide reduced-dichlorophenol indophenol (NADH-DCIP) reductase activity contained 50 μM DCIP, 50 μM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution in a total volume of

5.0 ml. The DCIP reduction was monitored at 595 nm using the extinction coefficient 90/mM/cm [6]. Aminopyrine N-demethylase (AND) activity was measured as reported elsewhere [13]. All enzyme assays were run in triplicate and average rates calculated. Protein concentrations were estimated by Biuret method.

UV-visible, HPLC and FTIR analysis of metabolites

The samples collected during decolorization at different time intervals were scanned between 400 and 800 nm, spectrophotometrically (Hitachi U-2800), for qualitative analysis of decolorization. For the analysis of dye metabolites, the metabolites were extracted from culture supernatants of different time intervals by adding equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. For high performance liquid chromatography (HPLC; waters model No. 2690) analysis, the crystals obtained were dissolved in small volume of HPLC grade methanol and sample run on C18 column (symmetry, 4.6×250 mm) with 1 ml/min flow rate and ultraviolet (UV) detector at 248/317 nm. The HPLC of authentic samples of 4-HBS acid (248 nm) and 4-A-6-NOH-2-S acid (317 nm) were carried out. Fourier transform infrared spectroscopy (FTIR; Perkin Elmer 783) analysis was done in the mid-IR region of $400\text{--}4000\text{ cm}^{-1}$ with 16-scan speed. The crystals obtained after rotary evaporation were mixed with spectroscopically pure KBr in the ratio 5:95, pellets were fixed in sample holder, and the analyzes were carried out.

Phyto- and microbial-toxicity studies

The phytotoxicity of the dye and its metabolites were tested in the concentration range of 200–3,000 ppm. Tests were carried out according to the method of Parshetti et al. [14] on two kinds of seeds, commonly used in the Indian agriculture: *Sorghum bicolor* and *Triticum aestivum*. Fifteen seeds of each plant were supplied daily with 10 ml dye or dye metabolites dissolved in water, for 7 days in petriplates, maintaining light and temperature (30°C) in a controlled environment. Germination inhibition of seeds was reported after 7 days. The microbial-toxicity of dye and its metabolites was accessed in the concentration range of 100–1,000 ppm by using some agricultural important microorganisms, viz. pd2112, *A. vinelandii* [15], *P. aeruginosa* [16] and biotechnologically important model organism, *Escherichia coli*. The zone of inhibition (diameter in cm) was recorded after incubation of 24 h at 30°C .

Statistical analysis

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

Results and discussion

Time course of decolorization and effect of co-substrates

Acclimatization of pd2112 reduced the time required for complete decolorization of 100 mg/l dye from 144 to 72 h. In nutrient medium the growth of pd2112 (0.45 g/l) and GHE4B decolorization (98%) was more at static as compare to shaking condition, which was 0.31 g/l growth and 63% decolorization. During decolorization, pH of medium was changed from 6.5 to 7.9 at shaking, and 6.8 to 8.0 at static condition. There was no shift in λ_{max} of dye in this pH range which indicates that the decolorization was due to microbial action. The COD was reduced by 65.91% at static and 33.39% at shaking condition (data not shown). The reason for less reduction in COD at shaking condition could be slow degradation to another cleavage product as oxygen and the azo compounds compete for the reduced electron carriers under aerobic condition [6]. There was no decolorization in abiotic control flasks.

Although ability of an organism to decolorize dye in effluent has great importance, the main limitation of such approach is that the process of decolorization is slow, typically requiring several days. Co-metabolism, by the addition of specific substrates, can induce the biodegradation process and reduce the overall process time [17]. The decolorization ability of pd2112 was accessed in synthetic effluent in the presence of different carbon and nitrogen sources. In synthetic effluent, within 10 days, 85 and 51% dye was removed at static and shaking condition respectively (Fig. 1A). Further studies were conducted at static condition as it favored maximum decolorization of GHE4B. Seventy-one to 80% dye was decolorized in the presence of urea and glucose. Addition of sucrose, peptone, or malt extract in synthetic effluent gave 100% decolorization within 7 days (Fig. 1B). The time required for complete decolorization was significantly reduced to 5 days in the presence of molasses (Fig. 1B). Microbial growth in molasses containing medium (0.55 g/l) was more as compare to growth in the presence of other co-substrates (data not shown). Molasses is widely used as a carbon source in fermentation industries because of its availability. In *Geotrichum candidum* Dec 1,

Fig. 1 (A) Decolorization of GHE4B in synthetic effluent at static and shaking conditions. (B) Effect of co-substrate addition on decolorization efficiency of *P. desmolyticum* at static condition.

molasses at concentrations ≥ 10 g/l was found to increase the decolorization activity of the culture broth toward dye, Reactive Blue 5 [18].

Our preliminary studies revealed that pd2112 require additional carbon and nitrogen sources to co-metabolize the dye. Hence, the effect of individual carbon or nitrogen sources was evaluated for dye decolorization in NaCl containing medium. In the presence of glucose, sucrose and molasses, the decolorization was $\leq 22\%$ within 108 h, which was significantly soared up to 97% in the presence of

urea. A dramatic decrease of 36 h in decolorization time was achieved when cells were grown in the presence of peptone (Fig. 2). The growth of organism was more in the presence of urea and peptone as 0.40 and 0.49 g/l, respectively (data not shown). Peptone is also reported to induce anoxic biodecolorization of Reactive Brilliant Blue (KN-R) by *Rhodocyclus gelatinosus* XL-1 [19]. Here, 20 g/l peptone accelerated decolorization, reaching 100% within 48 hours. The peptone concentration ≥ 20 g/l did not repress the dye decolorization (data not shown).

Fig. 2 Decolorization of GHE4B in NaCl medium (N) in presence of different carbon and nitrogen sources. Increased decolorization performance of *P. desmolyticum* after addition of beef extract in the medium. The maximum standard deviation is within ± 3 . BE: Beef extract, Glu: Glucose, Suc: Sucrose, Mol: Molasses, Ur: Urea, Pep: Peptone.

pd2112, in nutrient medium gave maximum decolorization in minimum time (72 h), and beef extract is a major component of it, we had further studied the effect of beef extract (0.3%) addition on decolorization of GHE4B. Beef extract increased the decolorization in glucose, sucrose and urea containing medium by $\geq 98\%$, reducing the time required for complete decolorization to 96 hours in later two co-substrates (Fig. 2). There was no significant effect in peptone containing medium. All these finding suggest that decolorization of GHE4B by pd2112 is dependent on carbohydrate metabolism. The ability of pd2112 to use molasses and urea, as co-substrate was encouraging from a commercial point of view. Such co-metabolic nature was observed in *Pseudomonas* species [20] and *P. aeruginosa* [21]. Addition of co-substrates induces dye degrading enzymes [22].

Biotransformation enzyme activities during dye decolorization

Bacterial extra/intracellular lignolytic enzymes comprising mainly lignin peroxidase (Lip), laccase, and tyrosinase can attack a wide variety of complex aromatic structures including dyestuffs [9, 23]. The activities of biotransformation enzymes in pd2112 were significantly induced during decolorization of GHE4B (Fig. 3). Intracellular Lip activity was induced by 150% when complete dye decolorization accomplished (72 h). However, extracellular Lip activity was induced by 43% when dye decolorization reached to $\sim 90\%$ (in 48 h), which was gradually leveled off reaching to control status. In case of intracellular Lip, even though the activity was reduced after complete dye degradation, but still it was 50% induced as compare to its 0 h status. Intracellular laccase and tyrosinase activities were induced by 355 and 212%, respectively, till maximum dye removal took place. At 96 h, although intracellular laccase activity was decreased, it retained 236% induced (Fig. 3A). Extracellular laccase or tyrosinase activity was absent. AND- and DCIP-reductase activities were induced by 130 and 20%, respectively at 72 h incubation during decolorization. MG-reductase activity remained unchanged (Fig. 3B).

Enzyme induction pattern during GHE4B decolorization was different as compare previous reports on Direct Blue 6 [6] and Red HE 7B [9] decolorization. The induction of lignolytic enzymes and their sequence of induction are varied upon structure of dye and presumably on its first cleavage metabolites. In this study, polyphenol oxidases (laccase; 55% and tyrosinase; 78%) were induced significantly as compare to other biotransformation enzymes in initial 24 h of decolorization. During this period 50% dye was decolorized.

Fig. 3 Time course of dye degrading enzymes during decolorization of GHE4B in nutrient medium.

Fig. 4 UV-visible spectroscopic analysis of dye decolorization at different time interval by *P. desmolyticum*.

Fig. 5 HPLC chromatogram of original dye at 0 h (A), metabolites formed after 24 h (B), and 72 h of decolorization (C).

Analysis of dye decolorization and its metabolites

The UV-visible scan (400–800 nm) of supernatant of different time intervals showed decolorization and decrease in dye concentration from batch culture. The λ_{max} of original dye was shifted from 630 nm and showed three major peaks at 600, 570 and 440 nm after 24 h of incubation, which remained acquit up to 48 hours of incubation. The 100% decolorization of GHE4B exhibited as a peak at 440 nm after 72 h of incubation (Fig. 4). The HPLC analysis of 0 h extracted dye sample appeared as a peak at 2.24 minutes (Fig. 5A). As the decolorization progressed, the biodegradation of parent compound was conformed to seven detectable peaks in 24 h extracted sample (Fig. 5B; Rt: 2.48, 2.73, 3.42, 3.91, 4.12, 5.39 and 5.71 min). The metabolites formed at 24 hours were further degraded and only three detectable peaks were observed in the 72 h extracted sample, with retention time of 2.58, 2.80 and 5.37 minutes (Fig. 5C). The metabolites at retention time 2.48–2.58 and 2.73–2.80 min were identified as 4-HBS acid and 4-A-6-NOH-2-S acid, respectively (data not shown).

The FTIR spectrum of 24, 48 and 72 h extracted samples revealed the absence of peaks at 550–713 cm^{-1} (C–S stretching), 1046 and 1340 cm^{-1} (S=O stretching), 1017 cm^{-1}

(C–OH stretching) and 1574 cm^{-1} (–N=N– stretching of the azo bond) which were present in control dye sample. Here, biodegradation of parent dye compound by breakdown of azo bond as well as desulfonation by *P. desmolyticum* is evident (Fig. 6). In the 24 h extracted sample, peaks at 1656 and 3402 cm^{-1} indicate formation of amides. However, primary amines were identified in 48 and 72 h samples at 3247 cm^{-1} (symmetric N–H stretching). The appearance of peak for C=O stretching at 1667–1670 cm^{-1} in 48 and 72 h extracted sample indicates that the hydroxyl group attached to benzene ring.

Mechanism of biodegradation

Azo group N and aromatic ring C substituted with hydroxyl/amino group in sulfonated dyes is an acting site for lignolytic enzymes (laccases/peroxides) [24]. Such a site in GHE4B resulted in reactive intermediates, which underwent several redox reactions to produce more stable intermediates. Fig. 3A shows that lignolytic enzymes were prevalent in initial 24 h incubation, which played major role in initial breakdown. Induction of AND in later phase of decolorization (i.e. after 24 h) is marker for monooxygenase-based reactions (Fig. 3B). Desulfonated

Fig. 6 FTIR spectroscopic comparison of GHE4B control dye with metabolites extracted at different time interval (24–72 h) during degradation of dye.

products were identified as 4-A-6-NOH-2-S acid and 4-HBS acid by HPLC. Although, we did not find any metabolite of a complex intermediate, its degradation by reductase and monooxygenase type of enzymes is quite possible [25, 26], which were induced at later phase of decolorization (Fig. 3B).

Many sulfonated aromatic amines accumulate in the environment and other dye metabolites such as unsulfonated aromatic amines are relatively stable in aquatic conditions and are poorly degraded under anaerobic and aerobic waste water treatment conditions [27]. Degradation of GHE4B produced aromatic amines at static condition which were degraded aerobically. *P. desmolyticum* took 6 days to reach stationary growth phase when grown on GHE4B metabolites aerobically (data not shown). pd2112 degraded sulfonated reactive dye intermediates, 4-A-6-NOH-2-S and 4-aminobenzene sulfonic acid, aerobically at stationary phase of 15 and 27 days, respectively [9].

Phyto- and microbial-toxicity

Germination inhibition increased linearly with concentration of dye applied. Untreated dye (3000 ppm) gave 100% germination inhibition of *T. aestivum* and *S. bicolor*. At same concentration, GHE4B metabolites did not show any inhibition. Apparently, in both the plants, there was induction in shoot and root growth. We found 14 and 3% statistically significant induction in shoot and root length of *S. bicolor* as well as 50 and 3% induction in case of *T. aestivum*. (data not shown). In pd2112, zone of inhibition was 0.6 cm by untreated dye (10,000 ppm) and it was 0.8, 0.7 and 0.4 cm for *A. vinelandii*, *P. aeruginosa* and *E. coli*, respectively. Where as dye metabolites at same concentration did not show any growth inhibition in all four microorganisms studied (data not shown).

In conclusion, pd2112 decolorized and degraded GHE4B by lignolytic enzymes produced at static condition. Addition of co-substrates like molasses had significantly improved GHE4B decolorization ability of pd2112. pd2112 degraded dye metabolites aerobically. Phyto- and microbial-toxicity study brought into agreement the non-toxicity of dye intermediates to some agricultural crops and agricultural important microorganisms. This makes a promising appeal to use pd2112 for complete decolorization and detoxification of GHE4B by using combination of static and shaking conditions.

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