Red HE7B degradation using desulfonation by *Pseudomonas desmolyticum* NCIM 2112

Satish Kalme, Gajanan Ghodake, Sanjay Govindwar*

*Department of Biochemistry, Shivaji University, Kolhapur 416004, India*

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**Abstract**

Red HE7B (RHE7B, 100 mg l\(^{-1}\)), a sulfonated azo dye, was decolorized at static condition by *Pseudomonas desmolyticum* NCIM 2112 in 72 h with 71% reduction in chemical oxygen demand (COD). Extracellular lignin peroxidase (LiP) has played a crucial role in breakdown of the dye by asymmetric cleavage and reductases in the initial 24 h incubation to break azo bonds of some dye molecules. Dye also induced the activity of aminopyrine N-demethylase, one of the enzymes of mixed function oxidase system. Decolorization and degradation were analyzed by using UV–vis and high-pressure liquid chromatography (HPLC). The Fourier transform infrared spectroscopy (FTIR) analysis revealed that *P. desmolyticum* preferred C–N and S=O bonds to break down the RHE7B. GC–MS identification of 8-amino-naphthalene-1,3,6,7-tetraol and 2-hydroxyl-6-oxalyl-benzoic acid as final metabolites supports the degradation of RHE7B by desulfonation before and after ring cleavage. Aerobic degradation of amines and reduced phytotoxicity increased the applicability of this microorganism for dye removal.

**Scientific relevance of the paper:** This is the first report on degradation of Red HE7B by oxidative enzymes and on further degradation by desulfonation before and after ring cleavage.

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**Keywords:** Anoxic; Biodegradation; Desulfonation; *Pseudomonas desmolyticum*; Red HE7B

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1. **Introduction**

Reactive sulfonated dyes are very soluble by design. As a result, not all are used up by textile fibers during the dyeing process and, therefore, discharge from dye houses results. The sulfonic acid groups that are introduced to increase the water solubility of the dye and the azo group (chromophoric group) confer resistance to microbial attack, making them recalcitrant to oxidative decolorization (Coughlin et al., 1999).

There are some reports on aerobic desulfonation of sulfonated aromatic compounds (Kertesz et al., 1994; Kneifel et al., 1997) but most of the sulfonated azo dyes are hardly biodegraded under aerobic conditions. There are worm in the literature in decolorization of sulfonated dyes by chemicals, e.g., Reactive Black 5 by *Candida oleophila* (Lucas et al., 2005), Remazol dyes by *Trichophyton rubrum* LSK-27 (Yesiladali et al., 2006), Acid Red 151 and Acid Orange 12 by *Kerstesia* sp. (Vijaykumar et al., 2007) and Direct Red 81 by bacterial consortium (Junnarkar et al., 2006) but they report only on physico-chemical parameters involved. The studies reporting anaerobic degradation of Red HE7B (RHE7B) did not explain the metabolic pathway followed by the microorganism during degradation (Carliell et al., 1995; O’Neill et al., 2000). As initial breakdown of azo bond requires anaerobic/anoxic conditions, there is a need to study the fate of such sulfonated azo dyes in anoxic conditions. It has been reported that complete mineralization of dyes is possible only if anaerobic reduction is followed by aerobic oxidation of the amines formed in the reductive steps (Rajaguru et al., 2000). So, in the present study we have described the degradation of sulfonated dye RHE7B by *Pseudomonas desmolyticum* NCIM 2112 at static anoxic condition. Aerobic degradation of aromatic amines which are
produced after anoxic digestion, enzymes involved in the degradation and the degradation mechanism has been explained.

2. Material and methods

2.1. Dyestuff and chemicals

2.2. Microorganism and culture conditions

2.3. Decolorization experiments

2.4. Determination of enzyme activities

2.5. Decolorization and biodegradation analysis

Decolorization was monitored by UV–vis spectroscopic analysis (Hitachi U-2800) at different time intervals. For biodegradation analysis, 100 ml culture broth was taken at 24, 48 and 72 h intervals, centrifuged at 10,000 g and extraction of metabolites was carried out from supernatant using equal volumes of ethyl acetate. The extracts were dried over anhydrous Na2SO4 and evaporated to dryness in rotary evaporator. High-pressure liquid chromatography (HPLC) conditions applied were the same as reported earlier (Kalme et al., 2007) except the UV detector at 314 nm. The Fourier transform infrared spectroscopy (FTIR) analysis was done in the mid-IR region of 400–4000 cm<sup>-1</sup> with 16-scan speed. The samples were mixed with spectroscopically pure KBr in the ratio 7:93, pellets were fixed in sample holder, and the analyses were carried out. Rotary vacuum evaporated sample (extracted after 72 h decolorization period) was dissolved in methanol and GC–MS analysis of metabolites was carried out using a Hewlett-Packard 5890 B MS Engine, equipped with integrated gas chromatograph with an HP1 column (30 m long, 0.25 mm i.d., nonpolar). Helium was used as carrier gas at a flow rate of 1.1 ml min<sup>-1</sup>.

The injector temperature was maintained at 300 °C and too oven conditions were the following: 100 °C kept constant for 2 min; raised upto 250 °C at a rate of 10 °C min<sup>-1</sup>; raised up to 280 °C at a rate of 30 °C min<sup>-1</sup>. The compounds were identified on the basis of mass spectra, using the NIST library.

2.6. Aerobic degradation of aromatic amines

Five percent inoculum of <i>P. desmolyticum</i> was added (absorbance at 530 nm: 0.1) in 100 ml synthetic medium containing 0.1 g l<sup>-1</sup> individual aromatic amine and incubated at 30 °C at 150 rpm. At different time intervals, culture supernatant was collected (5000 g, 5 min) and diluted in 0.10 M sodium phosphate buffer solution (pH 7.0). The aerobic degradation of aromatic amines was analyzed spectrophotometrically (Hitachi U-2800) by measuring decrease in their concentration at their absorbance maxima (Tan et al., 2005): p-aminobenzene sulfonamide (p-ABSA, 260 nm), 4-aminobenzene sulfonic acid (4-ABS, 248 nm), 4-amino-6-naphthol-2-sulfonic acid (4-A-6-NOH-2-S, 317 nm) and RHE7B metabolites produced at static anoxic condition (315 nm). Growth of <i>P. desmolyticum</i> on aromatic amines was measured at 530 nm periodically.

2.7. Phytotoxicity study

This test was performed in order to assess the toxicity of the untreated dye in the concentration range 2000–10,000 ppm. As 10,000 ppm concentration of RHE7B dye showed maximum germination inhibition, the effect of dye metabolites was recorded at this concentration. Tests were carried out with two kinds of seeds commonly used in the Indian agriculture: <i>Sorghum bicolor</i>, <i>Triticum aestivum</i> as reported by Parshetti et al. (2006). Thirty-five individual seeds were used for the germination in each set and watered separately with 10 ml solution of RHE7B and its degradation product (10,000 ppm) per day for 7 days.

2.8. Statistical analysis

For analysis of the data one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test was used. Readings were considered significant when <i>P</i> was ≤0.05.

3. Results

3.1. Effect of static and shaking conditions

Acclimatization of the <i>P. desmolyticum</i> had reduced the time required for complete decolorization of 100 mg l<sup>-1</sup> dye from 168 to 72 h. Decolorization of RHE7B was 95% at
static condition and 53% at shaking condition with growth of microorganism. Growth was observed to be more at shaking (0.46 g l\(^{-1}\)) as compared to static condition (0.32 g l\(^{-1}\)) (Fig. 1). To confirm whether this decolorization was due to microbial action or change in pH, the change in pH was recorded, which was in the range of 6.8–7.8 at shaking and 6.8–8.0 at static condition. UV–vis spectra of RHE7B did not show any change at this pH range. Reduction in COD was observed by 71% at static and 43% at shaking condition (data not shown). There was no decolorization in abiotic control.

3.2. Enzyme activities during decolorization of RHE7B

In order to gain additional insight into the decolorization mechanism, screening of oxidative enzyme activities were also monitored over time. At 48 h, induction in extracellular LiP activity was observed by 143% (as compared to 0 h activity). The activity of intracellular LiP remained unchanged throughout the decolorization process. Induction in laccase and tyrosinase activities was observed up to 72 h incubation (270% and 94%, respectively). Even after complete decolorization (at 96 h), a noticeable induction in LiP (intracellular 40% and extracellular 86%), laccase (550%) and tyrosinase (150%) have been observed (Fig. 2). There were no activities of laccase and tyrosinase in culture supernatant. AND, MGR and DCIP reductase activities were induced in the time course of 96 h by 187%, 101% and 4.5%, respectively. Especially, MGR activity was highly induced (214%) up to 24 h incubation during the decolorization process (data not shown).

3.3. Biodecolorization and biodegradation analysis

UV–vis scan (400–800 nm) of supernatants at different time intervals showed decolorization and decrease in dye concentration from batch culture. Peaks observed at 552 and 500 nm (0 h) were decreased with shift in \(\lambda_{\text{max}}\) showing a new peak at 440 nm with complete decolorization of dye (72 h) (Fig. 3). HPLC analysis of RHE7B dye showed a peak at retention time 1.82 min and the sample extracted after 72 h showed three major metabolites at retention times 1.94, 2.16 and 2.54 min (data not shown). The FTIR spectral comparison between control dye and samples extracted at different time intervals showed degradation of RHE7B in different metabolites by \textit{P. desmolyticum}. FTIR spectra of RHE7B showed the presence of different peaks at 3421 cm\(^{-1}\) for N–H stretching. It also showed vibration of N–H deformation +C–N stretching as a peak at 1541 cm\(^{-1}\) and, C–H stretching at 2924 cm\(^{-1}\). The presence of sulfonic acid was confirmed by two peaks at 1206 cm\(^{-1}\).
(symmetric S=O stretching) and 1041 cm\(^{-1}\) (asymmetric S=O stretching). In 24, 48 and 72 h extracted samples, the appearance of new peaks at 1670, 3413, 1667 and 3402 cm\(^{-1}\) expressed the breakdown of RHE7B in various compounds showing C=O stretching in amides, free N–H stretching in pyrroles and amines, respectively (Fig. 4).

The GC–MS analysis of 72 h extracted sample showed 13 peaks. The compound at retention time 6.25 min was identified as \(N, N\)-bis-[1,3,5] triazin-2-yl-benzene-1,4-diamine, one of the metabolites generated after asymmetric cleavage of RHE7B by LiP (Table 1). Two more possible metabolites of asymmetric cleavage, 2-hydroxynaphthalene 1,5-disulfonic acid (2HN-1,5SA) and 6-diazenyl-5-hydroxy-4-imino-3,4-dihydronaphthalene 2,7-disulfonic acid (6Az-5H-4I-3,4HN-2,7SA) and 2-aminonaphthalene-1,5-disulfonic acid (2AN-1,5SA), generated after azo bond cleavage by reductase were not detected in the sample (data not shown). But their presence was anticipated on the basis of the metabolites detected after desulfonation, before and after ring cleavage.

### 3.4. Biodegradation mechanism

Desulfonation of 2HN-1,5SA before ring cleavage by dioxygenase enzyme produced naphthalene 1, 2, 3, 6, 7 pentaol (Table 1). 6Az-5H-4I-3,4HN-2,7SA was further degraded by desulfonation before ring cleavage. The desulfonation in hydrolysis product of 6Az-5H-4I-3,4HN-2,7SA, due to monoxygenase reaction, was identified as 5,7-dihydroxy-4-imino-6-nitroso-3,4-dihydronaphthalene-2-...
sulfonic acid (Fig. 5[1]), whereas oxidation product of 6Az-5H-4I-3,4HN-2,7SA was identified as 1-hydroxy-8-imino-3,6-disulfo-7,8-dihydronaphthalene-2-diazonium (Fig. 5[2]). The desulfonation of later intermediate due to monooxygenase enzyme was identified as 1,3-dihydroxy-8-imino-6-sulfo-7,8-dihydronaphthalene 2-diazonium (Fig. 5[3]). Further dioxygenase action and reduction in N2 had given a compound loosing both sulfonic groups, identified as 8-amino-naphthalene-1,3,6,7-tetraol (Fig. 5[4]). The desulfonation after ring cleavage in 2AN-1,5SA (azo bond cleavage metabolite) was identified as 2-hydroxy-6-oxalylbenzoic acid (Fig. 5[5]).

3.5. Aerobic degradation of amines

Aerobic degradation of aromatic amines by *P. desmolyticum* was confirmed by decrease in absorbance maxima of respective amines with respect to incubation time (30 days) and growth of microorganism in synthetic medium containing amines. *P. desmolyticum* took 15 days to reach stationary growth phase during growth on RHE7B metabolites and 4-A-6-NOH-2-S. The degradations of these amines were, 95% and 94%, respectively. In the presence of *p*-ABSA and 4-ABS, the stationary growth phase occurred after 21 and 27 days (91% and 99% degradation), respectively (data not shown).

3.6. Phytotoxicity analysis

After 7 days of incubation, untreated RHE7B (10,000 ppm) showed 99% and 98% germination inhibition in *S. bicolor* and *T. aestivum*, respectively. When metabolites formed after complete decolorization were applied at the same concentration, there was no germination inhibition in both the seeds. But the growth observed in presence of metabolites was not normal as compared to the growth in distilled water. The shoot and root lengths were decreased by 19% and 11%, respectively, in *S. bicolor*, whereas in *T. aestivum*, the shoot and root growths were affected by 14% and 57%, respectively (Table 2).

4. Discussion

In this study, the observations suggest that the decolorization performance of *P. desmolyticum* was better at static anoxic condition where depletion in oxygen content is followed. The physiology of the possible reactions that result in a reductive cleavage of azo compounds under anoxic conditions differs significantly from the situation in the presence of oxygen, because several redox active compounds (e.g. reduced flavins or hydroquinones) rapidly react either with oxygen or with azo dyes. Therefore, decreased decolorization at shaking condition is due to competition between oxygen and the azo compounds for the reduced electron carriers. The co-metabolic activity by *P. desmolyticum* during decolorization was biomass and supplemented source dependent.

The azo dye reduction in anaerobic incubation is a non-specific and extracellular process in which reducing equivalents from either biological or chemical source are transferred to the dye (Stolz, 1999). The involvement of fungal peroxidases and laccases for the oxidation of
Table 2
Phytotoxicity study of Red HE7B and its metabolites produced after complete decolorization (72 h)

<table>
<thead>
<tr>
<th>Dye concentration (ppm)</th>
<th>Plants studied</th>
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<tbody>
<tr>
<td></td>
<td><em>Sorghum bicolor</em></td>
</tr>
<tr>
<td></td>
<td>Germination inhibition (%)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.0</td>
</tr>
<tr>
<td>10,000</td>
<td>99±0.5</td>
</tr>
<tr>
<td>Metabolites (RHE7B) (10,000)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Values are mean of two experiments.

aSignificantly different from sample grown in untreated Red HE7B at P<0.001.
bSignificantly different from sample grown in distilled water at P<0.05.

sulfonated and unsulfonated azo dyes has been reported (Kandelbauer et al., 2004; Zille et al., 2005). In this study, there was induction in extracellular LiP during decolorization, and this is opposite of that in the earlier study of Kalme et al., (2007). Extracellular LiP has played a crucial role in breaking down the dye by asymmetric cleavage and reductases in the initial 24 h incubation to break azo bonds of some dye molecules. Even after complete decolorization, upto 96 h, enzyme activities (laccase and tyrosinase) remained increasing in the batch culture. The induction in AND activity showed the involvement of mixed function oxidase system. The induction in oxidative enzymes (LiP, laccase) and tyrosinase upto complete decolorization period (72 h) are presumably responsible for the degradation of RHE7B.

The elucidation of degradation pathways is of special interest considering health and environmental priorities. It was confirmed by Carliell et al. (1995) that 2-aminoanthra-

lène-1,5-disulfonic acid was present after anaerobic diges-
tion of RHE7B, thus showing that azo bond has been cleaved. In our study, we have observed the desulfonated product of 2-aminoanthra-
lene-1,5-disulfonic acid (Fig. 5b). Desulfonation of sulfonated aromatic compounds after/before ring cleavage (Kertesz et al., 1994; Kneifel et al., 1997; Stolz, 1999) and symmetrical splitting of azo linkage or asymmetrical cleavage of sulfonated azo dyes (Lopez et al., 2004) has been studied at aerobic condition. In this study at static condition, the oxidation–reduction reactions made possible the degradation of RHE7B by desulfonation before and after ring cleavage by *P. desmolyticum*. The FTIR analysis revealed that *P. desmolyticum* preferred C–N and S=O bonds to break down the RHE7B. This data was supported by GC–MS identification of 8-amino-naphthalene-1,3,6,7-tetraol and 2-hydroxy-6-oxalyl-benzoic acid as final metabolites of RHE7B.

Use of untreated and treated dyeing effluents in agriculture has direct impact on fertility of soil. Hence we considered assessing the phytotoxicity of the undecolorized and decolorized dyes important. The aromatic amines produced during static digestion of RHE7B were degraded aerobiocally and reduced phytotoxicity of metabolites produced by the action of *P. desmolyticum* enables this microorganism to be used in biological treatment of industrial effluents containing the sulfonated azo dye RHE7B.

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References


