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ORIGINAL PAPER

Degradation of the synthetic dye amaranth by the fungus *Bjerkandera adusta* Dec 1: inference of the degradation pathway from an analysis of decolorized products

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Abstract We examined the degradation of amaranth, a representative azo dye, by *Bjerkandera adusta* Dec 1. The degradation products were analyzed by high performance liquid chromatography (HPLC), visible absorbance, and electrospray ionization time-of-flight mass spectroscopy (ESI-TOF-MS). At the primary culture stage (3 days), the probable reaction intermediates were 1-aminonaphthalene-2,3,6-triol, 4-(hydroxyamino) naphthalene-1-ol, and 2-hydroxy-3-[2-(4-sulfophenyl) hydrazinyl] benzenesulfonic acid. After 10 days, the reaction products detected were 4-nitrophenol, phenol, 2-hydroxy-3-nitrobenzenesulfonic acid, 4-nitrobenzene sulfonic acid, and 3,4′-disulfonyl azo benzene, suggesting that no aromatic amines were created. Manganese-dependent peroxidase activity increased sharply after 3 days culture. Based on these results, we herein propose, for the first time, a degradation pathway for amaranth. Our results suggest that Dec 1 degrades amaranth via the combined activities of peroxidase and hydrolase and reductase action.

Keywords *Bjerkandera adusta* Dec 1 · Amaranth · Azo · Degradation · Peroxidase

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Introduction

Treatment of waste water that contains synthetic dyes is essential. Over 90% of 4,000 dyes tested in the Ecological and Toxicological Association of Dyestuffs Manufacturing Industry survey had LD_{50} values greater than 2×10^3 mg/kg (Robinson et al. 2001). Moreover, degradation of azo dyes by reduction in water produces several aromatic amines, which are more toxic and carcinogenic than are the dyes themselves. The permissible concentration of aromatic amines in waste water is regulated by legislation, especially in developed countries (Tauber et al. 2005). Aromatic amines enter the human body through the environmental chain and are next converted to acyloxyamine derivatives. Such compounds intercalate with and denature DNA (Takahashi and Hashimoto 2001). Therefore, reduction of azo dyes is not recommended, even though decolorization is achieved. To date, decolorization of such dyes by biological methods has attracted a great deal of research attention. Most decolorization pathways involve the action of azo reductases and its reduction often occurs under anaerobic conditions (Delee et al.

1998; Stolz 2001). However, aerobic degradation of several azo compounds has been proposed (Chivukla and Renganathan 1995; Goszczynski et al. 1994). The fungus *Trametes versicolor* produces ligninolytic enzymes including laccase, manganese peroxidase, and lignin peroxidase, and these enzymes have been reported to play important roles in decolorization, but no decolorization mechanism or products have been identified (Champagne and Ramsay 2005; Gavril and Hodson 2007; Gavril et al. 2007).

Strain Dec 1 (formerly classified as *Thanatephorus cucumeris*) is a fungus that effectively degrades several refractory materials including synthetic dyes, molasses, and pulp effluent (Kim et al. 1995; Kim and Shoda 1998; Shintani et al. 2002; Sugano et al. 2006). These materials contain compounds that act as xenobiotics. Martínez (CSIC, Madrid, Spain) has suggested that Dec 1 should be re-named *Bjerkandera adusta* (personal communication). Indeed, the ITS-5.8S rDNA sequence of Dec 1 (GenBank accession number: AB567717; registered in June 2010) is identical to the most recently deposited sequence from *Bjerkandera adusta* (GenBank accession number: FJ810147; registered in April 2009). On the other hand, the relevant sequence of Dec 1 is 99.5% homologous to that of *Thanatephorus cucumeris* (GenBank accession number: AF455461; registered in June 2003). Therefore, we will term the strain *Bjerkandera adusta* Dec 1.

Dec 1 decolorizes several synthetic dyes. Decolorization of molasses and pulp effluent has been clearly demonstrated, although the relevant enzymes have not been identified. However, decolorization of anthraquinone dyes apparently depends on the activities of two novel enzymes, DyP and TcVP1 (Sugano et al. 2006, 2007, 2009; Sugano 2009). Apart from these peroxidases, Dec 1 produces several other enzymes, such as a manganese peroxidase, that assist in the degradation of refractory materials (Shintani et al. 2002). Synthetic dyes are representative of compounds that are difficult to decompose, and such dyes cause the serious environmental problems mentioned above. As waste has been estimated to contain 15% (w/w) of total produced dye (Wesenberg et al. 2003), decolorization is required before waste water can be released to the environment. Amaranth is a versatile and widely used synthetic dye; however, concerns have been raised regarding amaranth toxicity. Notably, the dye cannot be employed in the USA, but use is not prohibited in other countries. It is clear that an efficient degradation process is required. However, such a system has not yet been established. In the present study, we show that *B. adusta* Dec 1 effectively decolorizes amaranth and we propose a degradation pathway. Our results show that the reaction products do not include any aromatic amines. This will assist in development of practical treatments for dye effluent.

Materials and methods

Microorganism, medium, and chemical reagents

B. adusta Dec 1 was employed for dye degradation. Potato dextrose medium (PD) was prepared as described previously (Sugano et al. 2006). Amaranth (Bordeaux S; analytical grade) was purchased from Wako Junyaku (Tokyo, Japan). All other chemical reagents were of analytical grade unless otherwise specified.

Culture

A *B. adusta* Dec 1 conidial suspension (10 μl per plate) was inoculated onto PD agar plates and incubated for 12 days at 29°C. Mycelia from six plates were collected and suspended in 10 ml of sterilized distilled water. The suspension was agitated vigorously for 7 min, filtered through gauze, and 5 ml amounts of filtrate were inoculated into 150 ml aliquots PD containing various levels of amaranth. Cultures were grown at 29°C with shaking at 120 rpm.

Peroxidase activity

We analyzed manganese-dependent peroxidase including versatile peroxidase (TcVP1) and dye-decolorizing peroxidase (DyP) activities. The manganese-dependent peroxidase activity was determined by the formation of Mn^{3+}-tartrate complex (ε_{238} = 6,500 M^{-1} cm^{-1}) from 0.1 mM $MnSO_4$ using 0.1 M sodium tartrate buffer (pH 5.0) and 0.2 mM H_2O_2. DyP activity was determined by the degradation of 0.1 mM Reactive blue 19 (ε_{593} = 8,600 M^{-1} cm^{-1}) which is a synthetic dye using 25 mM citrate buffer (pH 3.2) and 0.2 mM H_2O_2. Each assay was measured by spectrophotometer (UV2400; Shimadzu, Kyoto, Japan). One unit (*U*) of the manganese-dependent peroxidase activity and DyP activity was defined as the amount of enzyme required to

produce 1 μmol of Mn^{3+}-tartrate complex and the amount of enzyme to decolorize 1 μmol of RB19 respectively, per minute at 30°C.

Visible absorbance

Absorbance at 520 nm of twofold diluted culture supernatant was periodically noted with UV2400PC spectrometer (Shimadzu, Kyoto, Japan).

High-performance liquid chromatography (HPLC)

Amaranth and degradation products thereof were analyzed by HPLC. Cultures were centrifuged and supernatants recovered. Two volumes of ethanol were added to supernatants and the suspensions were filtered through 0.45 μm pore-sized acetyl cellulose membranes. HPLC conditions were as follows: a TSK-gel ODS-100 V column (4.6 mm ϕ × 150 mm); a detection wavelength of 254 nm; a flow rate of 0.4 ml/min; and 5 mM ammonium acetate:methanol = 90:10 (v/v) as eluent. Phenol and 4-nitro-benzene sulfonate as authentic reagents were measured under the same condition.

Electrospray ionization time-of-flight mass spectroscopy (ESI-TOF-MS)

ESI-TOF-MS was performed using a TOF CS JMS-T40 (JEOL, Tokyo, Japan). Each sample was continuously loaded at a constant rate of 0.2 ml/min in 5 mM ammonium acetate:methanol = 90:10 (v/v) as eluent. Analysis was performed in the positive ion (ES+) mode, and the scan interval was 1 s. The electrospray, orifice, and ring lens voltages were 2.0 kV, 85, and 15 V, respectively. The nebulizing and drying gas flow rates were 600 and 60 l/h, respectively. Nebulizing chamber and ion source temperatures were 100 and 80°C, respectively.

Results

Visible decolorization and spectrophotometric analysis

When the initial amaranth concentration of the medium was 94 mg/l, absorbance at 520 nm (Abs_{520}) gradually decreased after 2 days of culture (Fig. 1), although decolorization was not visually apparent until 3 days of culture. After 10 days of culture, 98% decolorization was achieved.

HPLC data

HPLC of culture medium just after inoculation showed a single peak at 6.7 min, corresponding to amaranth. After 3 days of culture, two sharp peaks at 4.6 and 6.7 min, and two broad and weak peaks at 3.3 and 5.3 min, were apparent. Amaranth had completely disappeared after 10 days. On the other hand, the broad and weak peak at 3.3 min remained, and another peak (corresponding to the 5.3 min peak seen on day 3) increased sharply. Although several compounds were mixture in each retention time, authentic phenol and 4-nitro-benzene sulfonate were detected at 2.9, and 5.2 min, respectively.

ESI-TOF-MS data on degradation products

To identify HPLC-separated components by ESI-TOF-MS, the initial medium amaranth concentration was adjusted to 910 mg/l. HPLC fractions obtained after about 3, 4, and 5 min of elution were analyzed by ESI-TOF-MS. After 10 days of culture, the peak with a retention time of 4.6 min had disappeared but the peaks at 3.3 and 5.3 min remained. These peaks were isolated and analyzed by ESI-TOF-MS. The material of retention time of about 3 min on HPLC,

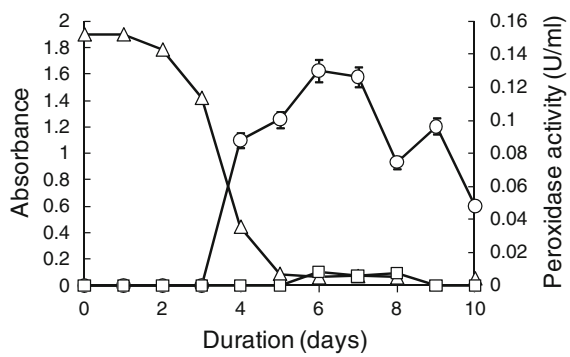

Fig. 1 Time course of absorbance at 520 nm and manganese-dependent peroxidase and DyP activities of the Dec 1 culture supernatant with amaranth (94 mg/l). *Circle*, manganese-dependent peroxidase activity; *square*, DyP activity magnified tenfold; *triangle*, absorbance at 520 nm

after 3 days of culture (3d-P3), showed an *m/z* value of 214 (Fig. 2a). The material of retention time about 5 min on HPLC, after 3 days of culture (3d-P5), yielded *m/z* values of 203, 198, 365, and 383 (Fig. 2b). The material of retention time about 4 min yielded a mixture of the ion peaks of the 3- and 5-min fractions. The material of retention time about 3 min on HPLC, after 10 days of culture (10d-P3), showed *m/z* values of 95, 140, and 242 (Fig. 2c). The material of retention time about 5 min on HPLC, after 10 days of culture (10d-P5), yielded *m/z* values of 203, 365, and 383 (Fig. 2d).

Peroxidase activity

Manganese-dependent peroxidase and DyP activities of the culture supernatant are shown in Fig. 1. The manganese-dependent peroxidase activity increased sharply after 3 days of culture and achieved maximum at the sixth day. On the other hand, low DyP activity was detected after 6, 7 and 8 days of the culture.

Discussion

Several decolorizing treatments involve dye adsorption by microbes (Zhou and Zimmermann 1993; Sumathi and Manju 2001; Drista et al. 2007). However, Dec 1 did not display this feature (Kim et al. 1995). This intriguing observation indicates that dye decolorization by Dec 1 is achieved not by adsorption but rather by degradation employing fungal enzymes.

After 3 days of culture, the highest 3d-P3 ion peak had an *m/z* value of 214. This compound is generated by degradation of the azo bond and replacement of a sulfonyl group by a hydroxyl. This substitution often occurs under oxygen-rich conditions (Kudlich et al. 1999). As the ESI-TOF-MS was operated in the

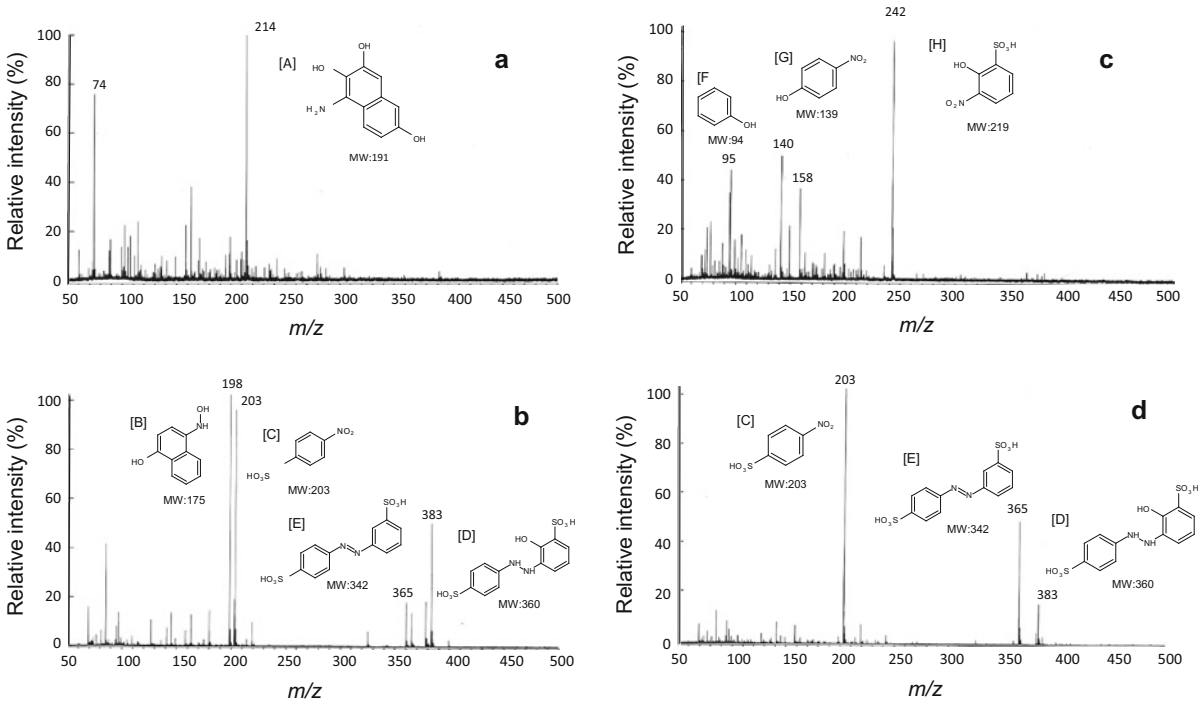

Fig. 2 ESI-TOF-MS data from various samples. **a** The spectrum of an HPLC fraction of culture supernatant obtained after 3 days (3d-P3), with a retention time of about 3 min; **b** The spectrum of an HPLC fraction of culture supernatant obtained after 3 days (3d-P5), with a retention time of about 5 min; **c** The spectrum of an HPLC fraction of culture supernatant obtained after 10 days (10d-P3), with a retention time of about 3 min; **d** The spectrum of an HPLC fraction of culture supernatant obtained after 10 days (10d-P5), with a retention time of about 5 min. The probable chemical structures and the molecular weights are shown

positive ion mode, the detected ion peaks reflect proton or sodium addition. Therefore, we deduce that this compound must be 1-aminonaphthalene-2,3,6-triol [A] $(M + Na)^+$. The highest peak at *m/z* 198 in the 3d-P5 sample must also be generated by degradation of an azo bond, at a region of amaranth other than that cleaved to give [A], and we deduce that the peak is 4-(hydroxyamino) naphthalene-1-ol [B] $(M + Na)^+$. The peaks at *m/z* values of 203 and 383 are deduced to be 4-nitrobenzenesulfonic acid [C] $(M - H)^+$ and 2-hydroxy-3-[2-(4-sulfophenyl)hydrazinyl] benzenesulfonic acid [D] $(M + Na)^+$, respectively. In particular, the retention time of authentic 4-nitrobenzenesulfonic acid by HPLC was identical to product [C]. The weak peak at *m/z* 365 is considered to be 3,4′-disulfonyl azo benzene [E] $(M + Na)^+$. These results indicate that compounds [A] and [B] must be mainly generated by reduction and hydrolytic degradation of azo bond of amaranth. Such degradation depend not only reductase action but also hydrolase action. A hydrazine compound [D] and a small amount of an azo product [E] were deduced to be generated by degradation of the corresponding naphthalene ring. Totally, these results suggest that the main degradation up to 3 days culture must involve attack on azo bond and the degradation of naphthalene rings is secondarily.

After 10 days of culture, three 10d-P3 ion peaks appeared at *m/z* values of 95, 140, and 242, indicating the presence of phenol [F] $(M + H)^+$, 4-nitrophenol [G] $(M + H)^+$, and 2-hydroxy-3-nitrobenzenesulfonic acid [H] $(M + Na)^+$, respectively. In particular, the retention time of authentic phenol by HPLC was completely identical to product [F]. Product [G] was deduced to be generated from product [B], and removal of a further nitro group yielded phenol. Product [H] must be generated from product [D]. The naphthalene derivatives [A] and [B] evident after 3 days of culture had completely disappeared by 10 days. The principal 10d-P5 ion peaks had *m/z* values of 203 and 365. The additional ion peak at *m/z* 383 was weak. Notably, the relative intensity ratio (*m/z* 365 vs. *m/z* 383) was reversed compared with that in 3-day culture supernatants, indicating that the unstable hydrazine compound [D] was easily oxidized by oxygen (Mohanty et al. 2006). In fact, after 10 days, the culture supernatant (initial amaranth level 910 mg/l) was slightly yellow in color, although the supernatant was colorless when the initial

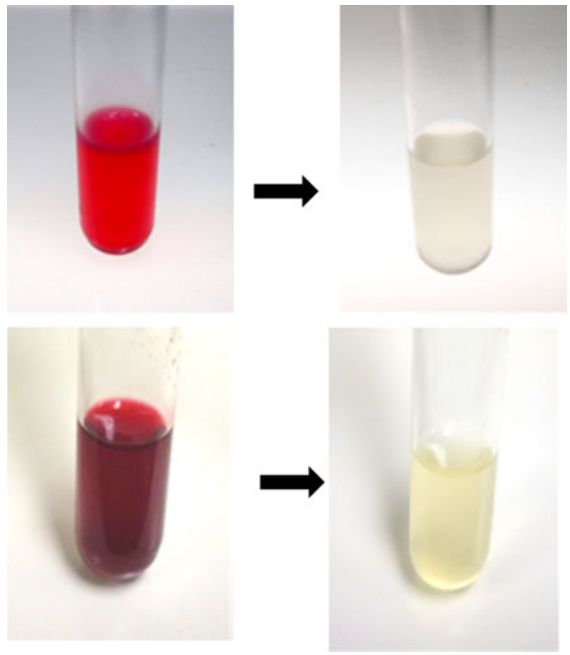

Fig. 3 Decolorization of different concentrations of amaranth. The tubes on the *left* contain samples of the initial medium and the tubes on the *right* samples of supernatant obtained after 10 days of culture. *Above*, initial amaranth concentration 94 mg/l; *below*, initial amaranth concentration 910 mg/l

amaranth concentration was 94 mg/l as shown in Fig. 3. Moreover, when the initial concentration of amaranth was 94 mg/l, no peak of 3, 4′-disulfonyl azobenzene was apparent after 10 days of culture. This suggests that 910 mg/l amaranth overloads the degradation capacity of the fungus. However, this will not be a problem in practice, because most dyehouse effluent has been reported to be 10–50 mg/l except very concentrated effluent (O'Neill et al. 1999).

In our earlier work, we indicated how Dec 1 degraded anthraquinone dyes (Sugano et al. 2006, 2009). In this process, an anthraquinone dye is converted to short-lived azo compounds via the action of DyP (Sugano et al. 2009), after which TcVP1 decolorizes the azo compounds (Sugano et al. 2006). Therefore, we also speculated that the degradation of amaranth must proceed via the action of several enzymes. Interestingly, decolorization started at day 2, while manganese-dependent peroxidase acitivity was nothing at that timing (Fig. 1). This means that initial stage of decolorization is not promoted by peroxidase. From the MS data, at initial

culture stages (until day 3), the azo bond must be degraded principally by reduction and hydrolysis. This is important, because no hydrolytic process decomposing aromatic azo compounds has yet been reported. Two naphthalene rings were also degraded, but this may be a secondary reaction. At the second stage (after day 3), nitro and hydroxyl products were main, indicating that oxidative reaction proceeded at this stage. DyP seemed not to contribute the decolorization of amaranth because DyP activity appeared at 6 days of culture. In this timing, the decolorization of amaranth was completed. Basically, DyP activity of Dec 1 culture in PD medium is very low (Kim and Shoda 1999). Moreover, DyP decolorizes azo dyes poorly, although it decolorizes anthraquinone dye very well (Sugano et al. 2000, 2006). Therefore, it is consistent that DyP is not important factor for amaranth decolorization. On the other hand, manganse-dependent peroxidase activity increased from 3 to 6 days. This is synchronized with the second stage decolorization of amaranth as shown in Fig. 1. Finally, the naphthalene structure disappeared completely after 10 days of culture. During degradation of the naphthalene ring, butadiene derivatives or related compounds must be generated. However, we did not detect such compounds under our experimental conditions, presumably because of compound volatility. Totally, we propose the degradation cascade of Amaranth as shown in Fig. 4.

The most important feature of this decomposition process is that no aromatic amines remain. To date, azo compounds have been degraded principally by the action of azoreductases using NADPH (Chen 2006). Unfortunately, such pathways frequently generate several types of aromatic amines (Hong et al. 2007). As aromatic amines are suspected to trigger cancer, azoreductase treatment is unsuitable if release of waste water to the environment is planned. Although several aromatic compounds were still present 10 days after culture of amaranth with Dec 1, they can be easily degraded by bacteria such as *Pseudomonas* and *Arthrobacter*, under natural conditions (Bagnéris et al. 2005; Jindorová et al. 2002; Alvarez and Vogel

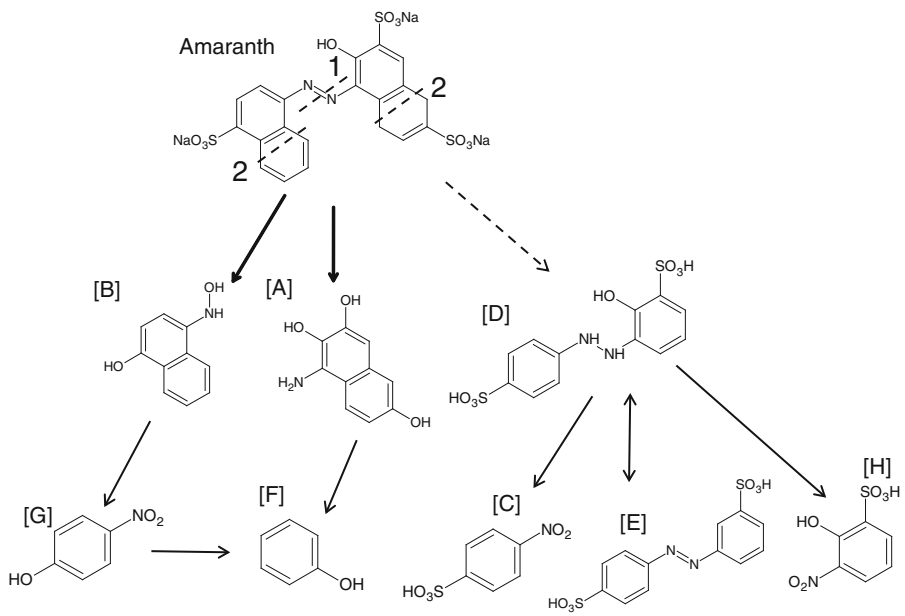

Fig. 4 A logical schematic for degradation of amaranth by Dec 1. The probable degradation sites of amaranth by Dec 1 are shown as three *broken lines* superimposed on the amaranth structure. The *number* shown in the figure is preference order of cleavage of each chemical bond. The degradation cascade is indicated by *arrows*. The *bold arrows* are the main initial cascade and *broken arrow* is the secondary cascade. A *double-headed arrow* indicates that the reaction may be reversible if the concentration of amaranth is high (>910 mg/l). [A], 1-aminonaphthalene-2,3,6-triol (MW:191); [B], 4-(hydroxyamino) naphthalene-1-ol (MW:175); [C], 4-nitrobenzenesulfonic acid (MW:203); [D], 2-hydroxy-3-[2-(4-sulfophenyl) hydrazinyl]benzenesulfonic acid (MW:360); [E], 3,4′-disulfonyl azobenzene (MW:342); [F], phenol (MW:94); [G], 4-nitrophenol (MW: 139); [H], 2-hydroxy-3-nitrobenzenesulfonic acid (MW: 219)

1991). Therefore, the present study suggests an attractive and promising method for practical waste water treatment, and the results assist in an understanding of the mechanism of azo dye degradation.

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