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Effects of reduction products of *ortho*-hydroxyl substituted azo dyes on biodecolorization of azo dyes

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ABSTRACT

The mediated effects of reduction products of some *ortho*-hydroxyl substituted azo dyes on biodecolorization were investigated. The results indicated that the addition of reduction products could effectively accelerate dye decolorization by *Shigella* sp. QRZ-1. The best accelerating effect was obtained with the addition of reduction products of Acid Red 14 (AR14), resulting in an over 3-fold increase in decolorization efficiency of many azo dyes. In sequencing batch reactor experiments, the accelerating effect of reduction products of AR14 was more obvious (1.5-fold) during the startup of the system. When the dye concentration was increased to 500 mg L^{-1}, the accelerated decolorization efficiency was still maintained around 95%. The presence of AR14 in the feed enhanced the decolorization performance of anaerobic sludge, indicating that the strategy may be beneficial for practical application. 1-Naphthol-2-amino-4-sulfonic acid, which is one of the reduction products of AR14, may function as redox mediator to speed up azo dye biodecolorization.

1. Introduction

Almost 10^6 tons of dyes are produced annually around the world, of which azo dyes, characterized by one or more azo groups (R_1–N=N–R_2) linking substituted aromatic structures, represent more than 50% by weight [1]. These dyes are widely used in a number of industries such as textile, food, cosmetics and paper printing. Approximately 10–15% of dyes are released into the environment during manufacturing and usage [2]. Azo dyes are generally recalcitrant to biodegradation in conventional activated sludge treatment due to their xenobiotic toxicity [3]. Many studies on biodecolorization of azo dyes using bacteria or fungi have been reported in recent years [4–8]. An effective strategy for azo dye degradation is the anaerobic/aerobic sequential treatment, in which azo dyes are firstly reduced to colorless aromatic amines under anoxic or anaerobic conditions, and then mineralized aerobically [3].

The mechanism of azo dye reduction is still a subject of debate. Some suggested that it should be a specific reaction by intracellular azoreductase while others reported it to be an extracellularly unspecific reduction process [9,10]. Intracellular azoreductase cannot reduce all types of azo dyes, especially those sulfonated azo dyes with high polarity and polymeric azo dyes with high molecular weight, which have limited membrane permeability [9].

The extracellular reduction of the azo dye is usually a slow process. However, it could be significantly enhanced by the use of redox mediators such as lawsone, riboflavin, and anthraquinone-2,6-disulfonate (AQDS) [11–13]. Quinone compounds used as redox mediators are reduced by cytoplasmic or membrane-bound quinone reductase. The hydroquinones formed can then reduce azo dyes in a purely chemical redox reaction [9,14]. However, the continuous dosing of redox mediators implies continuous expenses related to procurement of the chemical, as well as continuous discharge of biologically recalcitrant compounds resulting in secondary contamination. In addition, it is usually difficult to immobilize quinone redox mediators. The catalytic effects of immobilized mediators were not satisfying [15].

It was reported that monoazo dye Acid Orange 7 (AO7) has an autocatalysis effect. 1-Amino-2-naphthol, the constituent aromatic amine and degradation product of AO7 can accelerate the reduction process, possibly by mediating the transfer of reducing equivalents [16,17]. Similarly, it was reported that the decolorized supernatants of Direct Blue 71 and Direct Blue 53, two tri-azo dyes, increased the biodecolorization rate of Methyl Orange [13]. Such mediators can be generated during azo dye reduction, and mineralized by sequential aerobic treatment without secondary contamination. However, the rate constant of AO7 reduction mediated with 1-amino-2-naphthol was 10-fold lower than that obtained using AQDS [16]. Thus some better mediators generated from azo dye reduction are expected for practical application.

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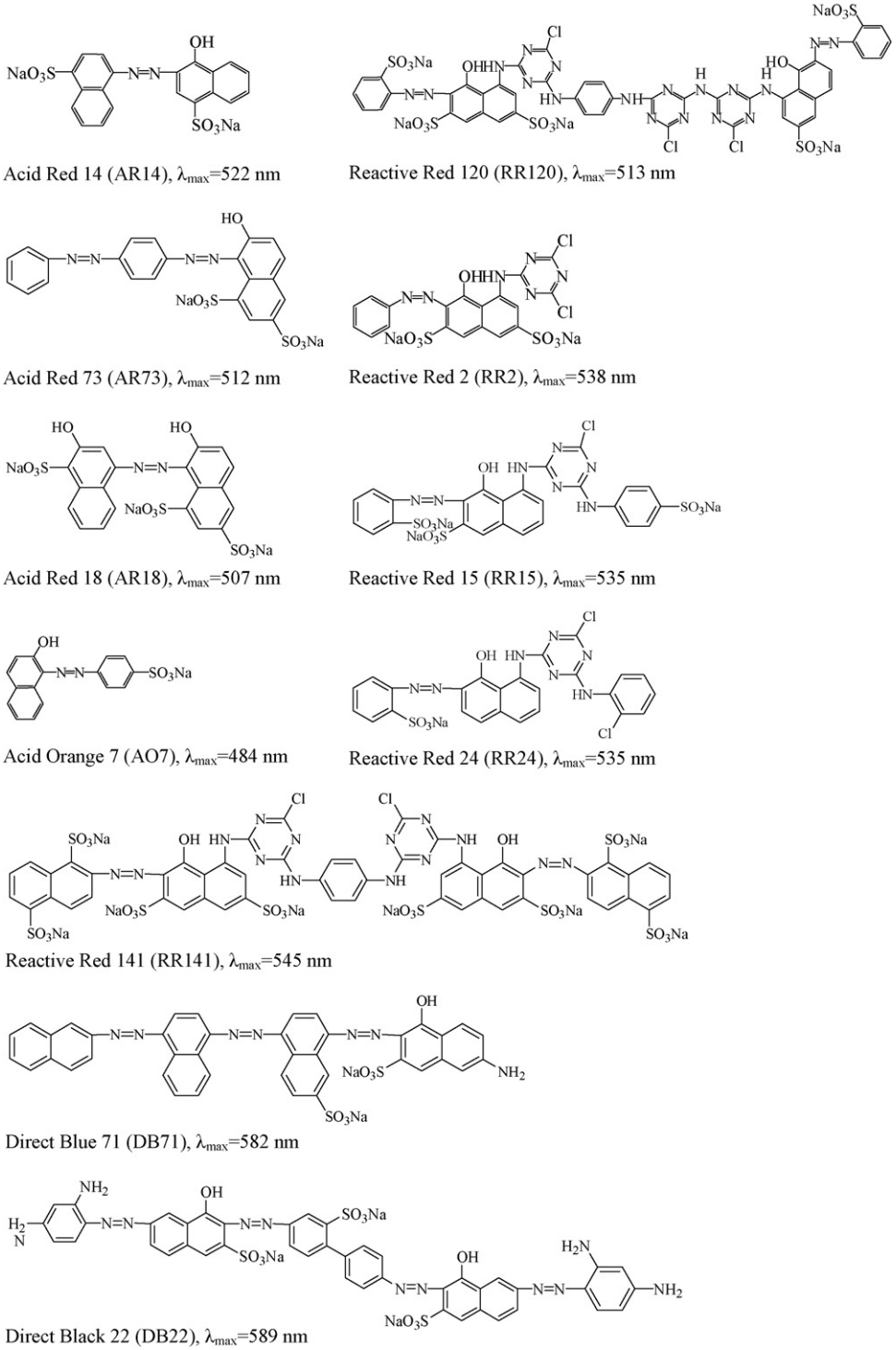

Fig. 1. Azo dyes used in this study.

In this study, the effects of reduction products of some *ortho*-hydroxyl substituted azo dyes on biodecolorization of azo dyes were investigated.

2. Materials and methods

2.1. Chemicals

Azo dyes used in this study are shown in Fig. 1. All the dyes were purchased from Tianjin Tianshun Chemical Dyestuff Co., Ltd. Other chemicals used in the study were all purchased from Sigma.

2.2. Activated sludge, strain, media and culture conditions

Three kinds of original sludge samples were taken from Dalian Chunliu river wastewater treatment plant, Dalian petrochemical company wastewater treatment plant and black sediment derived from Dalian Xishan reservior, respectively. The mixture (1:1:1) of these sludge samples was used for decolorization experiments.

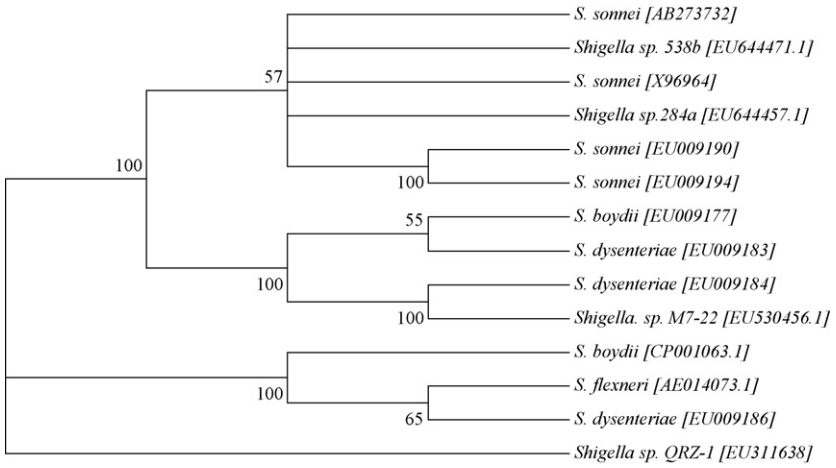

Fig. 2. Phylogenetic tree of strain QRZ-1 based on the 16S rRNA sequences using the MP method. The sources of sequences are shown as GenBank accession number in brackets. Only bootstrap (500 replicates) values above 50% are shown.

The quinone-reducing strain QRZ-1 used in this study was a native strain isolated from the sludge mixture. Mixed sludge was enriched using growth medium containing glucose as sole electron donor and AQDS as sole electron acceptor, respectively. After turbidity was observed, the enrichment was plated onto growth agar medium, and colonies were re-grown in suspension and re-plated until purity was achieved.

The growth medium which was developed in our lab for isolation and cultivation of quinone-reducing community and strain consisted of ($g\,L^{-1}$): $NaHCO_3$ 0.71, NH_4Cl 1.0, KH_2PO_4 0.5, K_2HPO_4 0.6, $MgCl_2{\cdot}6H_2O$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.05, $Na_2S{\cdot}9H_2O$ 0.037, glucose 0.5, and AQDS 0.15 [18]. The decolorization medium contained the same compositions as the growth medium except the removal of AQDS.

Strain QRZ-1 was inoculated to serum vials filled with sterilized growth medium. Then the vials were sealed with butyl rubber stoppers, and statically cultivated at 30 °C for 24 h to reach the exponential growth phase. Cells were harvested by centrifugation ($12{,}000 \times g$, 10 min) and washed twice with 0.1 M phosphate buffer (pH 7.0). Then the cells were resuspended in the same buffer to obtain condensed cell suspensions ($160\,g\,L^{-1}$).

2.3. Preparation of the reduction products of ortho-hydroxyl substituted azo dyes

Cell suspension (5 mL) was inoculated to 135 mL serum bottles filled with sterilized decolorization medium containing 1 M specific acid azo dye. Then the vials were sealed with butyl rubber stoppers, and statically cultivated at 30 °C until the complete decolorization of dyes. Cells were removed by centrifugation ($24{,}000 \times g$, 10 min), and the supernatants were kept at 4 °C before used as mediator in the following decolorization studies. The exact amounts of specific mediators in the supernatants were determined with high performance liquid chromatography before use.

2.4. Batch decolorization experiments

All batch decolorization experiments were conducted in 135 mL serum bottles filled with decolorization medium. Condensed cell suspensions or mixed sludge were transferred under anaerobic conditions to the bottles to reach $OD_{660} = 0.4$, or sludge concentration (MLSS) of $3.0 \pm 0.1\,g\,L^{-1}$. Azo dyes and reduction products used as mediators were added by syringe, respectively. The serum bottles were sealed with butyl rubber stoppers, and then statically cultivated at 30 °C. All the experiments were done in triplicate.

2.5. Sequential batch reactor (SBR) experiments

SBR experiments were conducted in 135 mL serum bottles filled with decolorization medium. RR120 and reduction product of AR14 (RAR14) were added by syringe, respectively. The serum bottles were sealed with butyl rubber stoppers and then statically cultivated at 30 °C. Each cycle consisted of 0.5-h fill, 7–30-h decolorization reaction at different stages, and approximately 1.5-h settle and discharge. The SBR experiments were divided into 4 different phases. The RR120 concentrations and the corresponding decolorization time were 100, 200, 300, and 500 mg L^{-1} and 7, 15, 25, and 30 h, respectively. The concentrations of sludge and RAR14 in each cycle were kept at approximately $3\,g\,L^{-1}$ and 0.1 mM, respectively.

2.6. Analytical methods

Absorbance was measured spectrophotometrically with spectrophotometer (Jasco V-560) at respective maximum wavelength of azo dyes. The concentration was calculated according to the corresponding calibration curves.

The cyclic voltammetric experiments were carried out using a PARSTAT 2273 electrochemical system, employing a three-electrode configuration: glassy carbon electrode ($d = 3$ mm) as the working electrode, a platinum wire as the auxiliary electrode, and

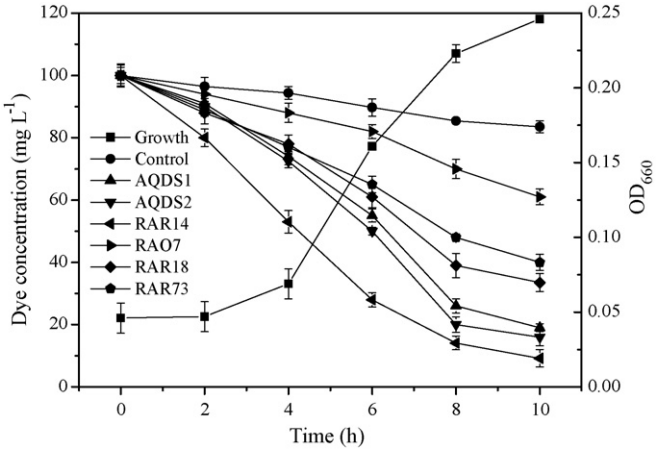

Fig. 3. Growth of strain QRZ-1 (OD_{660}) and biodecolorization of RR120 by strain QRZ-1 in the presence of AQDS1 (0.06 mM), AQDS2 (0.12 mM) and 0.12 mM reduction products of RAR14, RAO7, RAR18 and RAR73.

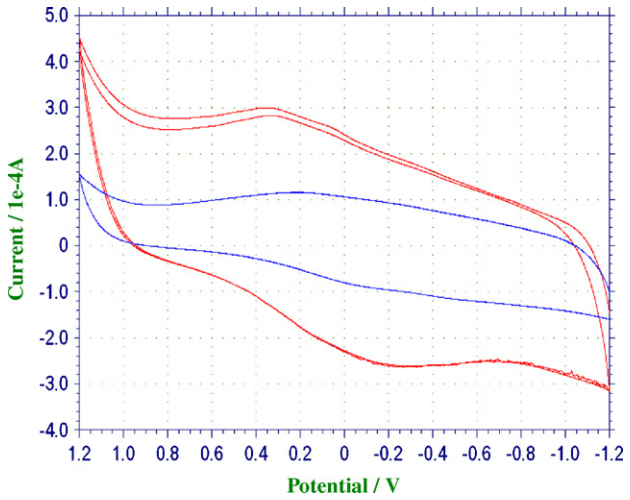

Fig. 4. Cyclic voltammograms of the system at 0 min (blue line) and when RAR14 was formed (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

saturated calomel electrode as the reference electrode. As electrolyte support, 0.1 M of Na_2SO_4 solution was used. The cyclic voltammetric scan was conducted at a rate of 100 mV s^{-1} within the potential interval from −1200 to 1200 mV. The test solutions were flushed with high-purity N_2 for 30 min to remove interfering oxygen.

The aromatic amines were detected with high performance liquid chromatography/mass spectrometry (HPLC/MS, Shimadu HPLC-2010A system). The chromatographic column is Shim-pack VP-ODS (150 mm × 2.0 mm, C18 column). The column temperature was 40 °C. For 1-naphthylamine-4-sulfonic acid, the mobile phase consisted of methanol and water containing dibutylamine (2.5 mM) and acetic acid (2.5 mM). The elution program began with 30% methanol and 70% water. The percentage of methanol was linearly increased to 70% over 10 min. The flow rate was 0.2 mL min^{-1}. For 1-naphthol-2-amino-4-sulfonic acid, the mobile phase consisted of methanol and water containing dibutylamine (4 mM). The elution program began with 60% methanol and 40% water. The percentage of methanol was linearly increased to 100% over 40 min. The flow rate was 1.0 mL min^{-1}. MS was performed under the following conditions: electrospray ionization source (ESI−), detector pressure 1.6 V, scan mode.

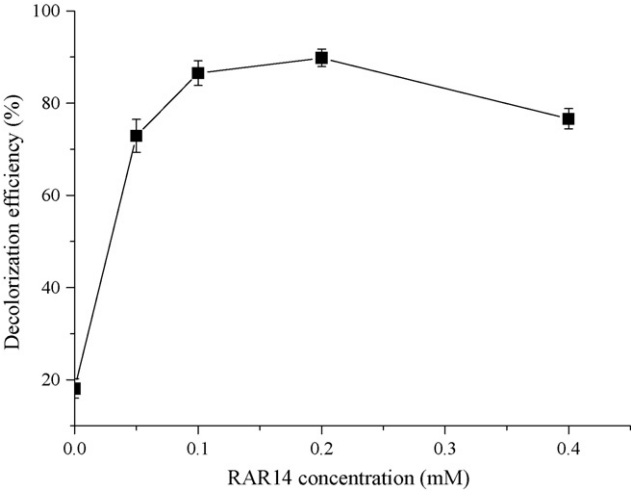

Fig. 5. Effects of RAR14 concentration (0–0.4 mM) on biodecolorization of RR120.

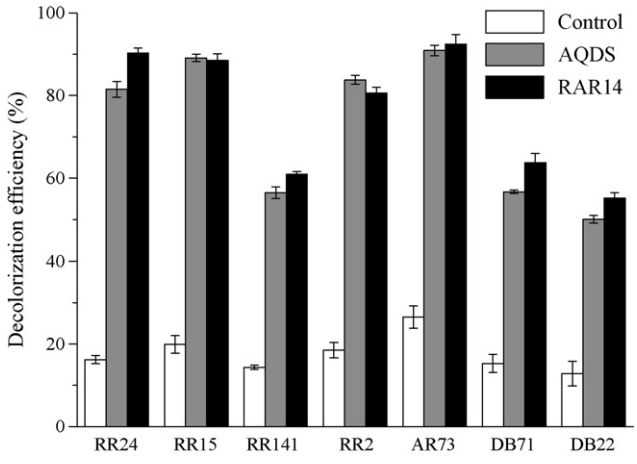

Fig. 6. Comparison of accelerating effects of RAR14 (0.12 mM) and AQDS (0.12 mM) on decolorization of different azo dyes by strain QRZ-1. Cultures without the addition of redox mediator served as control.

2.7. 16S rDNA sequencing and phylogenetic analysis

Genomic DNA was extracted according to a modified method used for the purification of DNA from Gram-negative bacteria [19]. The bacterial 16S rRNA gene was amplified by using primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1522R (5′-AAG GAG GTG ATC CAN CCR CA-3′). The sequencing of PCR product was done by TaKaRa Dalian. Multiple sequence alignment was performed with Clustal X [20]. The phylogenetic tree was constructed by maximum parsimony (MP) method using Molecular Evolutionary Genetics Analysis (MEGA) [21]. The sequence of the 16S rDNA gene of the strain QRZ-1 is available under the GenBank accession number EU311638.

3. Results and discussion

3.1. Characteristics of strain QRZ-1

A pure quinone-reducing isolate QRZ-1 was obtained from the sludge mixture after 2 months of enrichment at 30 °C in the growth medium. It is a Gram-negative, non-motile, rod-shaped, anaerobic bacterium (0.2–0.3 μm wide and 0.7–0.9 μm long). Colonies are mucoid, rounded and white when cultivated on solid growth medium. It is catalase-positive and produces acids from glucose. It can use glucose, maltose, sucrose, galactose, fructose, mannose,

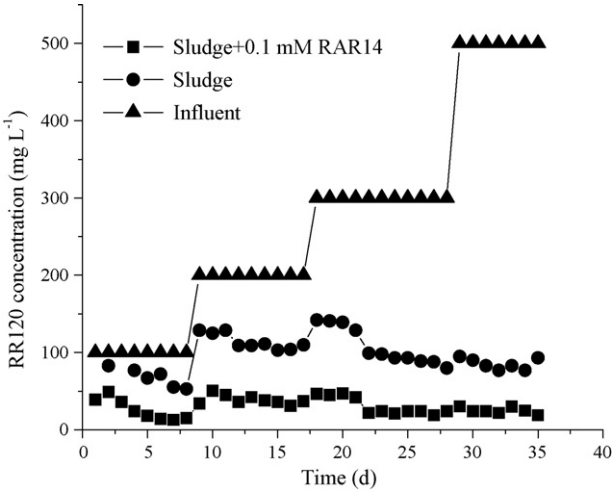

Fig. 7. Effects of RAR14 on RR120 decolorization by anaerobic sludge in SBR.

oxalate, lactate, D-xylose, α-lactose and sorbose as sole carbon source. However, citrate, acetate, malonate and succinate cannot be utilized by the strain. It is resistant to tetracycline and streptomycin. On the basis of 16S rDNA sequences comparison at NCBI and RDP II, QRZ-1 has higher homology (99%) with the genus *Shigella* within the family *Enterobacteriacea* of the gamma subclass of the *Proteobacteria*. The phylogenetic analysis demonstrated that QRZ-1 is distinct from other *Shigella* species (Fig. 2). According to the physiological and biochemical characteristics and 16S rRNA gene sequencing results, the strain QRZ-1 is a close relative of the genus *Shigella* and designated as *Shigella* sp. strain QRZ-1.

It has been found that many microorganisms are capable of reducing quinones, such as Fe(III)-reducers *Pantoea agglomerans* SP1, *Geobacter sulfurreducens*, and *Shewanella decolorationis* S12, uranium-reducer *Deinococcus radiodurans*, sulfate-reducer *Desulfovibrio* G11, halo-respiring bacterium *Desulfitobacterium dehalogenans*, fermentative bacterium *Propionibacterium freudenreichii*, and methanogenic archaea *Methanospirillium hungatei* [22–25]. However, to our knowledge, this is the first report on quinone reduction by genus *Shigella*. Genes encoding putative quinone oxidoreductases have been reported in genome of *Shigella* genus [26]. Previous studies using other bacterial strains demonstrated that quinoid redox mediators can be reduced by cytoplasmic or membrane-bound quinone reductase to speed up azo dye decolorization [9,14]. Thus QRZ-1 was used in the following mediated decolorization studies.

3.2. Decolorization experiments with strain QRZ-1

The effects of the reduction products of AO7, AR73, AR18 and AR14 (named RAO7, RAR73, RAR18 and RAR14, respectively) on decolorization of RR120 by strain QRZ-1 were studied. During the decolorization process we investigated, QRZ-1 kept its normal growth and almost no obvious growth difference was observed in the presence of different mediators. As shown in Fig. 3, without the addition of reduction products, only 17% RR120 was removed in 10 h. On the other hand, the addition of reduction products of acid azo dyes accelerated the decolorization of RR120 effectively. The addition of RAR14 resulted in removal of 91% RR120 in 10 h, which was the best accelerating effect and better than that obtained with the model redox mediator AQDS.

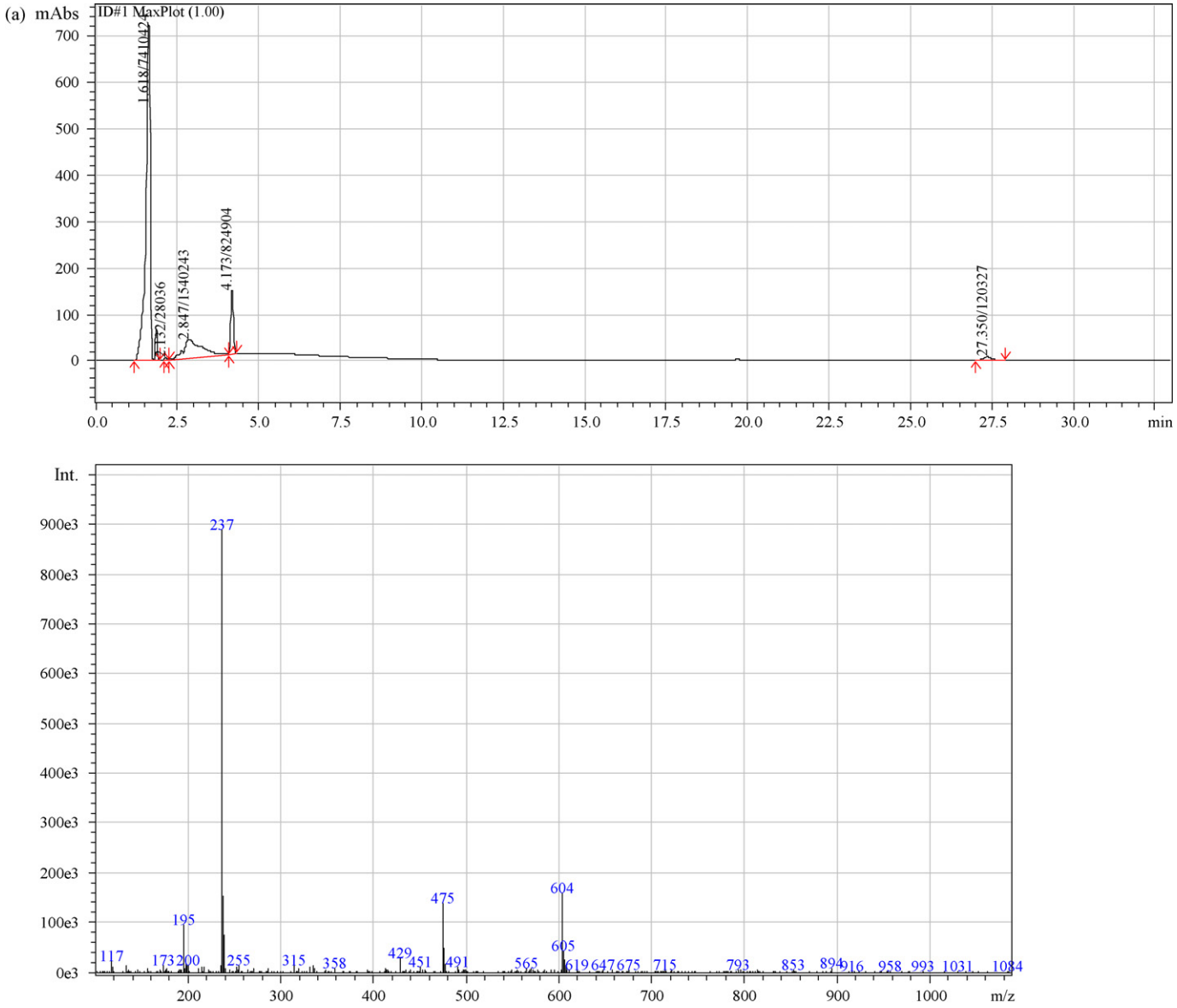

Fig. 8. HPLC/MS spectra of 1-naphthol-2-amino-4-sulfonic acid (a) and 1-naphthylamine-4-sulfonic acid (b) detected as reduction products of AR14.

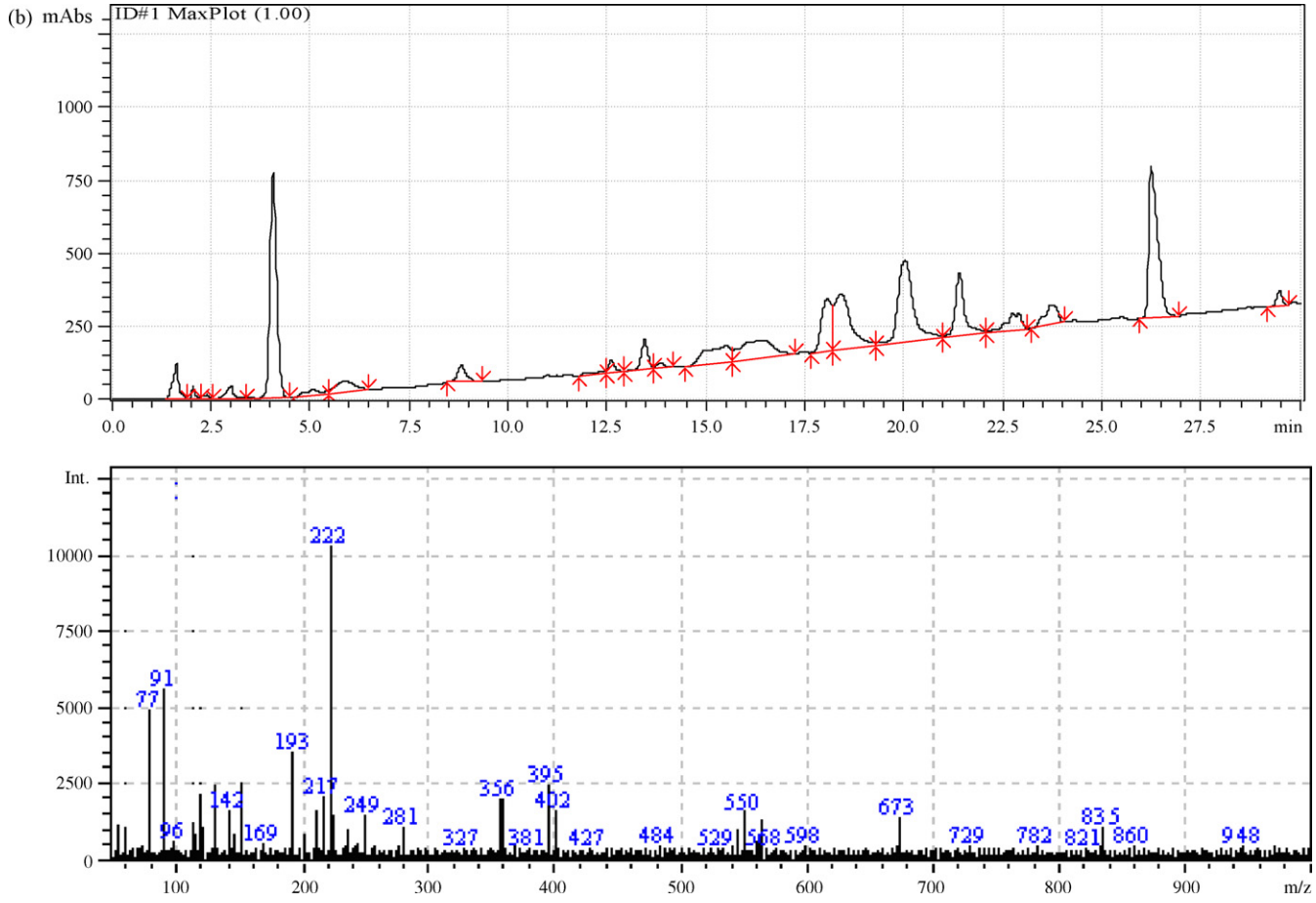

Fig. 8. (*Continued*).

Two current peaks of reversible oxidation and reduction were observed at potentials of approximately 338 and −209 mV, respectively which indicates the reversible redox process of RAR14. In contrast, no peak was found in voltammograms measured when no reduction products were formed at 0 min (Fig. 4). Thus RAR14 could function as redox mediator and was responsible for the accelerated decolorization performance. Similar current peaks were also observed with other reduction products (data not shown). It was reported that two flavoproteins from *S. dysenteriae* could act as azoreductase for the decolorization of some azo dyes *in vitro* [27]. However, due to their high polarity and large molecular weight, azo dyes such as RR120 are unlikely to pass through the cell membrane. The decolorization of RR120 might be a process occurred extracellularly. The reduction products of these *ortho*-hydroxyl substituted azo dyes possibly act like AQDS and RAO7 as redox mediators by mediating the transfer of reducing equivalents from the cell to extracellular azo dyes [16,17].

Effects of RAR14 concentration on decolorization of RR120 by strain QRZ-1 were investigated (Fig. 5). The results indicated that the decolorization efficiency was enhanced dramatically with the increase of RAR14 concentration. A 3.5-fold increase of decolorization efficiency was obtained with the addition of 0.05 mM RAR14. The addition of 0.1–0.2 mM RAR14 resulted in approximately 4.5-fold increase. However, with further increase of the RAR14 concentration over 0.3 mM, the stimulating effect on decolorization decreased correspondingly. When AQDS is used as redox mediator for azo dye decolorization, the enzymatic reduction of the AQDS is generally suggested to be the rate-limiting step [11,12]. Similar decrease of accelerating effects due to higher concentration of AQDS were reported previously [28].

Since the thermodynamics of different electron-donating half-reactions varied, the reaction rate may be influenced by the type of electron donor. Different carbon sources (0.5 g L^{-1}) were therefore chosen to determine their effects on RR120 decolorization by strain QRZ-1 in the presence of RAR14 (0.12 mM). The strain was able to grow on all tested carbon sources, whereas the decolorization activities varied. RR120 decolorization was observed after 10-h cultivation with glucose, sucrose, maltose, mannose, oxalate, lactate, and peptone as carbon sources, respectively. The maximum decolorization efficiency (91.3%) was obtained with glucose. Using lactate and oxalate as carbon sources resulted in less than 10% decolorization efficiency.

It was shown that AQDS can be used as redox mediator to accelerate the decolorization of many azo dyes [12]. Most of the azo dyes investigated in this study are characteristic of complex structure and high molecular weight (Fig. 1). It is believed that they cannot enter into the bacterial cells and are difficult to be biodecolorized [9,10]. As shown in Fig. 6, the addition of RAR14 could dramatically enhance the decolorization of these different azo dyes by strain QRZ-1.

3.3. Decolorization experiments with anaerobic sludge

SBR experiments with anaerobic sludge also demonstrated the enhancement of RR120 decolorization in the presence of RAR14 (Fig. 7). The accelerating effect was more obvious (1.5-fold higher) during the startup of the system. When RR120 concentration was increased to 500 mg L^{-1}, the decolorization efficiency was still maintained at approximately 95%, and the abiotic adsorption of dyes on the biomass was insignificant (data not shown). Moreover,

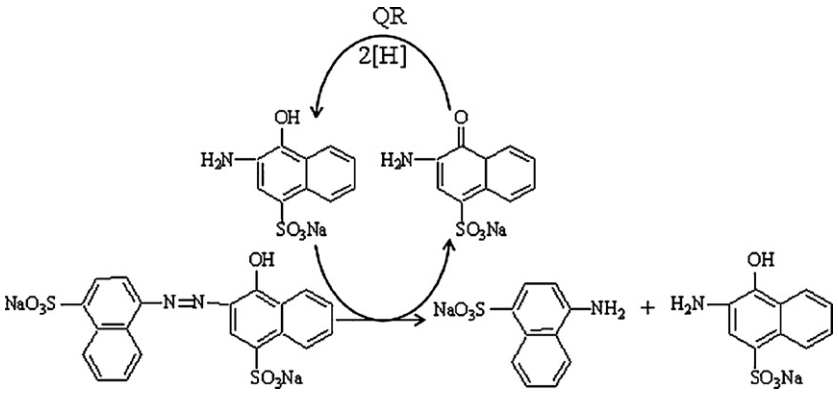

Fig. 9. Proposed mechanism of enhanced biodecolorization of AR14 mediated by its reduction product 1-naphthol-2-amino-4-sulfonic acid. QR, quinone reductase.

the decolorization of mixed dyes by anaerobic sludge was also speeded up in the presence of AR14 (data not shown). Thus RAR14 may act as redox mediator for various microorganisms in the sludge and be beneficial for the practical bioprocess of wastewater treatment.

3.4. Mechanism analysis

To investigate the accelerating mechanism, the metabolites formed in the decolorization process of AR14 by strain QRZ-1 were analyzed using HPLC/MS. The existence of 1-naphthol-2-amino-4-sulfonic acid was confirmed with a retention time of 3.42 min and *m*/*z* 237. 1-Naphthylamine-4-sulfonic acid was detected with a retention time of 4.17 min and *m*/*z* 222 (Fig. 8). Thus the decolorization of AR14 is originated by the breakage of azo bond.

During quinoid redox mediator-dependent biodecolorization of azo dyes, the mediators are reduced by membrane-bound or cytoplasmic quinone reductase, and then the hydroquinones formed reduce azo dyes in a purely chemical redox reaction [9,29]. 1-Amino 2-napthol, one of the constituent aromatic amines of the AO7, has redox mediating properties to enhance AO7 decolorization, possibly by mediating the transfer of reducing equivalents [16,17]. Having similar structure with 1-amino-2-naphthol, 1-naphthol-2-amino-4-sulfonic acid may act as redox mediator to enhance azo dyes decolorization. It is deduced that 1-naphthol-2-amino-4-sulfonic acid cycled between cells and extracellular azo dyes. After the chemically reduction of azo bond by 1-naphthol-2-amino-4-sulfonic acid, the oxidized form of 1-naphthol-2-amino-4-sulfonic acid was reduced by the quinone reductases of the cells, and the regenerated 1-naphthol-2-amino-4-sulfonic acid can function in the next redox cycle (Fig. 9).

1-Amino-2-naphthol-6,8-disulfonic acid was detected as one of the reduction products of AR18 and AR73 (data not shown). As an aromatic amine with an *ortho*-hydroxyl group, 1-amino-2-naphthol-6,8-disulfonic acid may also function as redox mediator to accelerate the bacterial decolorization performance. According to the results shown in Fig. 3, it seems that *ortho*-hydroxyl substituted aromatic amines having the electron-withdrawn sulfonate group (1-naphthol-2-amino-4-sulfonic acid of RAR14, and 1-amino-2-naphthol-6,8-disulfonic acid of RAR18 and RAR73) are better redox mediators than those without sulfonate group (1-amino 2-napthol of RAO7). In addition, higher mediated decolorization performances were observed when the sulfonate group of the *ortho*-hydroxyl substituted aromatic amine is present in the same benzene ring with the hydroxyl and amino groups (1-naphthol-2-amino-4-sulfonic acid of RAR14 over 1-amino-2-naphthol-6,8-disulfonic acid of RAR18 and RAR73, and possibly reduction product of Direct Blue 71 over that of Direct Blue 3 [13]). Further experiments are underway to confirm these findings.

Although it is well known that redox mediators could accelerate the anaerobic biotransformation of refractory pollutants, little is known about the enzymes responsible for the redox mediator reactions. Further studies will be necessary to characterize the enzymatic systems involved in the regeneration of 1-naphthaquinone-2-amino-4-sulfonic acid as redox mediator.

4. Conclusions

This study demonstrated that the reduction products of some *ortho*-hydroxyl substituted acid azo dyes, such as 1-naphthol-2-amino-4-sulfonic acid formed in AR14 decolorization by *Shigella* sp. strain QRZ-1 or anaerobic sludge could function as redox mediator and accelerate azo dyes biodecolorization. Compared to the synthetic mediator AQDS, RAR14 demonstrated better mediating effects. In addition, estimated from the results obtained in our studies, the use of metabolites generated from biological reduction possesses will save around $120–160 per ton of dye wastewater. The addition of such *ortho*-hydroxyl substituted acid azo dyes in the feed may be a promising and cost-effective strategy to improve the biodecolorization of azo dye wastewater.

Acknowledgment

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