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Humic Substances Act as Electron Acceptor and Redox Mediator for Microbial Dissimilatory Azoreduction by *Shewanella decolorationis* S12

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Abstract The potential for humic substances to serve as terminal electron acceptors in microbial respiration and the effects of humic substances on microbial azoreduction were investigated. The dissimilatory azoreducing microorganism *Shewanella decolorationis* S12 was able to conserve energy to support growth from electron transport to humics coupled to the oxidation of various organic substances or H_2. Batch experiments suggested that when the concentration of anthraquinone-2-sulfonate (AQS), a humics analog, was lower than 3 mmol/l, azoreduction of strain S12 was accelerated under anaerobic condition. However, there was obvious inhibition to azoreduction when the concentration of the AQS was higher than 5 mmol/l. Another humics analog, anthraquinone-2-sulfonate (AQDS), could still prominently accelerate azoreduction, even when the concentration was up to 12 mmol/l, but the rate of acceleration gradually decreased with the increasing concentration of the AQDS. Toxic experiments revealed that AQS can inhibit growth of strain S12 if the concentration past a critical one, but AQDS had no effect on the metabolism and growth of strain S12 although the concentration was up to 20 mmol/l. These results demonstrated that a low concentration of humic substances not only could serve as the terminal electron acceptors for conserving energy for growth, but also act as redox mediator shuttling electrons for the anaerobic azoreduction by *S. decolorationis* S12. However, a high concentration of humic substances could inhibit the bacterial azoreduction, resulting on the one hand from the toxic effect on cell metabolism and growth, and on the other hand from competion with azo dyes for electrons as electron acceptor.

Keywords: *Shewanella decolorationis* S12, humic reduction, azoreduction, electron acceptor, redox mediator

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Over 10,000 different dyes with an annual production of over 7×10^5 tonnes worldwide are commercially available [25]. Azo dyes, containing one or more azo bonds (-N=N-), account for 60–70% of all textile dyes used [34]. It is estimated that more than 10% of the dye-stuff used during the synthesis and dyeing processes was released into sewage treatment systems or into the environment, therefore bringing serious environmental pollution [23, 37]. The discharge in open waters not only presents an esthetic problem, but also causes a toxic impact on aquatic life and eventually affects human health through the food chain [1, 5, 6]. Compared with physicochemical methods, biological systems for the treatment of textile-printing wastewater are more favorable because of their cost effectiveness, lower sludge production and environmental friendliness. The anaerobic-aerobic treatment is the most effective method to decolorize and degrade azo dye-containing wastewaters [4, 31]. This strategy is based on the studies that various bacterial strains can reduce azo dyes under anaerobic conditions into corresponding aromatic amines, and subsequently mineralize them under aerobic condition [13, 22, 24, 26, 28, 35]. The first stage of the process, anaerobic azo dye reduction, has been considered an important reaction. Therefore, how to improve the rate of anaerobic azoreduction is a key factor to the treatment of wastewater contaminated by azo dyes.

Humic substances are the most abundant organic fraction in the biosphere, which are poorly biodegradable polymers formed during the decomposition of plant material in soil and sediments. Generally, humics are composed of quinone structural units [33]. Electron spin resonance measurements provided direct evidence that quinone moieties are the actual functional groups accepting electrons during the microbial reduction of humics [30]. Moreover, genetic evidence showed there is a common biochemical basis for quinone and humic reduction in *Shewanella putrefaciens*

MR-1 [21]. Thus, quinones are good analogs for the function of humics. To date, many quinone-reducing microorganisms have been confirmed; for example, the halorespiring bacterium *Desulfitobacterium* PCE1, the sulphate-reducing bacterium *Desulfovibrio* G11, and the methanogenic archaeon *Methanospirillum hungatei* JF1 [2], Fe(III)-reducing bacteria *Geobacteraceae* [7, 8, 18], *Pantoea agglomerans* SP1 [11], and *Thermoanaerobacter siderophilus* sp. nov [32]. It has recently been recognized that humic substances may play an important role in the anaerobic biodegradation and biotransformation of organic as well as inorganic compounds [3, 10]. Humus can serve as a terminal electron acceptor supporting the anaerobic oxidation of a variety of organic substrates by Fe(III)-reducing bacteria [18, 19]. Furthermore, humic substances are capable of serving as electron shuttlers, abiotically transferring redox equivalents from the cell membrane of the bacteria to azo dyes and stimulating the bacterial anaerobic azoreduction. However, these studies were based on enrichment cultures [10, 15, 29, 36]. Very few pure culture isolates have been reported about the organism that can use both humics and azo compounds as electron acceptors. *S. decolorationis* S12, previously isolated from activated sludge of a textile-printing wastewater treatment plant in Guangzhou, China [39], can effectively reduce azo dyes with many organic substances and H_2 as electron donors, which is a dissimilatory azoreduction bacterium [14]. In addition, this organism can respire with humic as the sole electron acceptor. In order to obtain a better understanding of how humic substances are involved in biological reduction of azo dyes, physiological studies were conducted to characterize humic reduction and to explore the link between humic and azo reduction.

MATERIALS AND METHODS

Humic Substances and Azo Dyes

Humics analogs, anthraquinone-2, 6-disulfonate (AQDS) and anthraquinone-2-sulfonate (AQS) used for studying humic reduction and amaranth, a model azo dye used in this study, were purchased from Sigma and Aldrich at the highest purity commercially available. Other azo dyes purchased from a dye-stuff factory in Guangzhou, China. Chemical structures of AQS, AQDS, and azo dyes are depicted in Fig. 1.

Organism, Media, and Cultivation

Shewanella decolorationis S12^T (CCTCC M 203093, IAM 15094) was isolated from activated sludge of a textile-printing wastewater treatment plant in Guangzhou, China [39]. The strain S12 was capable of growing under both aerobic and anaerobic conditions. Luria-Bertani (LB) medium was used for aerobic cultivation at 32°C with shaking at 150 rpm. The bacteria were cultivated under anaerobic condition with an anaerobic station (Ruskinn C0105) in a defined medium (pH 7.5) containing the following (mmol/l): succinate (10), Na_2HPO_4 (5.7), KH_2PO_4 (3.3), NH_4Cl (18.0), $MgSO_4$ (1.01), L-cysteine (20 μgml^{-1}), vitamin solution, and mineral solution [38].

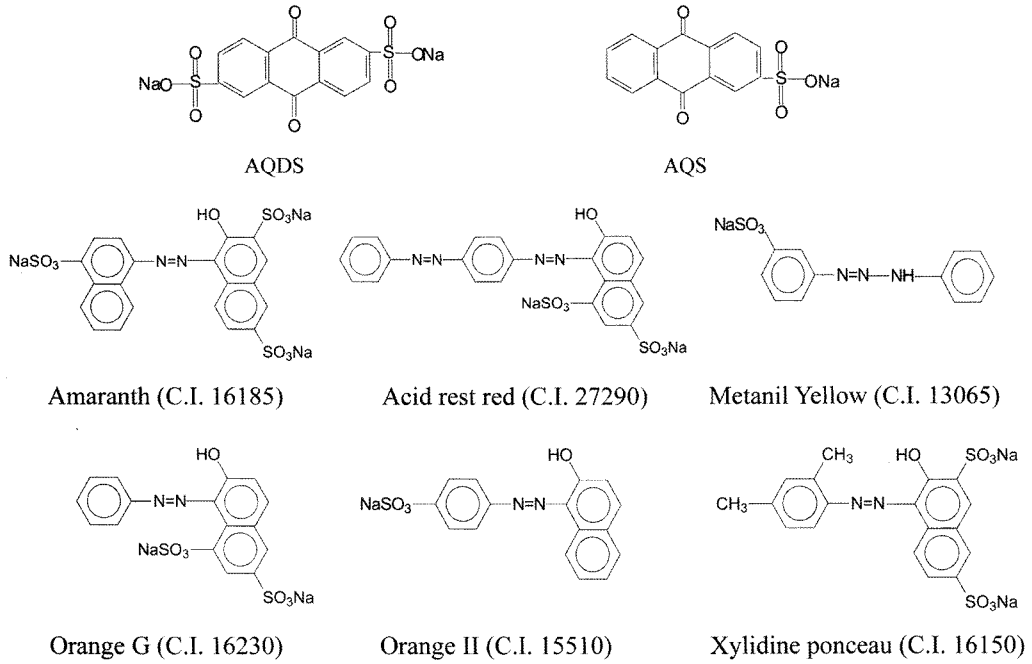

Fig. 1. Chemical structures of humic substances and azo dyes used in this study.

A modified Hungate technique [20] was used throughout the study for anaerobic cultivation. All batch experiments were conducted in 50-ml serum bottles. The medium was prepared by adding all the components from concentrated stock solutions in O_2-free distilled water and the preparation equilibrated with N_2-CO_2 (4:1), which was passed through a filter to remove bacteria. After flushed with the O_2-free gas for 5 min, the serum bottles were sealed with butyl rubber stoppers and incubated in an anaerobic station (Ruskinn C0105). H_2 was provided with 96 kPa unless otherwise noted. The initial cell concentration was 3.0–3.8×10^5 CFU/ml unless indicated otherwise.

The Oxidization of Electron Donors Coupled with the Reduction of Humics

Cells grown aerobically in LB medium were harvested by centrifugation, washed twice, and resuspended in the defined medium containing 1 mmol/l of azo dye amaranth and supplying 10 mmol/l of organic substance of either formate, lactate, aniline, toluene, or H_2 as electron donor. Control experiments were conducted to test whether the humic reduction can occur without adding any electron donors as well as by using heat-killed cells (incubated at 95°C for 30 min) with adding electron donors. These incubations were performed under anaerobic condition at 32°C.

Growth Assay with AQDS as Sole Electron Acceptor

Experiments were performed in the defined medium under anaerobic condition at 32°C, with 10 mmol/l of formate as electron donor. At selected intervals, each treatment was sampled and cell numbers of S12 were determined by direct plate counting on aerobic LB medium from duplicate experiments. The incubations without supplying formate or AQDS were used as control.

Effects of Humics on the Anaerobic Azoreduction

Strain S12 was grown aerobically under the conditions indicated above until they reached the late exponential growth phase. Cells were harvested by centrifugation (6,000×*g*), washed, and resuspended in phosphate buffer (20 mmol/l $Na_2HPO_4 \cdot 7H_2O$, 20 mmol/l $K_2H_2PO_4$, pH 8.0) to an optical density (OD_{600}) of about 0.5–0.8. The experiments were conducted in 50-ml serum bottles containing 20 ml of medium. The vials were filled with basal medium, organic primary electron donors, azo dyes, and different concentrations of AQS or AQDS. Next, the vials were flushed for 5 min with oxygen-free flush gas of N_2/CO_2 (80%/20%) and sealed with butyl rubber stoppers. In the AQDS or AQS gradient test, the rate of amaranth reduction was determined at five different AQDS or AQS concentrations, ranging from 1 to 5 mmol/l for AQS and 1 to 12 mmol/l for AQDS. The control experiment involved directly tested azoreduction by S12 without any humics. All experiments were performed in duplicate vials incubating in an anaerobic incubator at 32°C. At selected intervals, each treatment was sampled and the color was measured spectrophotometrically at the dye's wavelength of maximum absorbance (λ_{max}). The maximal wavelengths of Amaranth, Acid Great Red, Metanil Yellow, Orange G, Orange II, and Xylidine Ponceau are 520 nm, 510 nm, 414 nm, 483 nm, and 487.5 nm respectively.

The Chemical Reduction of Amaranth by Reduced AQS or AQDS

After AQS or AQDS were completely reduced by strain S12 with lactate or formate as electron donor, and the reduced humics solution was filtrated to remove the bacteria under the anaerobic condition. Different volumes of sterile aqueous solutions of amaranth (0.1 mol/l) were added to 10-ml serum bottles containing 2 mmol/l of AH_2QS or AH_2QDS, resulting in final concentrations of 0.1, 0.2, 0.3, and 0.4 mmol/l of amaranth and incubated at 32°C. When the amaranth was reduced completely, the AH_2QS or AH_2QDS was measured spectrophotometrically at 398 nm and 450 nm, respectively, under the anaerobic condition with a microplate reader located in an anaerobic chamber.

Toxic Test for AQDS and AQS

Both acute and long-term toxicities were tested. The acute toxicity of AQS and AQDS was determined by assaying the aerobic growth of strain S12 in LB medium under the condition of different concentrations of AQDS and AQS. The growth of strain S12 exposed to 5 mmol/l of AQS or 20 mmol/l of AQDS was as that for long-term toxicity of AQS and AQDS.

Analytical Methods

The concentrations of formate were measured by HPLC with separation on a polysulfonate ion exclusion column (Metrosep A Supp 5). The Azo dye color was measured spectrophotometrically with a spectrophotometer (BECKMAN DU640) at the dye's wavelength of maximum absorbance. Reduced AQDS (AH_2QDS) and reduced AQS (AH_2QS) were measured spectrophotometrically at 450 nm and 398 nm, respectively. Liquid-phase samples (1.0 ml) were centrifuged (2 min at 10,000 ×*g*) and diluted up to an absorbance of less than 1 with phosphate buffer. The buffer contained freshly added ascorbic acid (200 mg/l) to prevent autoxidation. Sampling and analysis were conducted in an anaerobic chamber under a 96%:4% N_2/H_2 gas. The extent of azoreduction and AQS and AQDS reduction was calculated according to the formulation as following at maximum absorbance, and all assays were done in duplicate. The adsorption of azo dye, AQS, or AQDS by bacterial cells was less than 1% and could therefore be neglected. Reduction extent=A–B/B×100%; A is the initial absorbance, B is the observed absorbance.

RESULTS

Humic Reduction by S12 Under Anaerobic Condition

Different organic substances were used as a primary electron donor for the reduction of AQS and AQDS by strain S12. In anaerobic condition, strain S12 was inoculated in the defined medium supplemented with each electron donor and AQS or AQDS. Experimental results showed that H_2, formate, lactate, and pyruvate were able to serve as favorable electron donors for reduction of AQS and AQDS. The reduction rate of AQDS was higher compared to that of AQS. After 30 h incubation, 1 mmol/l of AQDS was almost reduced completely, but reduction of 1 mmol/l of AQS required 50 h. In addition, aniline and toluene could also serve as electron donors for AQS and AQDS reduction, but the reduction rate was lower than that with H_2, formate, lactate, and pyruvate as electron donors. Strain S12 was not able to couple the oxidation of the following electron donors to AQS or AQDS reduction: acetate, propionate, salicylate, glycerin, glucose, carbinol, ethanol, sucrose, fructose, glucose, citrate, succinate, fumarate, and benzoate. In the medium lacking electron donor, there was less than 2% of AQS and AQDS to be reduced, indicating that the humic reduction must depend on the presence of electron donor. Humic reduction was an enzymatic process as either HB_{2B} or any of the organic electron donors was not able to reduce AQS or AQDS in the absence of the organism, or when cells were killed by incubation at 95°C for 30 min (Fig. 2).

To quantify the electron transfer from electron donor to humics, the oxidization of formate and the reduction of AQDS were determined simultaneously under formate limiting conditions, when there was excess AQDS (2.0 mmol/l). Once formate was exhausted, the reduction of AQDS stopped. Fig. 3 revealed the linear relation between consumption of formate and reduction of AQDS. These results showed clearly that reduction of AQDS by strain S12 completely depends on the consumption of formate when formate was used as the electron donor. The ratio of the number of moles of formate oxidized to the number of moles of AQDS reduced was 0.94. Because one molecular formate can provide two electrons and one molecular AQDS can accept two electrons for reduction, the ratio is 1.0 in theory. It was suggested that the electrons accepted by AQDS were almost completely transferred from formate. Formate consumption and AQDS reduction were in agreement with the following reaction: AQDS+ $COOH^-+H_2O \rightarrow AH_2QDS+HCO_3^-+2H^+$.

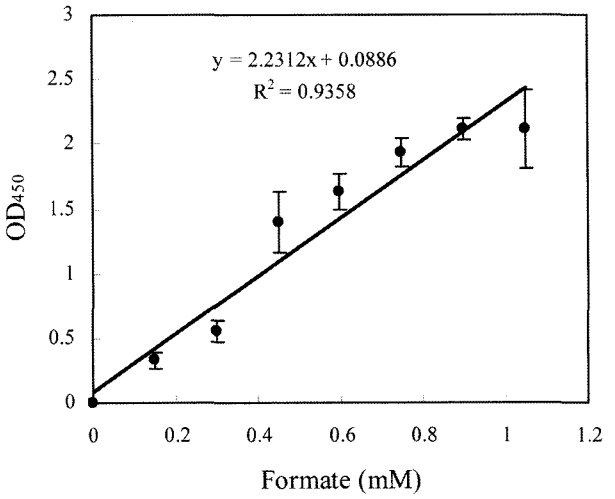

Fig. 3. The coupled relation between the humic reduction and oxidation of formate of *S. decolorationis* S12.
The experiments were performed at 32°C by addition of 2 mmol/l AQDS and different concentrations of formate. Data are the average from duplicate experiments. Error bars represent standard deviations of duplicate incubations.

Anaerobic Growth with Humics as the Sole Electron Acceptor

When *S. decolorationis* S12 was inoculated into the defined medium with formate as sole electron donor and

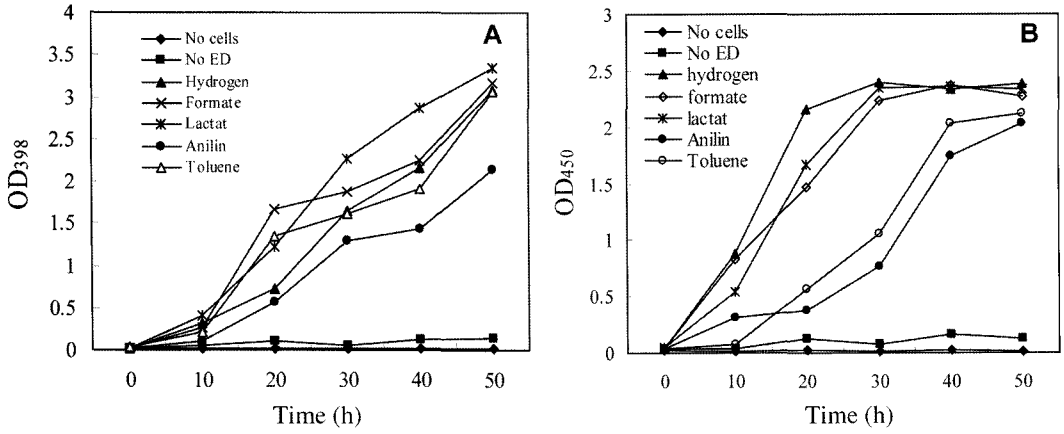

Fig. 2. The reduction of AQS (**A**) and AQDS (**B**) by *S. decolorationis* S12 with various substances as electron donor under anaerobic condition at 32°C for 36 h.

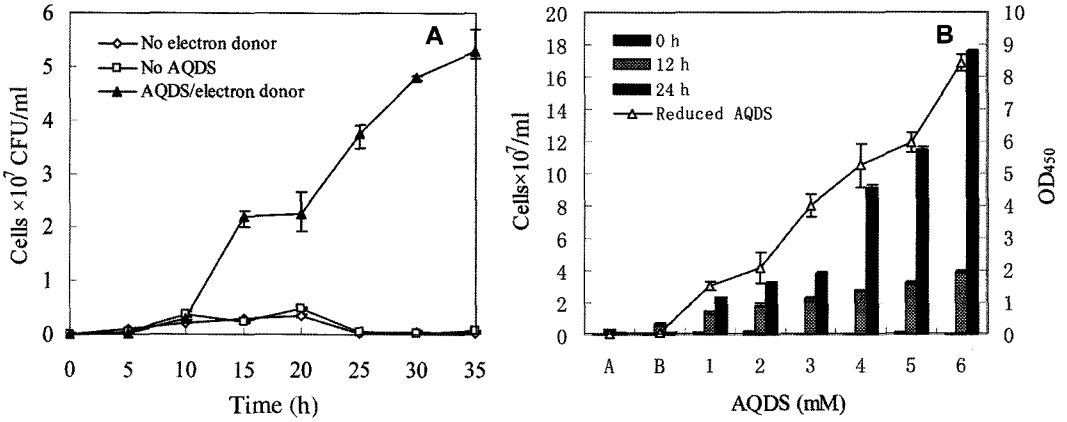

Fig. 4. Growth of *S. decolorationis* S12 using AQDS as the sole terminal electron acceptor with formate as the electron donor in a defined medium.

A. Growth in the presence of 2.0 mmol/l AQDS was assessed by increases in cell number with time process. **B.** Growth in the presence of different concentraton of AQDS was assessed by increases in cell number. A, no electron donor; B, no AQDS. Experiments were performed in the defined medium under anaerobic condition, with formate as electron donor. Cell numbers of S12 were determined by direct plate counting on aerobic LB medium from duplicate experiments. Error bars represent standard deviations of duplicate incubations.

AQDS as potential electron acceptor, AQDS was reduced to AH_2QDS with time (Fig. 1). Fig. 4 shows the anaerobic growth of *S. decolorationis* S12 coupled to the reduction of AQDS. The growth coincided with reduction of AQDS over time and stopped as AQDS became depleted. There was no growth or AQDS reduction if electron donors were absent from the medium, or if the electron donors were provided but the AQDS omitted. S12 grew to a maximal density of 1.8×10^8 cells per ml at 32°C by viable plate counting in the defined medium after compete reduction of 6.0 mmol/l of AQDS. These results indicate that AQDS as an electron acceptor, formate as an electron donor (energy source), and succinate as carbon source are essential for the anaerobic growth of *S. decolorationis* S12. Succinate alone did not support azoreduction and growth, suggesting that it was used only as a carbon source and not as electron donor for humic reduction. The pure culture can obtain energy for anaerobic growth by coupling oxidation of formate to reduction of AQDS. Energy conservation from the oxidation of formate was proportional to the AQDS reduction. This energy must be generated from the electron transport and oxidative phosphorylation, because there is no known mechanism to generate ATP through substrate level phosphorylation with formate as the substrate.

Effect of Humic Reduction on Anaerobic Azoreduction by S12

Previous study showed that *S. decolorationis* S12 was able to reduce various azo dyes in the defined medium with formate, lactate, pyruvate, or H_2 as electron donors under anaerobic condition [14]. To study the effect of

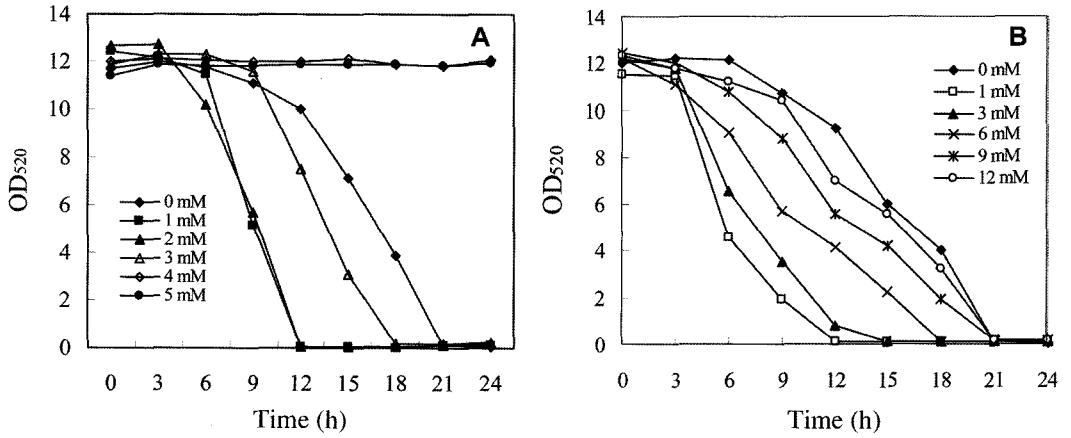

Fig. 5. Effect of AQS (**A**) and AQDS (**B**) on anaerobic reduction of amaranth by *S. decolorationis* S12.

The experiments were performed in the defined medium supplemented with 1 mmol/l amaranth and 10 mmol/l formate at 32°C.

Table 1. Effect of AQS and AQDS on anaerobic azoreduction of azo dyes by *S. decolorationis* S12. The experiments were performed at 32°C by addition of 1 mM azo dye as electron acceptor and 10 mM formate as electron donor under anaerobic condition. Data were obtained from separate duplicate experiments.

Azo dyes	Azoreduction (%)							
	Control	AQS 1 mM	AQS 3 mM	AQS 5 mM	AQDS 3 mM	AQDS 6 mM	AQDS 9 mM	AQDS 12 mM
Acid Great Red	16.5±2.7	99.4±0.1	21.3±2.8	3.2±0.5	96.9±0.2	97.8±0.2	92.6±1.8	90.0±2.2
Orange G	16.6±2.4	99.2±0.1	22.4±1.9	2.7±0.2	96.8±0.1	97.8±0.2	93.9±2.2	89.0±3.1
Xylidine Ponceau	15.8±1.7	96.8±0.2	23.2±0.9	1.7±0.3	96.8±0.2	94.8±1.6	77.1±5.4	70.1±5.7
Metanil Yellow	22.2±1.2	97.0±0.2	33.8±3.4	2.1±0.1	96.1±0.3	92.9±1.1	42.7±4.8	33.8±3.1
Orange 11	21.4±1.6	92.2±2.1	21.9±1.3	0.7±0.2	93.6±1.2	92.5±2.3	71.9±3.5	47.1±4.2

[a]Extent of azoreduction was measured spectrophometrically after 12 h incubation according to the following formulation: azoreduction (%)=(A-B)/A×100%; A, initial absorbance; B, observed absorbance.

humics on the microbial anaerobic azoreduction, AQS and AQDS were chosen as humic analogs for evaluating their effects on azoreduction by S12 with formate as electron donor.

The effect of AQS on anaerobic azoreduction by S12 is shown in Fig. 5A. After 12 h incubation, only 15% of 1 mmol/l of amaranth was reduced in the absence AQS, whereas 1 mmol/l amaranth was completely reduced in the presence of 1 mmol/l or 2 mmol/l of AQS. The corresponding first-order rate constants were 47.6 μmol/h in the absence of AQS and 83.3 μmol/h, 83.3 μmol/h, and 55.6 μmol/h with 1, 2, and 3 mmol/l of AQS, respectively. These values corresponded to a 75%, 75%, and 17% increase, respectively. With increase of the concentration of AQS, the stimulating effect on reduction of amaranth decreased correspondingly. Furthermore, the anaerobic azoreduction by S12 was completely inhibited in the presence of 4 or 5 mmol/l of AQS. The effects of AQS on anaerobic reduction of azo dyes such as Acid Great Red, Metanil Yellow, Orange G, Orange II, and Xylidine Ponceau were similar to that of amaranth (Table 1). With the increase of the concentration of AQS, the rate of azoredcution was decrease correspondingly. When the concentration of AQS was higher 5 mmol/l, anaerobic azoreduction by S12 was inhibited strongly.

Fig. 5B revealed the effect of AQDS on anaerobic azoreduction. After 12 h incubation, 1 mmol/l of amaranth was completely reduced in the presence of 1 mmol/l of AQDS, but there was only 23.6% reduction of same concentration amaranth in the absence of AQDS. The corresponding first-order rate constants were 50.6 μmol/h in the absence of AQDS and 111.1 μmol/h, 83.3 μmol/h, 66.7 μmol/h, 55.6 μmol/h, 47.6 μmol/h with 1, 3, 6, 9, and 12 mmol/l of AQDS, respectively. These values corresponded to a 133%, 75%, 40%, 17%, and no increase, respectively. The impacts of different concentration AQDS on azo dyes Acid Great Red, Metanil Yellow, Orange G, Orange II, and Xylidine Ponceau are shown in Table 1. Anaerobic azoreduction was accelerated in the presence of 3, 6, 9, or 12 mmol/l of AQDS. However, the acceleration declined with increase of the concentration of AQDS.

Rate of Electron Transfer to AQS, AQDS, and Amaranth Under Anaerobic Condition

Because *S. decolorationis* S12 can reduce both humics and azo dyes with various organic substances or H_2 as electron donor, it is necessary to determine the rate of electron transfer to AQS, AQDS, and amaranth under anaerobic condition. Table 2 shows the result of electron transfer rate experiments. The electron transfer rates of AQS and AQDS were 1,260±120 and 1,680±290 nmol of electrons/min/mg of protein, respectively. The electron transfer rate of amaranth was 2,220±390 nmol of electrons/min/mg of protein.

Toxic Test for AQDS and AQS

Growth of S12 exposed to 1 mmol/l of AQS did not significantly differ from that of the humics-free control, but the growth of S12 was completely inhibited when exposed to more than 5 mmol/l of AQS (Fig. 6A), indicating that AQS is a toxic compound affecting the metabolism and growth of S12 cell. However, no growth inhibition was observed even though AQDS was up to 20 mmol/l (Fig. 6B). Over a period of 5 d of aerobic incubation at room temperature,

Table 2. Rate of electron transfer to AQS, AQDS, and amaranth under anaerobic condition.

Electron acceptor	Number of electrons transferred	Rate of electron transfer (nmol of electrons/min/mg of protein)[a,b]
AQS	2	1,260±120
AQDS	2	1,680±290
Amaranth	4	2,220±390

[a]The rates were determined by assuming that 4 mol electrons were transferred for each 1 mol amaranth reduction, and 2 mol electrons were transferred for each 1 mol AQS and AQDS.

[b]The protein content of cell was determined with bovine serum albumin as a standard.

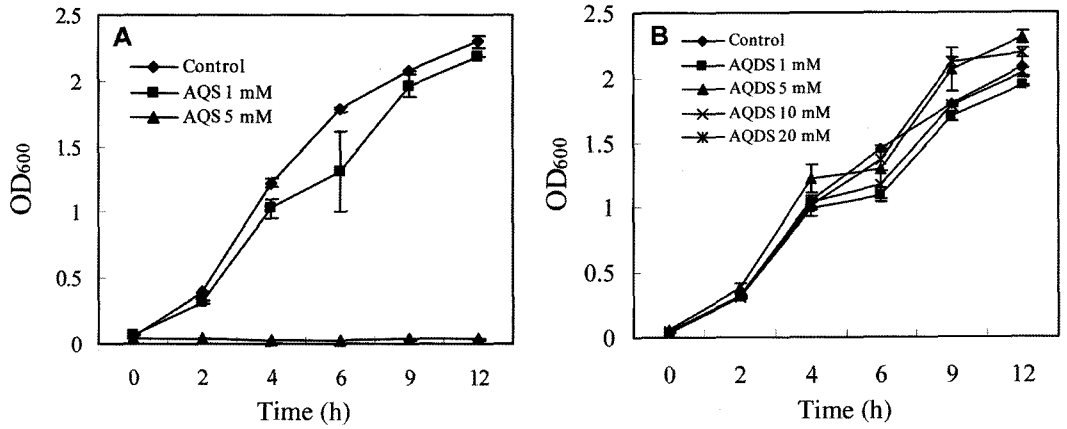

Fig. 6. Toxic effects of AQS (**A**) and AQDS (**B**) on growth of *S. decolorationis* S12.
The experiments were performed in LB medium at 32°C. Error bars represent standard deviations of duplicate incubations.

viable cell numbers dropped at a fast rate in the culture containing 5 mmol/l of AQS. However, there was no significant decrease in the culture containing 20 mmol/l of AQDS.

Analysis of the Chemical Reduction of Amaranth by the Reduced AQS or AQDS

The incubation of a fixed concentration of AH_2QS or AH_2QDS (2 mmol/l) with varying concentrations of amaranth demonstrated that the chemical reduction of 1 mole amaranth required 2.1 or 2.4 mole AH_2QS or AH_2QDS, respectively (Fig. 7). Obviously, only one quinone molecule was involved in the reduction reaction. The reduction rate of amaranth with AH_2QS was higher than that with AH_2QDS. The reduction rate is related to the redox potential of AH_2QS (-225 mV) and AH_2QDS (-184 mV).

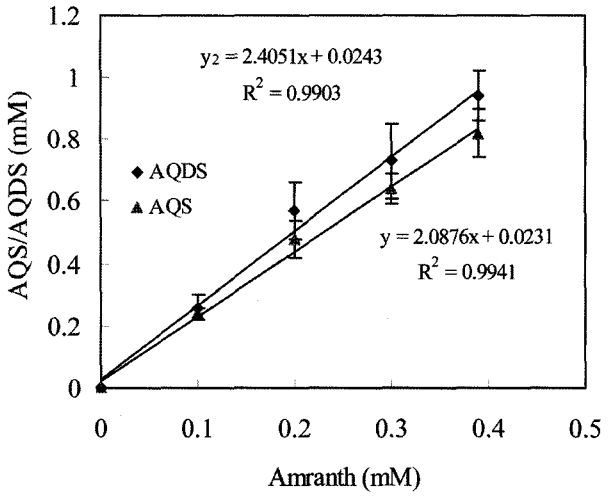

Fig. 7. Chemical reaction between reduced AQS or AQDS and amaranth.
Data are average from duplicate experiments. Error bars represent standard deviations of duplicate incubations.

It became evident that the AH_2QS was chemically a better reductant for the reduction of amaranth than AH_2QDS.

Discussion

Microbial humic reduction is an important form of anaerobic respiration of environmental relevance. Lovley *et al.* [18] firstly put forward the concept of humic respiration on evidence that *Geobacter metallireducens* and *Shewanella alga* both could grow with humics and AQDS as terminal electron acceptor when a wide variety of organic substrates or H_2 were oxidized. A great deal of research results showed that divers of microorganisms can use humics as electron acceptors for anaerobic growth. These bacterial humic reduction studies have been reviewed [10, 17]. Results of our study showed that strain S12 can also conserve energy for growth with humics as electron acceptor. The main evidence for this observation are outlined below: (i) humuic reduction is coupled to the oxidation of electron donors. When S12 was incubated with formate, lactate or H_2, reduction of AQDS or AQS was coupled to the oxidization of electron donor, whereas no reduction took place in controls where one of the ingredients (formate, lactate, or H_2) was omitted. (ii) The growth data provided here demonstrated that S12 is indeed capable of using AQDS as a terminal electron acceptor for energy conservation linked to the humic reduction under the growth condition used. It is evident that the microbial dissimilatory humic reduction by strain S12 is also a process of respiration.

Using an intermediate is an effective strategy to accelerate degradation of some environmental pollutants [12]. To date, there are many reports about the roles of humics in the microbial anaerobic azoreduction [10, 15, 16, 29, 36]. It was generally considered that humics can serve as redox mediators for microbial azoreduction. The proposed mechanism for

the redox mediator dependent reduction of azo compounds includes two independent reactions: first, the quinines (AQS, AQDS) are enzymatically reduced to the corresponding hydroquinone (AH_2QS, AH_2QDS) by organism; and second, the hydroquinones cleave the azo dyes in a purely chemical reaction [29]. Apart from the pure chemical reaction, however, there may be biological interaction between microbial anaerobic humic reduction and azoreduction, which was little known. Our previous study had shown that *S. decolorationis* S12 was capable of obtaining energy for growth, depending on the oxidation of various electron donors with azo dye as electron acceptor. As the previous study has shown, anaerobic azoreduction by strain S12 is a biochemical process that oxidizes the electron donors and transfers the electrons to the acceptors through a multi-component system related to the electron transport chain [14]. Thus, this strain provides a model to study the mutual interaction between dissimilatory humic reduction and azoreduction in a single microorganism.

Our results indicated that the low concentration of humics AQS and AQDS can effectively accelerate the reduction of azo dyes by strain S12. Strain S12 can transfer electrons from the oxidation of substrate to the electron acceptor, such as AQDS or AQS. The AH_2QS or AH_2QDS can then channel the electrons to azo dyes, as evidenced by the nonspecificity of the anaerobic azoreduction process; the AH_2QDS or AH_2QS was oxidized by the azo dye in a direct chemical reaction (Fig. 7). These observations suggest that a low concentration of AQS or AQDS can act as a redox mediator to stimulate the bacterial anaerobic azoreduction process.

However, the azoreduction was prominently inhibited when the concentration of AQS past a critical point of 5 mmol/l. Nonetheless, AQDS at 12 mmol/l could still have the acceleration effect, although the acceleration for azoreduction was gradually decreasing with the AQDS concentration increasing. These effects can be explained from two aspects: (i) the competition for electrons from primary electron donors between humic reduction and azoreduction had become stronger with the increase of the concentration of AQS or AQDS. Azo dyes accept electrons apart from redox mediator AQS or AQDS, mainly and directly from the organism. Because AQS, AQDS, and amaranth need to accept the electrons from a primary electron donor, there is inevitably competition for electrons between humic reduction and azoreduction. The rates of electron transfer to AQS, AQDS, and amaranth by strain S12 under anaerobic condition are determined by their standard redox potentials. The standard redox potential of AQS was closer to that of amaranth than AQDS [9], so the ability of competition for electron between AQS and amaranth was stronger when they were both present in the medium. When the concentration of AQS or AQDS was remarkably higher than that of azo dye, electrons are more apt to be transported to AQS or AQDS, resulting in the decrease of the azoredution rate. (ii) The toxicity of AQS or AQDS is an important factor effecting anaerobic azoreduction. Our result showed that AQS is toxic to the cell of strain S12, but AQDS had no effect on its growth. It seems likely that the inability of strain S12 to reduce azo dyes under the condition of high AQS concentration was due partly to its toxic effect. However, the decrease of azoreduction rate, when strain S12 was exposed to high concentration of AQDS, is not a result of its toxic effect but from the competition for electrons with azo dyes.

The addition of external electron donors is necessary to maintain the humic reduction. In the process of bacterial azoreduction, the accumulation of aromatic amines is certainly undesirable, because they also are more toxic environmental pollutants. The presence of humic substances, however, may contribute to attenuate the accumulation of these pollutants, since humics were shown to serve as electron acceptors, supporting the anaerobic oxidation of aromatic amines, such as aniline. Reduced humics can act as electron mediators to stimulate azo dye reduction.

In summary, our results indicated that the humics can serve as electron acceptors and redox mediators for bacterial anaerobic azoreduction. Low concentration of humics can stimulate the process of bacterial azoreduction owing to their electron shuttle function as redox mediator. However, a high concentration of humics can inhibit or slow the acceleration resulting from either toxicity or the competition affect. These findings may be significant for (i) understanding the link between bacterial dissimilatory azoreduction and humic reduction; and (ii) more rationally utilizing humics as a strategy for the treatment of azo dyes-containing waste-water and the remediation of environments contaminated by series of toxic organic compounds and azo dyes. We are currently exploring the molecular basis for understanding the link between bacterial dissimilatory humic reduction and azo reduction in depth.

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