Quinone-mediated decolorization of sulfonated azo dyes by cells and cell extracts from *Sphingomonas xenophaga*

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Abstract

The effects of various quinone compounds on the decolorization rates of sulfonated azo dyes by *Sphingomonas xenophaga* QYY were investigated. The results showed that anthraquinone-2-sulfonate (AQS) was the most effective redox mediator and AQS reduction was the rate-limited step of AQS-mediated decolorization of sulfonated azo dyes. Based on AQS biological toxicity tests, it was assumed that AQS might enter the cells and kill them. In the cytoplasmic extracts from strain QYY, AQS more effectively increased decolorization rates of sulfonated azo dyes than other quinone compounds. In addition, we found a NADH/FMN-dependent AQS reductase using nondenaturing polyacrylamide gel electrophoresis (Native-PAGE).

Key words: redox mediator; sulfonated azo dyes; *Sphingomonas xenophaga*; reductase

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Introduction

Azo dyes, characterized by one or more azo bonds (–N=N–), are extensively used in textile dyeing, paper, color photography, cosmetics and other industries (Robinson *et al*., 2001). Their discharge into open waters is problematic not only for aesthetic reason, but also some of these compounds and their decolorization products like aromatic amines are toxic and carcinogenic. Thus, the treatment of the effluents containing azo dyes has received much attention (Levine, 1991). In particular, biological processes have received more interests due to their cost-effectiveness, lower sludge production and environmental friendliness than physical and chemical methods. To date, various bacterial strains have developed enzyme systems for the decolorization and mineralization of azo dyes under aerobic, anaerobic and anoxic conditions (Banat *et al*., 1996; Bragger *et al*., 1997; Wuhrmann *et al*., 1980; Rafii *et al*., 1990; Stolz, 2001). Anaerobic decolorization is usually unspecific (Dos Santos *et al*., 2003). Moreover, compared with whole cells, cell extracts showed much higher decolorization rates. This has generally been assumed by the low permeability of the cell membranes for azo dyes, especially for sulfonated azo dyes (Russ *et al*., 2000).

It was reported that the addition of anthraquinone-2-sulfonate (AQS) greatly enhanced the decolorization rates of sulfonated azo dyes by *Sphingomonas xenophaga* BN6 under anaerobic condition (Kudlich *et al*., 1997). In this process, AQS acted as a redox mediators and was reduced

by quinone reductase, resulting in the formation of hydroquinone which subsequently reduced sulfonated azo dyes in a purely chemical redox reaction. Other quinone compounds, such as anthraquinone-2,6-disulfonate (AQDS), 2-hydroxy-1,4-naphthoquinone (lawsone), are also capable of increasing the decolorization rates of azo dyes under anaerobic conditions (Van der Zee *et al*., 2001). The effects of AQS and lawsone on the decolorization rate of sulfonated azo dyes by *S. xenophaga* BN6 and *Escherichia coli* K12 have been studied in great detail. Kudlich *et al*. (1997) demonstrated that the major AQS-reducing activity was presented in the cell membranes of strain BN6. However, Rau and Stolz (2003) reported that the lawsone reducing activity observed with whole cells of *E. coil* was caused by a soluble cytoplasmic enzyme. Shyu *et al*. (2002) showed that AQDS could enter *Shewanella oneidensis* MR-1 through genetic study. Thus, the location of quinone-reducing activity has not been fully understood.

In this study, quinone-mediated decolorization of sulfonated azo dyes by cells and cell extracts from *Sphingomonas xenophaga* QYY were investigated to further understand the effects of quinone reductases on the decolorization. It was also studied on the biological toxicity of effective redox mediator (AQS) to strain QYY for decolorization.

1 Materials and methods

1.1 Chemicals

Acid Red B (C.I.14720), Acid Red GR (C.I.27290),

1-aminoanthraquinone-2-sulfonic acid (ASA-2), and 1-amino-4-bromoanthraquinone-2-sulfonic acid (BAA) were obtained from Dye Synthesis Laboratory of Dalian University of Technology, China, and were purified before use. Acid Red 3R (C.I.16255), Amaranth (C.I.16185), AQS, AQDS, methanol, acetic acid, dibutylammonium, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma (USA). Bovine serum albumin (BSA) was purchased from TaKaRa Dalian (China). All other reagents used were of analytical grade.

1.2 Bacterial strain and cultivation conditions

Sphingomonas xenophaga QYY isolated from sludge samples in our previous study (Qu *et al.*, 2005) was used in this study. It was deposited in the China General Microorganism Culture Center (CGMCC) with the accession number 1172 (16S rDNA GenBank accession number AY611716). The composition of Luria-Bertani (LB) medium has been described previously (Qu *et al.*, 2005). The concentration of BAA in LB medium (LBB medium) was 0.2 g/L, LBB medium was autoclaved at 121°C for 20 min. The minimal medium used in this study contained (g/L): NaCl 1, NH₄Cl 1, $K_2HPO_4 \cdot H_2O$ 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, glucose 4 (pH 8.0). This medium was autoclaved at 115°C for 20 min.

1.3 Anthraquinone compounds mediated decolorization of sulfonated azo dyes by strain QYY

The strain was cultivated in 250 mL Erlenmeyer flasks containing 50 mL LBB medium at 150 r/min. Different anthraquinone compounds (0.05 mmol/L) were added to the minimal medium. The initial concentration of sulfonated azo dyes was 0.2 mmol/L. The effects of anthraquinone compounds on the decolorization rates of sulfonated azo dyes were investigated. The assays were performed in triplicate and the mean values of the data were presented.

1.4 Determination of the UV-Vis spectra during reduction of AQS

The 0.3 mmol/L AQS was reduced by strain QYY for 4 h in the minimal medium at 30°C under anaerobic conditions. UV/Vis spectra of the culture supernatants at 0 and 4 h were compared.

1.5 Toxicity test

Under aerobic growth conditions, strain QYY grew in LB medium containing AQS (0–7 mmol/L). Toxic effect on the growth of *S. xenophaga* QYY was monitored by cell counting method and measuring optical density at 660 nm using UV/Vis spectrophotometer (JASCO V-560, Japan). The assays were performed in triplicate and the mean values of the data were presented.

1.6 Analytical methods

The cell concentration was measured by optical density (OD) at 660 nm using UV/Vis spectrophotometer. The relationship between the bacterial cell concentration and OD_{660} was 1.0, OD_{660} = 0.9008 g cell dry weight/L (R^2 = 0.992). The concentration of azo dye in the medium supernatants was detected using high performance liquid chromatography (HPLC) (Lu *et al.*, 2008a). The efficiency of color removal (R) was expressed as: $R = (C_0 - C_i)/C_0 \times 100\%$, where, C_0 and C_i were initial and residual concentrations of azo dye, respectively.

1.7 Preparation of cytoplasmic extracts

The cells of strain QYY were grown in LBB medium at 30°C. At the end of exponential phase, the cells were harvested by centrifugation at 8000 ×g for 10 min at 10°C. The cell pellet was washed twice with 0.85% (*W/V*) NaCl and resuspended in 10 mmol/L phosphate buffer containing 5 mmol/L MgCl₂, then disrupted by ultrasonicator at 225 W, 4°C for 30 min (Ultrasonic processor CPX 750, USA). After sonication, the unbroken cells and cell debris were removed by centrifugation at 12000 ×g for 20 min at 4°C. Then the supernatant was obtained by ultracentrifugation at 150000 ×g for 1 h at 4°C and used as intracellular crude enzyme (Cozier *et al.*, 1999).

1.8 Determination of protein content

The protein content of cell extracts was determined according to the method of Bradford (Lowry *et al.*, 1981) with BSA as standard.

1.9 Enzyme assays

Azo reductase activity was assayed by measuring the decrease in optical density at suitable wavelengths with a UV/Vis spectrophotometer at 30°C. A typical reaction mixture (2.0 mL) contained Tris-HCl (10 mmol/L, pH 7.5), 0.045 mmol/L amaranth, 0.2 mmol/L NADH, 0.025 mmol/L FMN, and a suitable amount of crude enzyme. The reaction was initiated by adding NADH. The molar absorption coefficient for amaranth at 520 nm (22.6 L/(mmol·cm)) was used.

The quinone reductase activity was assayed by measuring the decrease in optical density at 340 nm. A reaction mixture (2.0 mL) contained Tris-HCl solution (10 mmol/L, pH 7.5), 0.2 mmol/L of NADH, 0.005 mmol/L quinone compounds, 0.025 mmol/L FMN and a suitable amount of crude enzyme. The reaction was initiated by the addition of NADH. The molar absorption coefficient for NADH at 340 nm (6.22 L/(mmol·cm)) was used.

The assays were performed in triplicates and the mean values of the data were presented.

1.10 Nondenaturing polyacrylamide gel electrophoresis (Native-PAGE)

Nondenaturing gel electrophoresis was used for the detection of quinone reductase or azo reductase activities in cell extracts (Rafii and Cerniglia, 1990). The basic composition of the gel was the same as that used for the SDS-PAGE but under omission of SDS. The gel electrophoresis was performed under aerobic condition. Samples were electrophoresed slowly until they had entered the stacking gel, approximately 65 V for 30 min, after

which the voltage was increased to 128 V. Electrophoresis continued at 128 V until dye front had reached the end of gel (Leo *et al.*, 2002). The gels were then stained with Coomassie Brilliant Blue R250 or amaranth for 3 h. After finishing the coloration, the gels were transferred to the anaerobic incubation chamber and washed with anaerobic Tris-HCl buffer (10 mmol/L, pH 7.5). The buffer was carefully removed and the gel was covered with 40 mL Tris-HCl buffer (10 mmol/L, pH 7.5) and 0.1 mmol/L FMN. After 20 min flushing with nitrogen gas, 1 mmol/L NADH with or without 0.5 mmol/L AQS was added. The active protein bands became visible as destaining zones in the gels after 30 min of incubation.

2 Results and discussion

2.1 Effects of different anthraquinone compounds on decolorization of sulfonated azo dyes by cells

It has been reported that strain QYY was not only capable of degrading BAA and ASA-2 under aerobic conditions, but also decolorizing sulfonated azo dyes under anaerobic conditions (Lu *et al.*, 2008b; Qu *et al.*, 2005). It was also demonstrated that the reduction rates of sulfonated azo dyes by *S. xenophaga* BN6 can be enhanced considerably by AQS and AQDS (Kudlich *et al.*, 1997). As the chemical structures of BAA and ASA-2 were similar with that of AQS, we decided to investigate the effects of these anthraquinone compounds on the decolorization rates of sulfonated azo dyes by strain QYY. The results demonstrated that these quinone compounds are effective redox mediators and increased the decolorization rates of sulfonated azo dyes. The results also showed that AQS was more effective than others. AQS mediated decolorization rate of Acid Red B by strain QYY was the largest among sulfonated azo dyes tested (Table 1).

2.2 Rate-limited step of AQS-mediated decolorization of Acid Red B

Rau *et al.* (2002) reported that the proposed mechanism for quinone-mediated reduction of azo dyes consisted of two independent reaction steps: first, the quinones are reduced by quinone reductase to the corresponding hydroquinones; second, the hydroquinones reductively cleave azo bond in a purely chemical redox reaction. In order to determine the rate-limiting step of AQS-mediated decolorization of Acid Red B, each reaction step was analyzed

and decolorization efficiencies of Acid Red B under different conditions were compared. As shown in Table 2, when strain QYY, Acid Red B, and AQS were mixed together at the beginning of the reaction, the decolorization efficiency was only 4.23% at 0.17 h, 30°C. However, when Acid Red B was added after strain QYY and AQS were mixed for 4 h at 30°C, the color removal efficiency was 52.36% at room temperature. Moreover, the reduced form of AQS (Fig. 1) was detected and the spectrum was consistent with the previous report. These results indicated that the enzymatic reduction of AQS was the rate-limiting step.

Thus, it was important to investigate the quinone reductases from strain QYY for quinone-mediated decolorization of sulfonated azo dyes.

2.3 Biological toxicity test of AQS to strain QYY

Previous studies have shown that AQDS was able to enter *Shewanella oneidensis* MR-1 (Shyu *et al.*, 2002). We further investigated whether AQS could enter *S. xenophaga* QYY from the view of biological toxicity. The results demonstrated that AQS was toxic at high level. As shown in Fig. 2, when the concentration of AQS was above 3 mmol/L, the growth of strain QYY was greatly inhibited. However, at low concentrations (below 0.5 mmol/L), no obvious inhibition was observed. In a separate experiment, strain QYY was resuspended in a nongrowth medium containing 7 mmol/L AQS and incubated aerobically at room temperature. Non-growth medium without AQS was used as control. The results showed that the cell numbers in the nongrowth medium containing AQS dropped faster than that without AQS. At the same time, the concentration of AQS in the culture supernatants decreased (Table 3). This phenomenon was similar with that reported by Shyu *et al.* (2002).

Table 1   Effects of anthraquinone compounds on the decolorization rates of sulfonated azo dyes*

Decolorization rate (μmol/(g·h))	Acid Red B	Acid Red 3R	Amaranth	Acid Red GR
Control	36.11	6.24	2.73	13.90
AQS	70.96	39.1	24.87	55.76
AQDS	54.72	17.69	15.24	44.65
ASA-2	52.08	10.12	4.55	31.65
BAA	37.60	8.29	3.68	21.08

* The concentrations of anthraquinone compounds and azo dyes were 0.05 and 0.2 mmol/L, respectively; the assays were performed in triplicate and the mean values were presented.

Table 2   Decolorization rate of Acid Red B under different conditions*

Reaction time	Color removal (%) under different conditions			
	Acid Red B[a]	Strain QYY Acid Red B[a]	Strain QYY Acid Red B[a] AQS[a]	Strain QYY AQS[a,b]
0.17 h	0	0.63	4.23	52.36

* The assays were performed in triplicate and the mean values were presented. [a] The final concentrations of Acid Red B and AQS were 0.15 and 0.3 mmol/L, respectively. [b] Strains QYY and AQS were mixed for 4 h at 30°C, then Acid Red B was added at room temperature.
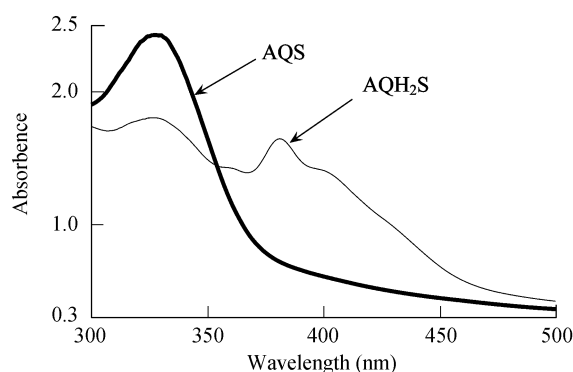

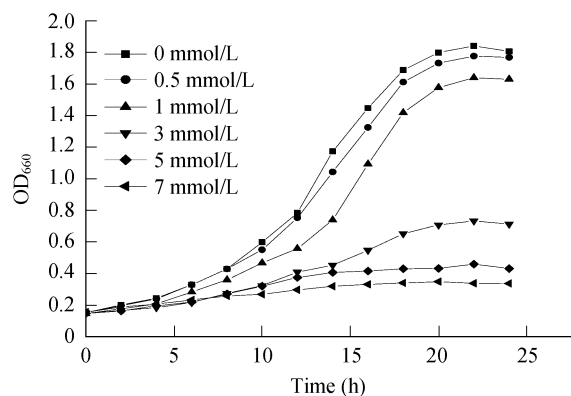**Fig. 1** UV/Vis spectra of AQS (solid line) and the reduced form of AQS (thin line).

Fig. 2 Effect of the concentration of AQS on the growth of strain QYY in LB medium under aerobic condition.

Table 3 Cell numbers and AQS concentration in different mediums*

Time (d)	0	2	3
Control	1.23×10^{10}	1.12×10^{10}	5.1×10^{9}
Medium with AQS	1.48×10^{10}	4.41×10^{9}	2.5×10^{8}
Concentrations of AQS (mmol/L)	7.01	6.04	5.84

* The assays were performed in triplicates and the mean values were presented.

Based on the results, it was asssumed that AQS probably penetrated the cell membranes and caused cells to die. At low concentration, however, the intracellular quinone reductases might play an important role in eliminating the toxicity. To answer this question, it was necessary to study the enzyme systems of the cytoplasmic extracts from strain QYY.

2.4 Flavin-dependent azo reductase of cell extracts

It was previously suggested that unspecific azo reductases acted via reduced flavin (Walker, 1970). This hypothesis has been proved by photochemical method (Fujita and Peisach, 1982). Flavin reductases can be subdivided into three groups according to their electron donors: NADH-preferring flavin reductases (FRD), NADPH-preferring flavin reductases (FRP), and general flavin reductases (FRG) (Tu, 2001). Up to now, most of the azo reducatases reported required FMN. However, a soluble FAD-dependent azo reductase was found in the cell extracts from strain BN6 (Haug *et al.*, 1991). To identify which form is involved with strain QYY, the effects of FMN, FAD, NADH, and NADPH on the decolorization of amaranth by the cytoplasmic extracts from strain QYY were investigated. The results showed that the major azo reductases in the cytoplasmic extracts were FMN/NADH-dependent azo reductases (Table 4). Without FMN, azo reductase activities can not be detected in the cytoplasmic extracts.

2.5 Effects of different quinones on the reduction of amaranth

There have been many studies on the role of redox mediators, such as AQS, AQDS and lawsone, in azo bond reduction by bacteria under anaerobic conditions (Kudlich *et al.*, 1997; Van der Zee *et al.*, 2001; Dos Santos *et al.*, 2003). It was recently suggested that quinone compounds

Table 4 Activity of azo reductases with different coenzymes*

Cofactor	Reduction rate (μmol/(min·g protein))	
	FMN (0.025 mmol/L)	FAD (0.025 mmol/L)
NADH (0.2 mmol/L)	3.02	0.29
NADPH (0.2 mmol/L)	0.64	0.06

* The assays were performed in triplicates and the mean values were presented.

with standard redox potentials (E'_0) between approximately –320 and –50 mV could function as effective redox mediators in the reduction of sulfonated azo dyes (Rau *et al.*, 2002). Therefore, we choose some quinone compounds to study their effects on the decolorization of amaranth by the cytoplasmic extracts from strain QYY. The results demonstrated that these anthraquinone compounds increased the decolorization rates of amaranth. Furthermore, anthraquinone compounds with a more negative redox potential led to higher reaction rate. As shown in Table 5, the addition of AQS (5 μmol/L) resulted in the highest decolorization rate of amaranth (4.88 μmol/(min·g protein)). Without FMN, amaranth was not reduced by the cytoplasmic extracts under the same conditions. Thus, it was reasonable to believe FMN-dependent quinone reductases exist in the cytoplasmic extracts from strain QYY.

2.6 Identification of quinone reductase in the cell extracts from strain QYY

Quinone reductases in the cytoplasmic extracts from strain QYY were further investigated. The results (Fig. 3) showed that the cytoplasmic extracts reduced AQS and AQDS. Without FMN, quinone reductase activities under the same conditions can not be detected. This indicated that the cytoplasmic extracts might contain some FMN-dependent quinone reductases. Moreover, the major enzyme was FMN-dependent AQS reductase.

2.7 Visualization of azo reductase and quinone reductase by Native-PAGE

FMN-dependent azo reductases and quinone reductases were visualized by Native-PAGE. After Native-PAGE, the proteins in lanes 1 and 2 were firstly treated with amaranth, then with FMN and NADH under anaerobic conditions. The results (Fig. 4) showed that only one distinct protein band was detected in lane 1, which also appeared in lane 2. This result indicated that a single protein was likely involved in the reduction of azo dyes. However, when the

Table 5 Effects of different quinones on the decolorization rates of amaranth by the cytoplasmic extracts*

Quinonesa	E'_0 (mV)	Decolorization rate (μmol/(min·g protein))
Control	/	3.02
AQS	–225	4.88
AQDS	–184	3.17
Lawsone	–137	2.36

* The assays were performed in triplicate and the mean values were presented; the final concentration of each quinone in the reaction was 5 μmol/L.
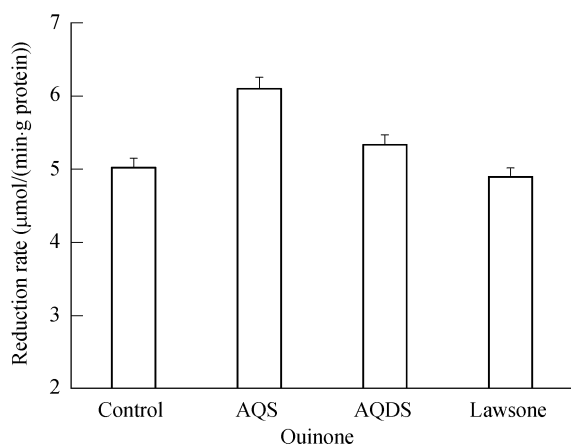
Fig. 3 Quinone reductase activity in the cell extracts from strain QYY. Error bars indicate the standard deviation obtained in triplicate experiments.

proteins in lane 2 were further treated with AQS under anaerobic conditions, another distinct decolorization band appeared. This result strongly indicated that there exist a FMN-dependent azo reductase and also a FMN-dependent AQS reductase in the cell extracts from strain QYY.
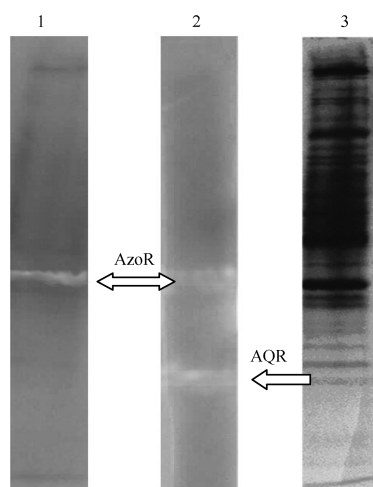

Fig. 4 Detection of azo reductase (AzoR) and AQS reductase (AQR) in the cell extracts from strain QYY by nondenaturing gel electrophoresis. Protein 0.21 mg protein was transferred to each lane of anondenaturing polyacrylamide gel (10% (*W/V*) polyacrylamide). After electrophoresis, the protein lanes were separated and two lanes were stained with amaranth (3 mmol/L); one lane was stained with Coomassie Brilliant Blue R250. After 3 h, lane 1 was transferred to an anaerobic chamber 1 and lane 2 was transferred to an anaerobic chamber 2. The proteins in lane 1 were treated with FMN (0.1 mmol/L) and NADH (1 mmol/L); the proteins in lane 2 were treated with FMN (0.1 mmol/L), AQS (0.5 mmol/L), and NADH (1 mmol/L). The reductase activities were detected by the decolorization of the amaranth stained gels. Lane 3 showed all soluble proteins under aerobic conditions with Coomassie Brilliant Blue R250.

3 Conclusions

In the present work, we demonstrated that AQS, AQDS, BAA, and ASA-2 increased the specific decolorization rates of sulfonated azo dyes by *Sphingomonas xenophaga* QYY. The results demonstrated that AQS was the most

effective redox mediator among the quinone compounds tested. Further research indicated that AQS reduction was the rate-limiting step of AQS-mediated decolorization of Acid Red B. Moreover, when concentration is higher than 3 mmol/L AQS inhibited the growth of strain QYY. In the cytoplasmic extracts from strain QYY greatly, AQS was more effective to increase decolorization rate of amaranth than other quinone compounds tested. Furthermore, a NADH/FMN-dependent azo reductase and a NADH/FMN-dependent AQS reductase were found by Native-PAGE.

The results of AQS biological toxicity tests indicated that AQS probably entered the cells of strain QYY. More evidence is needed and the further research on the function of AQS reductase is currently underway in our laboratory.

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