**Bioelectrochemical reduction of an azo dye by a *Shewanella oneidensis*
MR-1 formed biocathode**

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Abstract

Presently there is great interest to develop pure culture cathodes in bioelectrochemical systems (BES) for achieving decolorization/reduction of azo dyes. In this study, we investigated the decolorization of a model azo dye, acid orange 7 (AO7) in a biocathode inoculated with a model electrogenic microorganism *Shewanella oneidensis* MR-1 (MR-1). The decolorization efficiency of AO7 reached $95.8\pm4.6\%$, $86.4\pm2.0\%$, and $77.8\pm1.0\%$ in 46 h in the biocathode fed with lactate, in the biocathode without lactate, and in the abiotic cathode respectively. Thus, enhanced decolorization of AO7 occurred in the biocathode, compared to the abiotic cathode, regardless of the presence of lactate. To further investigate the AO7 reduction mechanism and electron transfer between the electrode and *S. oneidensis*, the cathode potential was controlled such that hydrogen was not produced. In the absence of lactate, the AO7 decolorization efficiency ($78.5\pm0.8\%$) and sulfanilic acid (SA) production efficiency ($70.9\pm1.5\%$) in the biocathode were higher than the AO7 decolorization efficiency ($63.7\pm3.6\%$) and the SA production ($56.1\pm1.9\%$) efficiency in the abiotic cathode. This suggests that the reduction of AO7, with electrode as the sole electron donor, was enhanced by electron transfer between the cathode and the *S. oneidensis* formed biofilm on the cathode surface. This study demonstrated that *S. oneidensis* enhanced the capture of electrons from the cathode electrode for the reduction of the organic pollutant AO7.

Keywords: AO7 reduction; biocathode; Bioelectrochemical systems; *Shewanella oneidensis* MR-1

1, Introduction

Azo dyes are compounds with aromatic structures characterized by containing one or more azo linkages (-N=N-) and they are widely used in the textile, leather, cosmetics, and food industries (Elisangela et al. 2009, Tony et al. 2009). Azo dyes can be water or oil soluble, and the presence of a sulfonated group results in the compounds being highly water-soluble. Consequently, azo dyes containing these groups are easily mobilized into water systems during production and applications (Tan et al. 2005). Environmental contamination by these azo dyes even at very low concentrations can cause water streams to become highly colored and give rise to undesirable aesthetic effects (Pandey et al. 2007, Lončar et al. 2012, Singh et al. 2015).

Azo dyes are generally persistent under aerobic conditions (Tan et al. 2013). However, under anaerobic conditions azo dyes can be reduced and decolorized, with the breakdown of azo bonds producing products that are easily mineralized (Tan et al. 2005, Wang et al. 2011). This anaerobic decolorization is a cost efficient process; however, it is usually very slow and requires an organic co-substrate to provide electrons for the reduction (dos Santos et al. 2005). The addition of the co-substrate adds to the costs and unwanted methane production since the dosage typically far exceeds the stoichiometric requirements (Van der Zee and Villaverde 2005). Thus, treatment of wastewater containing azo dyes is a challenging issue that requires improvement. Acid orange 7 (AO7) is an azo dye that is widely used in the textile, food and cosmetics industries, thus it is a significant component of wastewaters produced by these manufacturers that requires removal (Mu et al. 2009).

Recently, wastewater treatment capabilities of bioelectrochemical systems (BES) have been extensively explored for degradation of a number of organic and inorganic contaminants (Wang et al. 2015). Contaminants such as nitroaromatics (Wang et al. 2011, Liang et al. 2014, Feng et al. 2015), azo dyes (Liu et al. 2015), and halogenated aromatics (Liang et al. 2013, Kong et al. 2014) can be reductively eliminated under anaerobic conditions with acclimated biocathode communities. However, the biological reduction mechanisms of the pollutants at the biocathode are unclear and such details are required for optimization of the treatment processes. Bacterial reduction of azo dyes has been investigated using various pure cultures under cathodic conditions while adding specific carbon sources. Mostly these pure cultures are of the *Shewanella* genus and include *Shewanella decolorationis* S12 (Hong et al. 2007, Hong and Gu 2010, Yang et al. 2015), *Shewanella* sp. J18143 (Pearce et al. 2006), *S. oneidensis* (Pearce et al. 2006) as well as *Pseudomonas* sp. WYZ-2 (Wang et al. 2014). However, mostly these studies focused on the performance of the azo dye removal and the BES conditions rather than on determining details of the cathode electron transfer mechanisms for the reduction of the dye.

S. oneidensis exhibits very diverse respiratory capabilities. In addition to aerobic respiration it can conduct anaerobic respiration using a range of electron acceptors that include fumarate (Rosenbaum et al. 2011), nitrobenzene (Cai et al. 2012), Cr(VI) (Belchik et al. 2011), Fe(III) citrate (Rosenbaum et al. 2012), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Perreault et al. 2012) as well as azo dyes such as Naphthol Green B (Xiao et al. 2012). As a consequence of these diverse respiratory capabilities,

Shewanella species are often used in electrogenic studies for the reduction of organic pollutants in BES (Rosenbaum et al. 2012).

In this study, a dual chamber BES was used for the removal of azo dye AO7 at a biocathode in the presence of *S. oneidensis*. Removal efficiency of AO7 was compared between biocathodes in the presence and absence of the co-substrate lactate, as well as between the biocathode and the abiotic cathode. This work supports the feasibility of AO7 reduction mediated by the cathodic biofilm of *S. oneidensis*. To our best knowledge, this is the first report to demonstrate that *S. oneidensis* MR-1 enhances cathodic electron capture for the reduction of the organic pollutant AO7.

2. Materials and methods

2.1 Anode and cathode inoculum

The anodic microbial community was enriched from domestic wastewater that was used to inoculate to the BES as previously described (Wang et al. 2011). Sodium acetate was added to the anodic medium to ensure excess electron donor. A pure cultures of *S. oneidensis* was used to form biofilms in the cathodic chamber. *S. oneidensis* from glycerol stocks was firstly incubated in Luria-Bertani (LB) medium at 30°C, shaken at 150 rpm overnight. One loop of bacterial suspension was then streaked onto a LB plate and incubated overnight at 30°C. From the plate, one pink colony was transferred to liquid LB medium and incubated at 30°C, shaken at 150 rpm overnight. This suspension was used as the BES cathode inoculum. The cathode medium consisted of a nutrient solution that included Wolf's vitamins and trace elements (Wang et al. 2011). Additionally, when included in the cathode medium batch tests, lactate was added at 10

mM and AO7 was added at 0.28 mM in the presence of lactate and 0.147 mM in the absence of lactate. The cathodic medium was prepared by boiling and purging with N₂ gas (99.9% purity) for 20 min before autoclaving at 121°C for 30min.

2.2 BES reactor set up

Dual chamber polycarbonate BES reactors were constructed and assembled, using graphite fiber brush and carbon cloth as the anode and cathode materials respectively, as previously described (Liang et al. 2013). The working volume of each chamber was 85 mL. The carbon cloth was immersed in 1 M hydrochloric acid for 24 h and then in deionized water for another 24 h before use. The assembled reactors, excluding the carbon cloth, were covered with aluminum foil and autoclaved at 121°C for 30 min. Following that, the carbon cloth was assembled into the BES in sterile conditions before starting the batch experiments. An external power of 0.3 V was applied to assist the AO7 reduction in the abiotic and biotic cathodes, unless otherwise mentioned.

All the BES reactors had the same anode conditions. For one set of reactors the cathode was inoculated with *S. oneidensis* while in another set there was no inoculum and the cathode was abiotic. The cathode inoculum was centrifuged at 5000 rpm for 3 min, the supernatant was discarded, and then the pellet was dissolved in sterile cathode medium before transferring it to the cathode chamber. This was incubated under anaerobic conditions for 48 h to allow biofilm to develop on the cathode. Subsequently, the cathode liquid was removed and the cathode chamber was replenished with another batch of freshly prepared MR-1 inoculum and cathode medium. This procedure was

repeated six times to achieve adequate biofilm development on the cathode surface (Liang et al. 2013).

Two BES reactors with well-developed cathode biofilms were operated for three repeated batch experiments under particular conditions with AO7 as the electron acceptor. For each batch experiment fresh media was placed in the anode and cathode chambers and the BES reactions were monitored. Each batch experiment lasted approximately 60-80 h and was terminated when the current had nearly stopped decreasing (closed circuit mode). For each reactor, the initial batch experiments were operated in closed circuit mode, and then further batch tests were performed with the two reactors switched to opened circuit, thus operated without external electron supply. Another two BES reactors were operated as abiotic cathodes for three cycles initially with closed circuit, and then as open circuit mode. A summary of the overall batch test experiment design is provided in the results. All the BES batch experiments were performed at room temperature ($26 \pm 2^{\circ}\text{C}$).

2.3 Chemicals analysis

During the batch experiments samples taken from the cathode were immediately filtered through a 0.22 μm filter, and AO7 concentrations were measured using UV-vis spectrophotometer (Cary 50, Varian Inc., Australia) at 485 nm (Mu et al. 2009) over the wavelength range of 200 to 750 nm. The reduced product sulfanilic acid (SA) of AO7 was quantified by HPLC. The reverse-phase HPLC system (model e2695, Waters Co., Milford, MA) was equipped with a Semmetry C18 column (5 μm ; 5×250 mm, Waters

Co., USA) and a UV detector was used for SA measurement at 254 nm as described previously (Mu et al. 2009).

2.4 Cyclic Voltammetry analysis and SEM detection

The cyclic voltammetry (CV) analysis was conducted using an electrochemical workstation (model-660D, CH Instruments Inc., Austin, TX, USA) equipped with a three-electrode system. Cyclic voltammograms were recorded with the scan rate of 5 mV S⁻¹. The abiotic cathode/biocathode and bioanode of the BES reactors were designated as the working and counter electrodes, respectively. The saturated calomel reference electrode (SCE, 0.247 V vs standard hydrogen electrode (SHE), model-217, Shanghai Precise. Sci. Instru. Co., Ltd., China) was inserted into the cathode chamber. Concentrations of AO7 and lactate were 0.147 mM and 10 mM, respectively. All the cathode potentials were reported against the SHE. The biocathode biofilms were visualized by scanning electron microscopy (SEM) (HELIOS Nanolab 600i, Hillsboro, OR, USA) at 20 kV as described elsewhere (Xie et al. 2011).

3. Results and Discussion

3.1 Biocathode formation in the dual-chamber BES

Prior to the AO7 reduction experiments biofilms were formed on the cathodes over about a 12 day period by repeatedly inoculating the BES. During this biocathode biofilm formation process, the current of the BES reactors increased over time (Fig. 1A). After 12 days the current had increased from around 2.0 mA to be stable at 4.7 mA, indicating the occurrence of a stable MR-1 biofilm on the cathodic electrode (Fig. 1A). This was evident by scanning electron microscopy images of the cathode in the

191 absence of lactate after the biofilm formation period (Fig. 2 A&B). The biofilm on
192 cathode is not quite thick due to at least two possible reasons: 1) all the experiments
193 were conducted under strictly anaerobic conditions which produce much less biomass
194 than that of aerobic conditions; 2) electrode served as the potential sole electrode donor
195 and inorganic bicarbonate as the carbon source in this study.

196
197 For the last two periods of catholyte replacement, further inoculum of *S. oneidensis*
198 was not used. As expected AO7 (absorbance at 484 nm) and SA (absorbance at 254 nm)
199 were not detected at the end of batch test (Fig. 1B), indicating the reduction of AO7 in
200 cathode chamber.

201 202 3.2 Reduction of AO7 in the BES

203 The batch experiments for BES AO7 reduction were conducted in three groups: Group
204 I: AO7 removal in the presence of the co-substrate lactate; Group II: AO7 removal in
205 the absence of lactate; and Group III: AO7 removal without lactate with the cathode
206 potential held at -408 mV to avoid hydrogen production (vs. SHE). It was seen that
207 current was produced in the batch experiments in the presence or absence of lactate
208 (Fig. 3 A and B). However it was higher and more rapidly declined in the presence of
209 lactate. The reduction in current during the batch tests (Fig. 3) was likely due to the
210 decreasing levels of AO7 as it was being reduced. Electrode potentials and current
211 generation of cathode were recorded to evaluate the performance of the BES reactors
212 (Fig. 3). The cathode potentials were almost the same for the biocathode and abiotic
213 cathode (Fig. 3A and B). This was possibly due to the similar conditions in anode
214 chamber which resulted in a similar anode potential, then similar cathode potentials

occurred with the same applied extra power voltage. Nevertheless, current generation in the closed circuit biocathode in the presence of lactate was higher than that of the closed circuit biocathode in the absence of lactate (Fig. 3A and B). The higher the current in the closed circuit biocathode, the more efficient the electrons transfer from electrons to *S. oneidensis*, and the more efficient the AO7 reduction.

In Group I batch experiments there were three modes of reactor operation: (i) biocathode BES operated with a closed circuit and an external power source of 0.3 V (this called the closed circuit biocathode), (ii) biocathode BES operated as an opened circuit (opened circuit biocathode), and (iii) BES having an abiotic cathode with a closed circuit and an external power source of 0.3 V (abiotic cathode). For all three modes, the initial cathode AO7 and lactate concentrations were 0.285 and 10 mM, respectively.

In the batch experiments the reduction of AO7 was observed in all three modes with the highest removal obtained in the closed circuit biocathode (Fig. 4 A, C and E). The removal efficiency of AO7 in the closed circuit biocathode, opened circuit biocathode and abiotic cathode were $95.8 \pm 4.7\%$, $73.0 \pm 5.9\%$ and $77.8 \pm 1.0\%$, respectively after 46 h incubation. The removal efficiency of AO7 in the abiotic cathode was higher than that of the opened circuit biocathode, indicating that the electron supply from the anode and applied external power supply of 0.3 V did promote the AO7 reduction (Fig. 4A). SA is a product of AO7 reduction (Bay et al. 2014) and its production was evident during the removal of AO7 (Fig. 1B), consequently supporting that AO7 reduction occurred in the BES. SA was detected in all three modes (closed circuit biocathode, opened circuit

biocathode and abiotic cathode) (Fig. 4B), and its production was inversely correlated with AO7 level. So the production rate of SA in the biocathode was much higher than the opened circuit and the abiotic cathodes. The production efficiency of SA in the closed circuit biocathode, opened circuit biocathode and abiotic cathode at 46 h were $95.2\pm0.6\%$, $60.1\pm5.9\%$ and $41.7\pm18.5\%$, respectively. The removal efficiency of AO7 was consistent with the production efficiency of SA, this also indicating that there was no consumption of SA in the biocathode when lactate was supplied. Moreover, the kinetic constants under different operating conditions were further compared to determine whether the biocathode enhanced AO7 reduction due to the fact that different initial AO7 concentration was applied for Group I (in the presence of lactate) and Group II & III (in the absence of lactate) experiment (Table 1). The kinetic constant for closed circuit biocathode of Group I was 0.063 ± 0.002 , which was higher than 0.0479 ± 0.0035 in closed circuit biocathode of Group II. However, the kinetics constant for closed circuit abiotic cathode was comparable in Group I and Group II, which was 0.0272 ± 0.0027 and 0.0324 ± 0.002 respectively, although the initial AO7 concentration was different. Also it is obvious that the kinetics constant (0.0479 ± 0.0035) of biocathode in Group II was higher than that (0.0324 ± 0.002) of abiotic cathode in Group II. In conclusion, the presence of lactate enhanced the AO7 reduction in the cathode chamber and AO7 could be more efficiently reduced in the biocathode rather than in the abiotic cathode.

The electrons for the AO7 reduction by the MR-1 biocathode in Group I were provided by the anode and the lactate in the catholyte. In contrast, the AO7 removal and SA production in both the opened circuit biocathode and the abiotic cathode were much

lower than that detected for the closed circuit biocathode (Fig. 4A and B), thus, indicating that the biofilm was critical for the BES enhanced reduction of AO7.

To further investigate whether *S. oneidensis* could enhance the reduction of AO7 with the cathode as the sole electron donor, BES batch experiments were performed in the absence of lactate in the cathode (Group II). Two different modes were tested for comparison in Group II: the biocathode and the abiotic cathode. For both modes, the initial AO7 concentration was around 0.14 mM and this was reduced during the BES test, while simultaneous production of SA occurred (Fig. 4C and D). Although, these activities of AO7 reduction and SA production were somewhat greater for the biocathode in comparison to the abiotic cathode, indicating that the cells were enhancing the AO7 reduction while using electrons provided from the cathode. However, in comparison to Group I (Fig. 4A and B), the AO7 reduction efficiencies under the Group II conditions were much lower. For example, $93.6 \pm 1.0\%$ of AO7 was reduced in the biocathode without lactate within 120 h (Fig. 4C), while $95.16 \pm 0.59\%$ of AO7 was reduced in the biocathode in the presence of 10 mM lactate within 46 h (Fig. 4A). Consequently, it was evident that lactate was also important for providing electrons for the BES biocathode reduction of AO7.

In BES hydrogen gas can be produced at the cathode and this can facilitate electron transfer from the electrode to the bacteria (Croese et al. 2011). Experiments were conducted to determine whether H_2 was influencing the AO7 reduction (Group III). These were performed by controlling the cathode potential at -408mV (vs. SHE), at which hydrogen gas was not produced (Wang et al. 2011). In the absence of the external

electron donor and the possible electron facilitator, H_2 , in the biocathode, AO7 reduction was faster than that of in the abiotic cathode (Fig. 4E). Thus, indicating that *S. oneidensis* could still enhance the reduction of AO7 without the electron facilitator. At 120 h, the AO7 reduction efficiency of the biocathode in the Group III experiment was around $92.1 \pm 0.3\%$ (Fig. 4E), which was close to that of $93.6 \pm 1.0\%$ in the biocathode in Group II (Fig. 4C), demonstrating that H_2 had a negligible effect on AO7 reduction by *S. oneidensis*. Additionally, the production profiles of SA (Fig. 4F) were in good agreement with the reduction profiles of AO7 (Fig. 4E) and supported the conclusions drawn here. It was also seen that when the cathode hydrogen production was limited, the current generation of the biocathode was higher than that of the abiotic cathode (Fig. 3C), this further supporting that *S. oneidensis* could accelerate the AO7 reduction in the absence of the electron transfer facilitator.

3.3 Role of *S. oneidensis* in the biocathodic reduction of AO7

CV analysis of the closed biocathode and abiotic cathode in the presence of AO7 was performed (Fig. 5). In comparison to the CV profile of the abiotic cathode, the profile of the closed circuit biocathode has more positive onset potentials together with greater levels of cathodic current (Figure 5). This demonstrates that the AO7 reduction by *S. oneidensis* contributed to the enhanced current. The AO7 reduction peaks occurred at about $-0.66V$ for the closed circuit biocathodes and at about $-0.75V$ for the abiotic cathode (Fig. 5). The reduction peak of the closed circuit biocathode with lactate was higher than that of the closed circuit biocathode without lactate, thus indicating that the presence of the external electron donor facilitated the AO7 reduction. It has been reported that the biodegradation of AO7 was enhanced by *S. oneidensis* through co-

metabolism with the addition of pyruvate in microbial fuel cell (Fernando et al. 2012). Thus there is a possibility that AO7 could be co-metabolically reduced by *S. oneidensis* MR-1 through the addition of lactate. Moreover, the first-order kinetic constant of AO7 reduction decreased from around 0.25 to about 0.13 from when lactate is present to absent.

It has been proposed that these redox peaks result from the reversible reaction of the aromatic amine intermediates that form from the AO7 reduction (Mu et al. 2009, Kong et al. 2014). The variation in CV profiles on the closed circuit biocathode and abiotic cathode indicate that the electron transfer pathways are different, and this has been observed previously (Kong et al. 2014). From the CVs lower over-potentials for the AO7 reduction at the closed circuit biocathode were evident in comparison with that of the abiotic cathode, regardless of the presence of lactate. This also supports that the closed circuit biocathode was more efficiently catalyzing the AO7 reduction in comparison to the abiotic cathode.

4. Conclusions

This work demonstrated that the *S. oneidensis* formed biocathode could efficiently enhance the reduction of AO7 with the production of SA. Pure culture formed biocathode could offer an opportunity for unraveling the unclear electron transfer mechanisms between the electrode and microorganisms. Therefore, this study provides fundamental knowledge of the interaction between the cathodic electrode and microorganisms, thus benefiting the understanding of biocathodic electron transfer pathways and corresponding molecular mechanisms.

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344 **References**

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Table 1. Kinetic constants of AO7 reduction and SA production in the presence and absence of lactate

Initial (mM)	AO7 concentration	Kinetics constant of AO7 reduction (κ)	Correlation coefficient of AO7 reduction (R^2)	Final production concentration (mM)	SA	Kinetics constant of SA production (κ)	Correlation coefficient of SA production (R^2)
In the presence of lactate							
Closed circuit biocathode	0.2485±0.0038	0.063±0.002	0.9986	0.2565±0.0075		0.0503±0.004	0.994
Abiocathode	0.2531±0.0010	0.0272±0.0027	0.990	0.2351±0.0095		0.0171±0.0020	0.993
Open circuit biocathode	0.2500±0.0089	0.0239±0.0022	0.984	1.4561±2.0663		0.0021±0.0032	0.986
In the absence of lactate							
Closed circuit biocathode	0.1301±0.0049	0.0479±0.0035	0.9914	0.1254±0.0022		0.0467±0.0025	0.9973
Abiocathode	0.1286±0.0039	0.0324±0.002	0.9937	0.1050±0.0041		0.0396±0.0043	0.9901

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Figure 1. Current change during the biofilm formation of *S. oneidensis* on the BES cathodic electrode prior to use in the batch tests (A), and detection of AO7 in the cathode was evident only during the batch experiment (absorbance at 484 nm) (B).

Figure 2. Scanning electron microscopy (SEM) micrographs of the carbon cloth cathode in the BES after the *S. oneidensis* biofilm formation period, grown in the absence of lactate; at 10000 times (A) and 2000 times (B) amplification.

Figure 3. Cathodic potentials and current generation with lactate at the closed circuit biocathode (A), without lactate at the closed circuit biocathode (B), and when the cathode potential was controlled at -408 mV without lactate (vs SHE) to limit hydrogen gas production (C).

Figure 4. Reduction of AO7 and production of SA under different operational conditions in the batch BES tests: in the presence of lactate in three different operational modes (biocathode, opened circuit biocathode, abiotic cathode) (A and B); in the absence of lactate in two different operational modes (biocathode and abiotic cathode) (C and D); and in the absence of lactate when cathode potential was controlled at -408 mV (vs SHE) in two different operational modes (biocathode and abiotic cathode) (E and F).

Figure 5. Cyclic voltammograms of the BES biocathode with or without lactate, and of the abiotic cathode.

Figures