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Enhanced biotransformation of nitrobenzene by the synergies of *Shewanella* species and mediator-functionalized polyurethane foam

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HIGHLIGHTS

- *Shewanella* and AQS-PUF had synergistic effect on promoting nitrobenzene reduction.
- AQS-PUF can induce *Shewanella* species to secrete more flavins.
- EPS of *Shewanella* species was involved in direct bio-reduction of nitrobenzene.
- EPS could also interact with secreted flavins to mediate nitrobenzene bio-reduction.

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ABSTRACT

The performance and mechanism of anaerobic treatment of nitrobenzene using the combination of *Shewanella* species and anthraquinone-2-sulfonate-modified polyurethane foam (*Shewanella*/AQS-PUF) were investigated. The results showed that *Shewanella*/AQS-PUF significantly accelerated nitrobenzene bio-reduction (95.6%) and aniline formation (94.3%) with nitrobenzene removal rate up to 0.13 mM h^{-1}. Moreover, there were synergistic effects between *Shewanella* species and AQS-PUF on promoting nitrobenzene biotransformation with 5-fold increase in first-order rate constant compared to that without AQS-PUF. During this process, AQS-PUF could induce *Shewanella* species to secrete more flavins (0.335 μM) as redox mediator for nitrobenzene bio-reduction. Meanwhile, it was also found that the bound EPS of *Shewanella* species could act as biocatalyst for nitrobenzene reduction and the addition of flavins enhanced its catalytic activity. This indicated that the EPS of *Shewanella* species was not only involved in direct bio-reduction of nitrobenzene, but also interacted with secreted flavins to mediate nitrobenzene bio-reduction.

1. Introduction

Nitrobenzene is widely used in the production of anilines, dyes, explosives, pesticides and drugs [1,2]. As nitrobenzene is a highly toxic and recalcitrant chemical, it has been listed as a priority pollutant. The sequential anaerobic-aerobic biological methods were regarded as one of the most effective technologies for nitrobenzene wastewater treatments [3]. However, the anaerobic reduction of nitrobenzene was usually rate-limiting step in the conventional anaerobic process, resulting in lower biodegradation rate of nitrobenzene, and the presence of a more toxic metabolite nitrosobenzene in effluent [3,4]. Although it has been found that some quinone compounds such as anthraquinone-2,6-disulfonate (AQDS) and anthraquinone-2-sulfonate (AQS), could function as redox mediators and increase the anaerobic bio-reduction rates of nitroaromatics by accelerating the electron transfer from electron donor to terminal electron acceptor, continuous dosing of these quinone compounds during the practical application might result in the increase of running cost and the secondary pollution [5,6]. Therefore, it has been suggested that quinones as redox mediators need to be immobilized and then applied in anaerobic bioreactor for enhancing reductive biotransformation of xenobiotics including nitroaromatics. It was reported that AQDS adsorbed on anion exchange resins preserved its catalytic property, and could increase the rate of decolorization of azo dyes up to 8.8-fold compared to controls lacking quinones [5]. When AQDS was immobilized on activated carbon felt by electropolymerization, it could increase over 5-fold on the reduction rates of nitroaromatics [4]. In addition, it has been demonstrated that AQS covalently immobilized in polyurethane foam with macropores (AQS-PUF) exhibited high catalytic activity and good stability for potential applications [7].

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However, it was found that the catalytic activities of these immobilized quinones were lower than that of their soluble forms.

Shewanella species are facultative anaerobic bacteria with a unique respiratory versatility. It has been demonstrated that they could use insoluble Fe (III), Mn (IV) and anode in microbial fuel cell (MFC) as electron acceptors [8]. Moreover, different reduction mechanisms of these insoluble electron acceptors have been proposed to explain the extracellular electron transfer: the direct transfer to solid electron acceptors through the nanowires or redox components on cell surface (such as outer membrane *c*-type cytochromes), and the indirect transfer by using secreted soluble redox mediators as electron shuttles between the cell and solid electron acceptors [9,10]. Generally, electrochemically active bacteria can produce electrochemically active components or secrete redox mediators only in the presence of the solid electron receptors [11]. Thus, it could be an ideal option to combine *Shewanella* species and quinoid mediators-modified biocarrier such as AQS-PUF. *Shewanella* species are capable of excellently reducing solid quinones on biocarriers, which is the prerequisite for the catalytic performance of AQS-PUF. In addition, AQS-PUF could also induce *Shewanella* species to produce electrochemically active components or to secrete redox mediators, which might also enhance reductive transformation of recalcitrant organics in water. Therefore, it is expected that the synergistic effects of *Shewanella* species and AQS-PUF might be observed in the reductive transformation of nitroaromatics.

The objectives of the present study were to investigate the performance and mechanism of the combination of *Shewanella* species and AQS-PUF in the anaerobic treatment of nitrobenzene in water.

2. Experimental

2.1. Chemicals, bacterial strain and growth conditions

Nitrobenzene was purchased from Tianjin Dongnan Chemical Co., Ltd. Anthraquinone-2-sulfonic sodium salt (AQS) and riboflavin were purchased from Sigma-Aldrich. All other chemicals were of analytical grade. *Shewanella* sp. XB (GU001720) was isolated from a quinone reducing consortium, and *Shewanella* oneidensis MR-1 (ATCC 700550) was obtained from ATCC. Both strains were routinely cultured at 30 °C in Luria–Bertani (LB) medium under aerobic conditions, or in anaerobic medium, which contained (mM): 10 sodium lactate, 9.0 NH_4Cl, 5.7 $K_2HPO_4{\cdot}3H_2O$, 3.3 KH_2PO_4, 2.0 $NaHCO_3$, 1.01 $MgSO_4{\cdot}7H_2O$, 0.485 $CaCl_2$, and trace elements. The trace elements contained (μM): 67.2 $Na_2EDTA{\cdot}2H_2O$, 56.6 H_3BO_3, 10.0 NaCl, 5.4 $FeSO_4{\cdot}7H_2O$, 5.0 $CoCl_2$, 3.87 Na_2MoO_4, 1.26 $MnSO_4$, 1.04 $ZnCl_2$, 0.2 $CuSO_4{\cdot}5H_2O$. For pre-immobilization of *Shewanella* sp. XB, 0.2 g AQS-PUF and PUF, respectively, were placed in a 250 mL flask containing 100 mL normal saline, and 20 mg cells (dry weight) was added as inocula and cultured for 12 h at 30 °C in a rotary shaker (150 rpm).

2.2. Preparation of AQS-PUF

Polyurethane foam (PUF) was cut into cubes (about 0.5 cm × 0.5 cm × 0.5 cm), washed with distilled water, and then dried in oven at 60°C for following use as biocarriers. AQS-PUF was prepared by a two-step grafting procedure with diethylenetriamine and anthraquinone-2-sulfonyl chloride (ASC) as described by Lu et al. [7]. ASC was synthesized using AQS as described by Feng et al. [12]. The concentration of the immobilized AQS was about 0.1 mmol g^{-1} PUF.

2.3. Batch biotransformation of nitrobenzene

Shewanella strains of XB and MR-1 aerobically grown in LB medium overnight were harvested by centrifugation (10 min, 10000 g), washed twice with phosphate buffer (pH 7.0), and then resuspended in the same buffer. The experimental systems were sterile 100 mL serum bottles containing anaerobic medium, nitrobenzene (initial concentration of 1.63 mM), AQS-PUF (AQS concentration of 0.2 mM) and 10 mM lactate as electron donor. Biotransformation studies were started by addition of about 20 mg cells (dry weight). All the experiments were carried out in an anaerobic chamber at 30 °C in triplicate.

Transformation efficiency was expressed as the percentage of nitrobenzene reduction (%) = $(A_0 - A_t)/A_0 \times 100\%$, where A_0 and A_t were nitrobenzene concentrations at time zero and time t (h), respectively. A pseudo first-order model ($A_t = A_0 e^{-kt}$) could be applied to describe the kinetics of nitrobenzene biotransformation. The first-order rate constant k (h^{-1}) was determined.

2.4. Biotransformation of nitrobenzene by resting cells

Shewanella sp. XB was anaerobically grown in above anaerobic medium until they reached exponential growth phase. Cells were harvested by centrifugation (10 min at 10000 g), washed twice with phosphate buffer (0.05 M, pH 7.0), and then resuspended in the same buffer. Resting cells experiments were conducted in sterile 100-mL serum bottles containing phosphate buffer, nitrobenzene (0.24 mM) and lactate (0.1 mM). Initial concentration of cells was 200 mg L^{-1}. The concentrations of bound EPS, riboflavin and AQS in some cases were 10 mg L^{-1}, 0.1 mg L^{-1} and 0.1 mM, respectively. All the experiments were carried out in an anaerobic chamber at 30 °C for 24 h in triplicate.

2.5. Preparation and characterization of extracellular polymeric substances

Cells in batch biotransformation systems after 24 h were harvested by centrifugation at 10000 g for 10 min at 4 °C. Bound EPS was extracted from the cell pellet using EDTA method as described by Eboigbodin et al. [13]. The concentration of proteins in EPS was determined using the Lowry method with bovine serum albumin (BSA) as a standard [14]. The carbohydrate content was measured by the anthrone method using glucose as a standard [15]. UV–visible and fluorescence spectroscopy were used for the identification of cytochromes in EPS. The cytochromes were oxidized by gently pipetting EPS samples several times and the reduction of cytochromes was conducted by adding sodium dithionite to EPS and mixing for 2 min. Then the absorbance of samples was measured by UV–visible spectroscopy (300-700 nm), using Tris-HCl (pH 8, 10 mM) buffer as a control [16]. The fluorescence emission spectra (360–650 nm) of oxidized and reduced cytochromes were performed at 350 nm excitation wavelength [17].

2.6. Analytical methods

Samples taken from the experimental system were centrifuged at 10000 g for 10 min. The concentrations of nitrobenzene and aniline were measured using high performance liquid chromatography (Shimadzu LC-20AT) equipped with a elite hypersil BDS C18 column (25 μm, 4.6 × 250 mm) for separation at 40 °C and a diode array detector (DAD) (SPD-M20A, Japan) for measurement at 254 nm. The mobile phase consisted of 55% methanol and 45% deionized water was used at a flow rate of 1 mL/min. The identification of nitrobenzene metabolites was performed by using LC-MS with atmospheric pressure chemical ionization (APCI) mode. The