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Decolorization mechanism of 1-amino-4-bromoanthraquinone-2-sulfonic acid using *Sphingomonas herbicidovorans* FL

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Abstract

Sphingomonas herbicidovorans FL decolorized 1-amino-4-bromoanthraquinone-2-sulfonic acid and grew with it as the sole carbon source. The maximum rate of decolorization was achieved during the exponential growth phase of the bacterial strain. Of the total organic carbon ~52% could be removed, coupled with the partial release of ammonia, bromine and sulfate. Analysis of metabolites using gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry showed that phthalic acid was the metabolic intermediate and which may serve as the growth substrate for the bacteria. The end product was either 2-amino-3-hydroxy-5-bromobenzenesulfonic acid or 2-amino-4-hydroxy-5-bromobenzenesulfonic acid. A possible metabolic pathway is proposed.
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1. Introduction

Many natural and synthetic anthraquinone derivatives are toxic [1,2]. It is generally recognized that anthraquinone dyes are ligands with highly flexible molecules that have nucleotide-mimetic properties [3], and inhibit numerous nucleotide-dependent and nucleotide-utilizing enzymes [4].

1-Amino-4-bromoanthraquinone-2-sulfonic acid (bromoamine acid, BAA) is a major synthetic intermediate of acid and reactive anthraquinone dyes. Approximately 20 steres of wastewater are discharged per ton of BAA produced, whereas the traditional activated sludge treatment is ineffective. The biological persistence of BAA is due to its stable conjugated structure and high water solubility. Although the anthraquinone nucleus can be transformed to dihydroxyanthracene under anoxic/anaerobic conditions [5], the major fragments of the original molecule remain intact and the products easily oxidize when exposed to air [6]. Various physical and chemical treatments such as TiO_2-assisted photocatalytic oxidation and ozone oxidation [7] have been used to remove BAA, but they are not cost-effective. Hence, the screening of BAA-degrading bacterial strains and the application of bioaugmented reactors [8] have become popular research fields. Several BAA-degrading bacterial strains have been isolated, such as *Flavobacterium* BX26 [9], *Zoogloea* HP3 [10] and *Sphingomonas xenophaga* [11,12]. Qu et al. [8] supplemented suspended and immobilized cells of *S. xenophaga* QYY to enhance BAA removal in activated sludge sequencing batch reactors. The results of ribosomal intergenic spacer analysis (RISA) indicated that the strain QYY persisted in the augmented systems.

This paper deals with degradation of BAA by a newly isolated strain of *Sphingomonas herbicidovorans*, which has previously been used by other researchers to degrade phenoxyalkanoic acid herbicides [13,14]. A series of experiments are carried out to investigate the metabolic pathway of BAA.

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2. Materials and methods

2.1. Chemicals and growth conditions

BAA of commercial purity (91.77%) with an appearance of red acicular crystal was provided by the DanKong Industry & Trade Group Co., Ltd (Taizhou, Zhejiang, China). Phthalic acid and 2,5-dihydroxybenzoic acid (>99%) were purchased from the Sigma–Aldrich Corp. (Saint Louis, Missouri, USA). Acetonitrile of chromatographical purity was purchased from the Merck Corp. (Darmstadt, Germany). All other chemicals were of analytical grade.

Basal salts medium (BSM) was used for the isolation of the BAA-degrading strain. It contained 2.2 $g\,l^{-1}$ of Na_2HPO_4, 0.8 $g\,l^{-1}$ of KH_2PO_4, 0.4 $g\,l^{-1}$ of $(NH_4)_2SO_4$, 0.01 $g\,l^{-1}$ of $MgSO_4 \cdot 7H_2O$, 0.01 $g\,l^{-1}$ of $FeSO_4 \cdot 7H_2O$, and 0.005 $g\,l^{-1}$ of $CaCl_2 \cdot 2H_2O$. The pH of the medium was adjusted to 7.0. The medium was autoclaved at 121 °C for 20 min.

2.2. Microbial strain

The microbial strain designated as FL was isolated from a microbial consortium provided by the Department of Microbiology, Nankai University. The consortium was obtained from BAA-contaminated soil collected from a chemical plant by an enrichment culture technique using a mineral salts medium amended with BAA as the sole source of carbon [11]. The consortium was then inoculated on an agar plate containing BSM added with BAA and the colonies were transferred to fresh agar plates several times in order to obtain a pure strain which showed consistent decolorization.

The strain FL was a motile Gram-negative, rod-shaped, yellow-pigmented aerobic bacteria, 0.8–1.3 μm long and 0.5–0.6 μm wide. The strain FL is oxidase and catalase-positive, but ornithine decarboxylase and lysine decarboxylase negative. It ferments glucose, maltose, lactose, and sucrose but not inositol. It lyses esculin but not gelatin. The strain FL was identified as *S. herbicidovorans* by comparing its 16S rRNA gene sequence (GenBank accession no. EF065102) with the sequences in the GenBank/EMBL/DDBJ nucleotide sequence databases using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/). The closest neighbor was *S. herbicidovorans* (AB022428) showing 99% homology.

2.3. Decolorization of BAA

The decolorization experiments were carried out in the BSM supplemented with BAA at 30 °C on a shaker at 150 rpm. The stock cultures were prepared by growing a single colony in a conical flask (500 ml) containing 200 ml BSM with 500 $mg\,l^{-1}$ of BAA, and the biomass was harvested by centrifugation at 4500 rpm for 10 min and washed twice with 0.2 $mol\,l^{-1}$ phosphorous buffer solution. The cell pellets were re-suspended in washing buffer for decolorization experiments.

2.4. Analytical methods

During the reaction, samples were taken at regular intervals and the biomass concentration was determined by optical density (OD) at 660 nm. The samples were then centrifuged at 4500 rpm for 10 min, and the concentrations of BAA in the supernatants were measured at the maximum absorbance wavelength (485 nm) using a UV–vis spectrophotometer (Shimadzu UV1700, Japan). The total organic carbon (TOC) was measured by the combustion–infrared method using a TOC analyzer (Jena 3100, Germany).

The release of Br^- and SO_4^{2-} was determined by ion chromatography (Dionex ICS-1500, USA) using an AS9 analytical column and an AG9 guard column. The separation was performed using 9 $mmol\,l^{-1}$ Na_2CO_3 eluent. NH_4^+ was examined by the Nesslerization method.

The metabolites of BAA were analyzed using GC–MS after extraction and HPLC–MS directly. The supernatants (50 ml) were acidified to pH 2 with 2 $mol\,l^{-1}$ HCl and extracted three times with half volume of ethyl acetate. The extracts were combined, dried with anhydrous Na_2SO_4, and concentrated to 0.3 ml. The GC–MS analysis of metabolites was performed in the EI mode (70 eV) on a 6890N GC with a 5975 MSD (Agilent, USA). An HP-35 type 30 m long capillary column (250 × 0.25 μm) was used as the separation column. During analysis, the column temperature was first maintained at 50 °C for 2 min, then raised to 300 °C at a rate of 10 °C min^{-1}, and finally kept at 300 °C for 2 min. The mass scan range was 35–1000 *m/z*. HPLC–MS analysis of the metabolites was performed in the ESI mode using a Finnigan LCQ DecaXP ion trap mass spectrometer (Thermo, USA) equipped with a Hypersil Gold C18 column (150 × 2.1 mm). The eluent consisted of 10% acetonitrile and 90% water with 0.01 $mol\,l^{-1}$ ammonium acetate. The flow rate was 0.15 $ml\,min^{-1}$. Ionization was achieved in the negative mode. In the scan mode masses were detected from 120 to 800 *m/z*. In the Selected Ion Monitoring (SIM) measurements, the $[M-H]^-$ anions of the analytes were monitored: 165, 265.5–268.5, 379–385 *m/z*.

2.5. Growth on other aromatic compounds

Growth of the strain FL on aromatic substrates including phthalic acid, catechol, salicylic acid, benzoic acid, 2,5-dihydroxybenzoic acid and 3,4-dihydroxybenzoic acid was tested using BSM containing each aromatic compound (100 $mg\,l^{-1}$) as the sole carbon source. The liquid cultures were inoculated with 1 ml cell suspension induced by BAA and then incubated at 30 °C on a shaker at 150 rpm. The biomass concentration was determined at regular intervals and the UV–vis absorption spectra of the supernatants were examined after incubation for 5 days.

3. Results

3.1. Decolorization of BAA

The red color of BAA is caused by the conjugated structure of anthraquinone nucleus and the amino group. The color of

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