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ScienceDirect
Dyes and Pigments 78 (2008) 34–38
DYES and PIGMENTS
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Decolorization mechanism of 1-amino-4-bromoanthraquinone-2-sulfonic acid using *Sphingomonas herbicidovorans* FL

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Received 18 August 2007; received in revised form 8 October 2007; accepted 10 October 2007
Available online 22 October 2007

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.

Keywords: Bromoamine acid; Anthraquinone; Synthetic dyes; Biodegradation; Decolorization

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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doi:10.1016/j.dyepig.2007.10.004

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

the reaction mixture changed from red to colorless with time. Since the strain FL grew with BAA as the sole source of carbon, the anthraquinone nucleus of BAA cleaved and the primary chromophore was destroyed during the metabolism. Fig. 1 shows that the growing cells rapidly removed BAA from the reaction mixture. The decolorization reached its maximum rate of 12.68 mg l^{-1} h^{-1} during the exponential growth phase of the strain. At this instance, BAA was removed according to zero-order kinetics with $k = 9.8$ ($R^2 = 0.97$) in the test. Although the biomass concentration was low in the BSM, the decolorization percentage reached 99% within 14 h. During the decolorization of 93.49 mg l^{-1} of BAA, TOC decreased from 38.65 mg l^{-1} to 18.40 mg l^{-1} and remained unchanged until the end of the reaction. The fact that 52.4% TOC was removed showed that the microorganism metabolized only part of the organic carbon and BAA was not completely mineralized.

During the biodegradation of amino-, chloro- and sulfo-substituted aromatic compounds, the release of ammonia, chloride and sulfate often occurred [15–17]. In the present study, as the decolorization of 191.78 mg l^{-1} of BAA proceeded, the release of NH_4^+, Br^- and SO_4^{2-} was observed. Fig. 2 indicates that the concentrations of NH_4^+, Br^- and SO_4^{2-} increased gradually, reached a maximum, and then decreased with time. The maximum instantaneous concentrations of NH_4^+, Br^- and SO_4^{2-} were 0.81 mg l^{-1}, 8.58 mg l^{-1} and 5.22 mg l^{-1}, which were much lower than the theoretical values if the amino, bromo and sulfonate groups of BAA were completely removed. Therefore, it could be presumed that the molecular ring was only partially degraded. The reductions in concentrations of NH_4^+ and SO_4^{2-} were probably due to their utilization by the strain, whereas the decrease in concentration of Br^- might be because of its adsorption by the biomass.

3.2. Analysis of decolorization products

Only one metabolite, phthalic acid ($t_R = 13.9$ min), was identified in the acidified extract from the reaction mixture with GC–MS (Fig. 3). It was confirmed by comparing the retention time and the mass spectra with those of the authentic

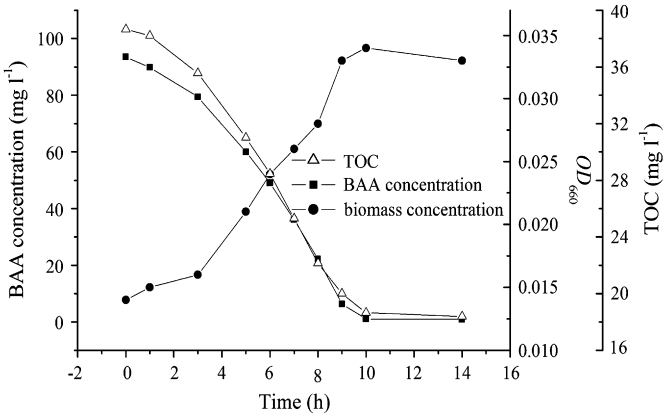

Fig. 1. Changes of TOC and biomass concentration during the degradation of BAA.

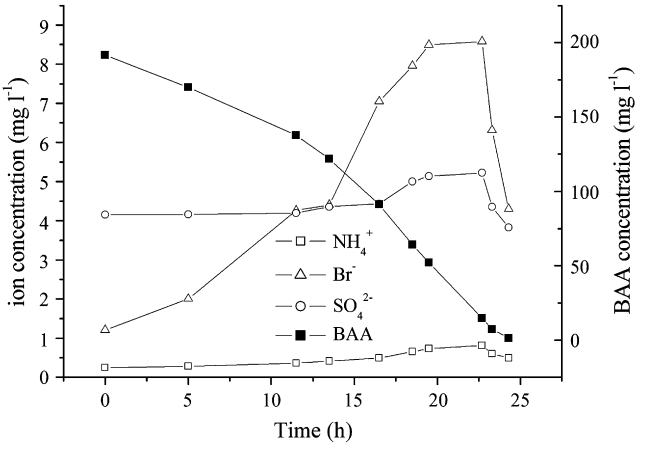

Fig. 2. Release of ammonia, bromine and sulfate during the degradation of BAA (0.01 g l^{-1} of $NaNO_3$, 0.001 g l^{-1} of $MgSO_4 \cdot 7H_2O$ and 0.001 g l^{-1} of $FeCl_3$ were used in the BSM instead of 0.4 g l^{-1} of $(NH_4)_2SO_4$, 0.01 g l^{-1} of $MgSO_4 \cdot 7H_2O$ and 0.01 g l^{-1} of $FeSO_4 \cdot 7H_2O$, respectively).

standard. Under the operating conditions, phthalic acid was easily converted into the corresponding anhydride: the *m/z* value of 148 was equivalent to the molecular weight of phthalic acid with loss of H_2O. The formation of phthalic acid was followed by consumption of BAA. During the degradation of 1000 mg l^{-1} BAA, the concentration of phthalic acid increased gradually with incubation time, reached a maximum of 19.7 mg l^{-1} after 22.5 h, and then decreased. Considering that the end products possessed nearly half of the TOC of BAA, it is believed that ring cleavage occurred between 9-C and 11-C as well as between 10-C and 12-C of BAA, with the end products comprising sulfonated derivatives, which cannot be detected with GC–MS [18].

With HPLC–MS scan mode, BAA and two metabolic products were detected in the reaction mixture. BAA ($t_R = 12.48$ min) had *m/z* values of 380 and 382. Metabolite 1 ($t_R = 2.8$ min) which had an identical molecular ion $[M - H]^-$ (165 *m/z*) was phthalic acid. Metabolite 2 ($t_R = 3.93$ min) had *m/z* values of 266 and 268, and their abundance ratio was approximately 1:1 (Fig. 4). The SIM measurements showed that the concentration of metabolite 2 increased with the degradation of BAA, and remained unchanged after decolorization. The end product, metabolite 2, was estimated to have amino, bromo and sulfonate groups since the amounts of NH_4^+, Br^- and SO_4^{2-} released during the decolorization were not stoichiometric with reference to the constituent amounts of these groups added, and the abundance ratio of the mass spectra accorded with that of bromine isotope. Based on the results listed above, the end product was estimated to be either 2-amino-3-hydroxy-5-bromobenzenesulfonic acid or 2-amino-4-hydroxy-5-bromobenzenesulfonic acid. Further degradation by the strain proved to be difficult, perhaps due to the molecular steric effect, which inhibited the contact between the enzymes and the substrates. HPLC–MS analysis has been demonstrated to be more useful for the detection of the polar sulfonates described here, unlike GC–MS which could not identify them.

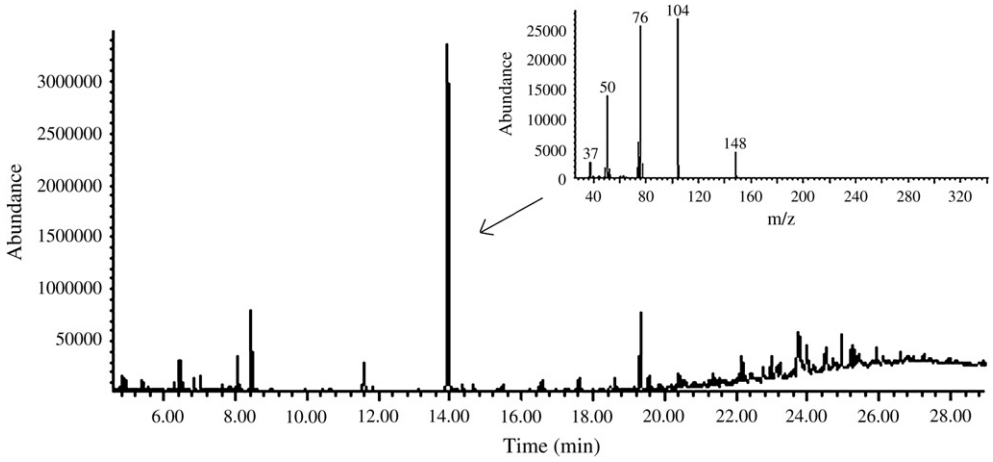

Fig. 3. GC–MS analysis of metabolites of BAA degradation (TIC: 22.5 h extract).

3.3. Growth on various aromatic compounds

The biodegradation of polycyclic aromatic hydrocarbons (PAHs) as sole carbon sources has been often coupled with the growth of the organisms by utilizing the intermediate products [19–21]. In the present study, several possible intermediates were tested to see if they could serve as growth substrates. Phthalic acid and 3,4-dihydroxybenzoic acid facilitated high rates of growth. No growth was observed on catechol, salicylic acid, benzoic acid and 2,5-dihydroxybenzoic acid. Although the increase of OD_{660} was observed during the incubation with catechol, it was caused by the brownish polymer resulting from the oxidation of catechol. The UV–vis spectra analysis confirmed that the strain could not utilize catechol. Qu et al. [22] reported that *S. xenophaga* QYY flourished in a mineral salts medium amended with catechol as the sole source of carbon, and believed that the degradation of BAA was mediated by the catechol dioxygenase.

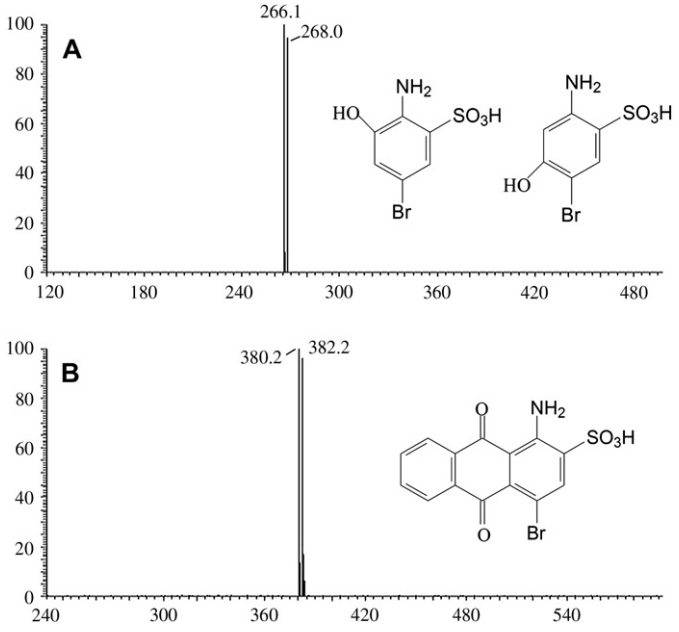

Fig. 4. Mass spectra of metabolite 2 (A) and BAA (B) in HPLC–MS analysis.

4. Discussion

The present study indicates that the isolated strain *S. herbicidovorans* FL could degrade BAA as the sole carbon source. Previously, *Sphingomonas* strains have been used to degrade a broad range of PAHs and related compounds [23].

There are a few reports in the literature about the biological oxidation pathway of anthraquinone compounds. Fujii et al. [24] described enzymatic degradation of an anthraquinone derivative, questin, by oxygenase from *Aspergillus terreus*. Itoh [25] studied the oxidative degradation of 1,4-dihydroxyanthraquinone (PV 12) by *Coriolus versicolor*. Itoh proposed that an attack of oxygenase from *C. versicolor* on PV 12 led to the formation of a lacton intermediate, which produced phthalic acid via hydrolytic cleavage of the carbon–oxygen bond. Subsequently, benzoic acid was formed by decarboxylation of phthalic acid. Hammel [26] found that the oxidation product of anthracene, 9,10-anthraquinone, was cleaved by *Phanerochaete chrysosporium* to give the ring-fission metabolite, phthalic acid. In the present study, phthalic acid was also detected in the reaction mixture of BAA degradation. This demonstrates that bacteria could also degrade anthraquinone compounds through the phthalate route.

HPLC–MS was shown to be a viable method to determine the nonvolatile end product and BAA, which could not be examined using GC–MS. Considering that phthalic acid was confirmed to be one of the metabolites, and the end product was estimated to be either 2-amino-3-hydroxy-5-bromobenzenesulfonic acid or 2-amino-4-hydroxy-5-bromobenzenesulfonic acid, a probable pathway of BAA degradation is proposed, as illustrated in Fig. 5. It is believed that BAA was attacked by oxygenase and then cleaved through hydrolyzation to give the final product. Since amino- is an electron-donating group while bromo- is an electron-withdrawing group, the electron cloud density on 9-C and 11-C was relatively higher than that on 10-C and 12-C; thus the oxygenase might first attack 9-C and 11-C. This pathway is further supported by the utilization of phthalic acid as the sole carbon source by the strain.

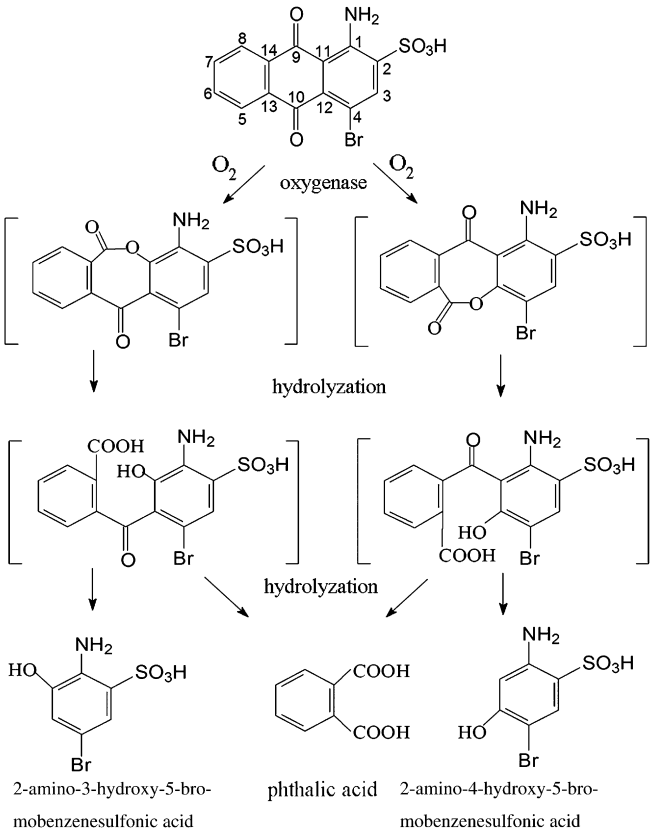

Fig. 5. Proposed metabolic pathway for the degradation of BAA by *Sphingomonas herbicidovorans* FL.

5. Conclusions

BAA belongs to the derivatives of anthraquinone. Its stable chemical properties and high water solubility permit BAA to persist in the environment. The present study has described the degradation of BAA as the sole carbon source by a strain of *S. herbicidovorans*. During the degradation, over half of the organic carbon was mineralized, and partial release of ammonia, bromine and sulfate was observed. Phthalic acid was identified as the intermediate metabolite and the end product was estimated to be either 2-amino-3-hydroxy-5-bromobenzenesulfonic acid or 2-amino-4-hydroxy-5-bromobenzenesulfonic acid.

Acknowledgements

We thank Professor B.L. Cai (Nankai University, China) for helping us to isolate the strain and Professor A.G.L. Borthwick (Oxford University, UK) for his help in editing the language.

This work was supported by the Presidential Foundation of the Shenzhen Graduate School of Peking University (Project 2006013).

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