International Biodeterioration & Biodegradation xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

# Microbial biodegradation of aniline at low concentrations by *Pigmentiphaga daeguensis* isolated from textile dyeing sludge

Jiaxing Huang, Jiayin Ling, Chaozhi Kuang, Jinliang Chen, Yanbin Xu*, Yuxin Li

*School of Environmental Science and Engineering, Guangdong University of Technology, Guangzhou 510006, China*

ARTICLE INFO

*Keywords:*
Aniline
*Pigmentiphaga daeguensis*
Textile dyeing wastewater
Low concentration
Biodegradation

ABSTRACT

Biodegradation with some predominated strains would be an effective and economical approach to eliminate aniline selectively from textile dyeing wastewater. In this study, an aniline-degrading bacterial strain called AN-4a was isolated from textile dyeing sludge and identified as *Pigmentiphaga daeguensis*. Strain AN-4a eliminated 10 mg $L^{-1}$ aniline within 15 h while shaking at 180 rpm at 30 °C. The optimal pH, temperature and NaCl concentration for aniline biodegradation by AN-4a was pH 7.0, 30 °C and 0.1% NaCl (w/v). Additional glucose and ammonium chloride inhibited the biodegradation of aniline by strain AN-4a. Aniline was initially converted to catechol and then metabolized to *cis,cis*-muconic acid (ccMA) by the catechol 1,2-dioxygenase (C12O) of strain AN-4a. To our knowledge, this is the first determination that *Pigmentiphaga daeguensis* can degrade aniline.

## 1. Introduction

Aniline is a toxic organic compound that is extensively utilized as a raw and processed material in dyestuffs, medicines, biocides, plastics, and other industries (O'Neill et al., 2000). It falls into Category 3 of the International Agency for Research on Cancer (IARC) list for its toxicity, mutagenicity, and carcinogenicity that threatens human health and the environment (Walpole and Williams, 1958). Although aniline often occurs as micropollutants in wastewater from the production of textiles, pesticides or pharmaceuticals, it can have deleterious effects on aquatic ecosystems even at low concentrations in sewage (Gabet-Giraud et al., 2010; Huber et al., 2005; Tekle-Roettering et al., 2016). In addition, a new national emission standard named "Wastewater Pollutants Discharge Standard for Textile Printing Industry" (GB4287-2012) stipulated that aniline must not be detected in the effluents from dyeing, which challenges China's current treatment techniques. Although several physical and chemical treatment methods such as oxidation by Fenton (Liu et al., 2016), ozonation (Tekle-Roettering et al., 2016), and electrochemical (Benito et al., 2017) and photochemical catalysis treatments (Tang et al., 2010) have been studied in wastewater treatment systems that contain aniline, both high capital and operational costs limit their applications, as well as their tendency to produce secondary pollutants. Therefore, a biological process utilizing a predominant strain that can degrade aniline would be an ideal technique (Meng et al., 2017). Previous studies have reported that several species including *Acinetobacter* (Wyndham, 1986), *Burkholderia* (Lee et al., 2016a), *Rhodococcus* (Zhuang et al., 2007), *Delftia* (Xiao et al., 2009), *Frateuria* (Kayashima et al., 2013), *Pseudomonas* (Jiang et al., 2016), *Comamonas* (Boon et al., 2000), *Moraxella* (Zeyer et al., 1985) and mix culture (Cui et al., 2017) can degrade aniline. Though some species can biodegrade aniline efficiently in wastewater containing a high concentration of aniline in alkaline conditions or at low temperatures (Jiang et al., 2016; Jin et al., 2012; Liu et al., 2015), there has been little research on the effective degradation of aniline at low concentrations.

This study aimed to isolate an effective strain to strengthen the existing wastewater treatment system and remove aniline at the low concentration level at which it usually exists in wastewater. A novel bacterial species with high aniline biodegradation efficiency was isolated from textile wastewater sludge. The physiological-biochemical characteristics and degradative conditions of the isolate were investigated as well as the biodegradation pathway of aniline.

## 2. Materials and methods

### 2.1. Reagents and media

Aniline, as well as chromatographic grade methanol, was purchased from ANPEL Laboratory Technologies, Inc. (Shanghai), China. All the other chemical reagents in this study were of analytical grade.

The mineral salt medium (MSM) was utilized as the selective medium in this study. The MSM medium (per liter) was composed of

* Corresponding author. School of Environmental Science and Technology, Guangdong University of Technology, No. 100 Waihuan Xi Road, Guangzhou Higher Education Mega Center, Panyu District, Guangzhou 510006, PR China.
*E-mail address:* hopeybxu@163.com (Y. Xu).

https://doi.org/10.1016/j.ibiod.2018.01.013
Received 26 October 2017; Received in revised form 6 January 2018; Accepted 22 January 2018

NaCl 1.0 g, $K_2HPO_4$ 1.0 g, $MnSO_4$ 1.69 mg, $ZnSO_4{\cdot}7H_2O$ 1.15 mg, $FeSO_4$ 3 mg, $CuSO_4{\cdot}5H_2O$ 0.38 mg, $H_3BO_3$ 1.16 mg, $KH_2PO_4$ 1.0 g, and $CoCl{\cdot}6H_2O$ 0.24 mg with the pH adjusted to 7.0. Luria-Bertani (LB) medium was utilized as the culture medium to amplify the bacterial mass for the aniline biodegradation experiment. Both the aniline solution and the MSM media were utilized after sterilization at 121 °C for 20 min.

### 2.2. Isolation and identification of the predominant strain

Textile dyeing activated sludge was collected from an aerobic tank at some textile Company, Zhongshan, China. Two grams of activated sludge was inoculated into the MSM containing aniline (50 mg $L^{-1}$) and incubated in a rotary shaker at a speed of 150 rpm at 30 °C for 7 days. After sufficient mixing time, 10% of the culture was inoculated in fresh MSM containing aniline (100 mg $L^{-1}$). Then, this step was repeated utilizing MSM containing aniline (150 mg $L^{-1}$). The culture was diluted and spread onto MSM agar plates containing aniline (100 mg $L^{-1}$) to isolate and purify the strains. The isolated strains were stored at −80 °C. The stock culture was subcultured twice before additional experiments. The isolated bacterial strain was identified by physiological and biochemical experiments and its 16S rRNA gene sequence. The universal PCR primer pair of 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3), were utilized in this experiment. The resulting 1420-bp gene sequence was compared with the genes in GenBank using the BLAST program (Zhao et al., 2016). Phylogenetic analysis utilized the MEGA 5.0 version.

### 2.3. Degradation of aniline with low concentration by the predominant strain

The predominant strain was inoculated into 100 mL LB liquid medium with a shaking speed of 180 rpm at 30 °C for 36 h. Cell pellets were collected when the logarithmic growth of the microorganisms was nearly complete with an $OD_{600}$ value of approximately 0.5. The bacteria that were harvested through centrifugation at 10,000 rpm for 15 min were washed twice with fresh MSM and then resuspended in 10 mL of fresh MSM. The cells were inoculated into a 250 mL-conical flask with 50 mL of MSM supplemented with aniline (10 mg $L^{-1}$) and cultured on a shaker at 180 rpm at 30 °C. Samples were removed at regular time intervals. The aniline concentration was analyzed by HPLC (Prominence LC-20A) equipped with an Eclipse XDB-C18 (4.6 * 250 mm, 5 μm) and a UV detector at 230 nm. The working conditions were the mobile phase consisting of methanol and $H_2O$ (50:50, v:v) at a flow rate of 1.0 mL $min^{-1}$ and a 10 μL injection volume. The bacterial growth was monitored spectrophotometrically at 600 nm ($OD_{600}$) by a UV-Vis spectrophotometer (UV-9600).

### 2.4. Influence of the initial concentrations on aniline biodegradation

Ten milliliters of concentrated bacterial suspension was inoculated in 250 mL flasks with 50 mL MSM liquid medium containing aniline (8.17, 17.53, 73.17, 98.76 and 198.87 mg $L^{-1}$) and then incubated under the conditions described previously. Samples were collected at the reaction time intervals of 12 h and then centrifuged at 10,000 rpm for 15 min. The supernatants were obtained and filtered through a 0.22 μm nylon filter for the aniline analysis.

### 2.5. Influence of environmental factors on aniline biodegradation

Ten milliliters of bacteria suspension was inoculated in 250 mL beaker flasks with 50 mL MSM containing 10 mg $L^{-1}$ aniline with pH, NaCl, glucose, and ammonium chloride and cultured at 180 rpm for 24 h at 30 °C or a temperature designed for the experiment on temperature effects. Samples were removed every 4 h to analyze the amount of aniline.

To investigate the effect of pH on aniline biodegradation, the pH of the MSM solution was adjusted with NaOH and HCl ranging from 5.0 to 10.0 with increments of 1.0 pH units. The effects of temperature were evaluated in a temperature range of 15.0 °C–40.0 °C at 5 °C intervals. To determine the influence of the NaCl concentrations on aniline biodegradation, 0.1–0.7% (w/v) of NaCl was added in MSM medium contained 10 mg $L^{-1}$ aniline.

MSM solution containing 10 mg $L^{-1}$ aniline was supplemented with glucose (20, 40 and 80 mg $L^{-1}$) or ammonium chloride (5 and 10 mg $L^{-1}$) to investigate the effects of external carbon and nitrogen sources on aniline biodegradation utilizing representative compounds that would be found in actual dyeing wastewater (Ashrafi et al., 2013; Singh and Pakshirajan, 2010). All the experiments were conducted in triplicate under aseptic conditions.

### 2.6. Analysis of the aniline biodegradation pathway

The predominant strain was inoculated into MSM medium with aniline (10 mg $L^{-1}$) at 30 °C and 180 rpm. One hundred milliliters of the culture was sampled at 12 h and then concentrated to 1 mL by vacuum rotary evaporation (IKA RV10C S96) at 40 °C. The solutions were prepared following the method described in Section 2.3. An SBC 18 column (4.6 * 150, mm 5 μm) was utilized to separate the intermediate products. The aniline biodegradation metabolites were identified by HPLC and HPLC-MS/MS (Agilent Technologies, USA). Mass spectrometry analysis was conducted in the negative mode (electrospray ionization (ESI) source). The operational parameters were as follows: fragmentor 125 V, capillary voltage 3.5 kV, nitrogen (P99.99%) as the desolvation gas with a flow rate of 10 L $min^{-1}$, nebulizer pressure 40 psi, temperature 350 °C, scan range of m/z 50–400 with argon (P99.99%) gas.

Bacterial solutions were prepared following the method mentioned above and sampled to test for enzymatic activity. Supernatants containing the crude extracellular enzymes were harvested by centrifugation at 15,000 rpm for 30 min, and then, the bacteria were washed twice with 50 mM phosphate buffer saline (PBS, pH 7.0). The bacteria were resuspended in 50 mM PBS (pH 7.0) and disrupted in an Ultrasonic Biomixer (JY92-IIN, China) at 400 W. Supernatants that represented the crude intracellular enzymes were harvested by centrifugation at 15,000 rpm for 30 min. C12O and catechol 2,3-dioxygenase (C23O) activity were measured using a UV spectrophotometer (UV-9600) at 260 nm and 375 nm (Lee et al., 2016a,b).

## 3. Results and discussion

### 3.1. Identification of the isolated strain

Since strain AN-4a possessed the highest aniline degradation efficiency of all the isolates, it was selected to use in the following experiments after enrichment and screening. The colonies of strain AN-4a were milky white with a round edge and ordered and convex morphology when cultivated on solid MSM supplemented with aniline. The phylogenetic analysis of the 16S rRNA gene sequences demonstrated that strain AN-4a grouped among the *Pigmentiphaga daeguensis* sp. and was most similar to *P. daeguensis* K110 (Yoon et al., 2007). The 16S rDNA gene sequences was deposited in Genbank and accession number was KY645966. Some *Pigmentiphaga* species were reported to be able to utilize aromatic hydrocarbons such as furans (Lee et al., 2016b), benzoxazolinone (Dong et al., 2016), chlorothalonil (Wang et al., 2013), fenoxaprop-ethyl (Dong et al., 2015), acetamiprid (Yang et al., 2013) and naphthalene disulfonate (Uchihashi et al., 2003) as their sole carbon and energy source. However, the aniline degradative ability of *Pigmentiphaga* sp. had not been reported previously. The relationship between strain AN-4a and other closely related members is shown in Fig. S1.

Physico-biochemical characterization tests were performed to identify strain AN-4a. The results showed that strain AN-4a was Gram-