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Analysis of biodegradation by-products of nitrobenzene and aniline mixture by a cold-tolerant microbial consortium

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HIGHLIGHTS

- NB and AN degrading consortium isolated from the contaminated site was cold-tolerant.
- Composition of the microbial consortium was analyzed by PCR-pyrosequencing method.
- By-products of NB and AN mixture degradation by this consortium were first reported.
- New by-products were generated by biodegrading of NB and AN simultaneously.

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ABSTRACT

A cold-tolerant microbial consortium, which can use nitrobenzene (NB) and aniline (AN) as sole carbon, nitrogen and energy sources, was isolated from an NB and AN contaminated site. Pilot 454 pyrosequencing analysis of the consortium showed that it was mainly made up of *Pseudomonas* spp. (98%). At 10 °C, the consortium degraded the mixture of 50 mg/L NB and 50 mg/L AN at a similar rate as those achieved at 20 °C and 30 °C. The biodegradation by-products with different initial NB and AN concentrations at 10 °C were analyzed. Azobenzene, azoxybenzene and acetanilide were observed in NB and AN mixtures degradation. These by-products are generated by the reaction between different intermediates resulting from the NB and AN degradation as well as the parent compounds. To the best of our knowledge, this is the first report confirming the by-products of NB and AN mixture biodegradation by a cold-tolerant microbial consortium.

1. Introduction

Nitrobenzene (NB), which is listed as a priority pollutant by the United States Environmental Protection Agency due to its carcinogenetic and mutagenetic properties [1], is widely used in the synthesis of organic products such as aniline (AN), lubricating oils, dyes, drugs, pesticides, and synthetic rubber [2]. Its daughter product, AN, is also widely used in the synthesis of chemical products such as herbicides and dyes [3]. In the past decades, industrial usage and accidental spills released large amounts of both NB and AN to the environment [4], which posed a serious threat to environmental health.

The biodegradation of NB, AN, and their pathways has been extensively studied separately [5–9]. However, all of the degradation studies are conducted as a single contaminant [10–12], and no attention has been paid to the degradation of NB and AN as a mixture. NB and AN are typically found in the same contaminated site because NB is the primary starting material for the manufacture of AN, which leads to the co-occurrence of these compounds in an accidental spill. For example, an explosion that occurred on November 13, 2005 at an AN-production factory in China caused serious NB and AN contamination of the Songhua River and the adjacent groundwater. Moreover, AN is the by-product of NB biodegradation under anaerobic condition [13–16], which likewise leads to their co-existence. Therefore, it is necessary to study the degradation of NB and AN in the same system from a practical perspective.

NB and AN contamination often exists at the subsurface, which requires a cold-tolerant consortium for their remediation under relatively low temperature. Generally, the adoption of cold-tolerant microorganisms may greatly reduce the remediation cost of the contaminated sites [17]. However, most studies on NB and AN degradation via microorganisms are at or above 30 °C [12,18–20], and little information is available about NB and AN biodegradation

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at low temperature. Therefore, the isolation and incubation of a cold-tolerant consortium is necessary.

This study aims to isolate a cold-adapted consortium from a contaminated site that could degrade both NB and AN at the same time under relative cold conditions, as well as to investigate the degradation ability of an NB and AN mixture at low temperature (10 °C, which is the average groundwater temperature throughout the year in this contaminated site in Northeastern China). The by-products of the NB and AN mixture biodegradation (with different initial concentrations, at 10 °C) were likewise analyzed to determine the possible degradation pathway. The results presented in this paper may benefit the remediation practices of NB and AN contaminated sites in cold areas.

2. Experimental methods

2.1. Chemicals

NB and AN (99% purity) were purchased from the Beijing Chemical Company, China. Solvents including methanol and dichloromethane (Fisher Scientific, USA) were of high-performance liquid chromatographic quality. All of the chemicals used in the medium preparation were of analytical grade.

2.2. Cultivation and growth medium

The NB and AN degradation consortium was grown in mineral saline medium (MSM) containing the following ingredients in 1 L of distilled water: $Na_2HPO_4{\cdot}12H_2O$ (3.8 g), KH_2PO_4 (1.0 g), KCl (3.0 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), and 10 mL trace elements solution. Trace elements solution was prepared with: $CaCl_2{\cdot}2H_2O$ (5.0 g), $EDTA{\cdot}2H_2O$ (1.0 g), $FeSO_4{\cdot}7H_2O$ (1.0 g), $MnCl_2{\cdot}4H_2O$ (0.16 g), $ZnSO_4{\cdot}7H_2O$ (0.04 mg), H_3BO_3 (0.03 mg), $CoCl_2{\cdot}2H_2O$ (0.04 mg), $CuCl_2{\cdot}2H_2O$ (0.04 mg), $NiCl_2{\cdot}H_2O$ (0.005 g) and $NaMoO_4{\cdot}2H_2O$ (0.04 g) in 1 L distilled water and preserved at 4 °C.

2.3. Consortium isolation

The NB and AN degradation consortium was isolated from the soil of the Jilin Chemical Industry Corporation (JCC) explosion site in an AN production factory (Jilin, China). The soil (10 g) was inoculated into a 250 mL autoclaved flask containing 100 mL normal saline solution, and shaken (at 200 rpm) for 5 min. 10 mL of the supernatant was inoculated to 100 mL liquid MSM. NB and AN (50 mg/L each) were provided as the sole carbon, nitrogen, and energy source. The cultures were incubated in a shaker (120 rpm) at 10 °C, and then transferred to fresh MSM containing AN and NB after 7 days of incubation. This process was repeated five times.

2.4. V1–V3 16S rRNA genes pyrosequencing of microbial consortium

Total genomic DNAs were extracted as previously described by Gurtner et al. [21]. PCR for amplicon pyrosequencing was performed with barcode primers (27Fmod AGRGTTTGATCMTGGCTCAG, bac 519RGWATTACCGCGGCKGCTG), which targeted the 16S rRNA gene V1–V3 regions. Pyrosequencing was performed using the Genome Sequencer FLX System (Roche 454) [22].

2.5. Quantitative analysis of NB and AN

Liquid samples (1.0 mL) were collected and mixed with 4 mL methanol in sterile syringes (5 mL). The mixture was filtered by a Nylon filter (0.2 μm, 13 mm, MilliPore, Billerica, MA). A mixed sample (5 μL) was injected into an HPLC (Agilent Technologies, Palo Alto, CA) equipped with fluorescence and UV detector. Mobile phase of the column (Agilent Eclipse SB-C18 column, 150 mm × 4.6 mm, 5 μm) was a mixture of acetonitrile and pH buffer solution (65:35 by volume). The pH buffer solution contains ammonium acetate (3.85 g/L) and acetic acid (3 g/L). The flow rate was 1 mL/min. NB and AN were detected by the Fluorescence Detector ($\lambda_{ex}/\lambda_{em}$ = 280/340 nm) and the UV Detector (λ = 362 nm), respectively. The detection ranges for NB and AN are from 0.018 mg/L to 50 mg/L, and from 0.021 mg/L to 200 mg/L, respectively.

2.6. Qualitative analysis of by-products

The consortium was inoculated in MSM that contains different concentrations of NB and AN (as shown in Table 2), after 10 days of cultivation, 100 mL culture was centrifuged at 5000 rpm for 20 min, the supernatant was transferred to 125 mL separating funnel and extracted for the metabolites for three times after adjusting the pH to 7.0, 2.0, and 11.0. The extracts were condensed to 1 mL via a rotary evaporator (LabTech, EV311), transferred to 1 mL Agilent bottles, and then stored at 4 °C for GC/MS analysis.

The by-products of NB and AN were monitored by the GC/MS (Agilent 6890/5973 N), equipped with a HP-5ms column (30 m × 0.25 mm, 0.25 μm). The oven temperature gradient program was: 37 °C for 2 min, 37 °C to 180 °C (10 °C/min), 180 °C to 250 °C (30 °C/min), and 250 °C for 2 min. Helium was used as carrier gas at a flow of 1 mL/min (split: 10:1). The temperatures for the injector and ion source were 250 °C and 230 °C, respectively. Mass scan range was 50–550 amu.

2.7. NB and AN biodegradation

Batch NB and AN degradation experiments were individually performed in flasks, which were sealed by butyric rubber stopper (250 mL) and contained a mixture of various NB and AN concentrations (1.15 mg/L and 0.76 mg/L, 4.58 mg/L and 3.29 mg/L, 57.90 mg/L and 38.64 mg/L, 132.22 mg/L and 124.00 mg/L, as well as 583.53 mg/L and 412.38 mg/L) in 100 mL of sterilized MSM. All experiments were conducted in triplicates. The flasks were inoculated with 2% (v/v) of the consortium and incubated aerobically at 10 °C with agitation at 120 rpm. Samples were taken at intervals to analyze the NB and AN concentrations.

3. Results and discussion

3.1. Composition of the cold-tolerant microbial consortium

As shown in Table 1, 19 species of *Pseudomonas* were detected, which accounted for approximately 98% of the total microbial populations. *Pseudomonas* spp. can grow in a wide temperature range of 4–42 °C. Several species, such as *Pseudomonas mandelii* (37.7%) and *Pseudomonas syringae* (23.0%), were reported as cold-adapted bacteria [23,24]. This consortium was cultivated in relatively low temperature (10 °C), so low temperature could lead to the enrichment of these species of cold-adapted *Pseudomonas*. Also, *Pseudomonas* spp. are mainly degraders of nitro-aromatic compounds and aromatic amino compounds [25–28], so nitrobenzene, and aniline could result in the enrichment of degraders of *Pseudomonas*. *Aachromobacter*, *Acidovorax*, *Afipia*, *Brevundimonas*, *Chryseobacterium*, *Delftia*, *Methylobacterium*, *Rhizobium*, *Rhodanobacter*, *Sphingobacterium*, *Sphingomonas*, and *Stenotrophomonas* were likewise detected in this cold-tolerant microbial consortium. Although their percentages were quite low (2%), certain species, such as *Delftia*,