Isolation and characterization of benzene-degrading *Acinetobacter* sp. strain B2 from industrial wastewater

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Abstract

Benzene is a carcinogenic chemical used in petrochemical industry. Intensive use generates benzene-containing waste/wastewater and subsequent environmental contamination. Although benzene can be treated through biological remediation, the efficiency is often limited by the degradation ability of the microbe used. Microbial bioaugmentation is typically applied to enhance the treatment efficiency, but generally suffers from low activity and microbial instability in such environments. This study aimed to isolate benzene-degrading bacteria which can degrade benzene at a high rate, to be added in the treatment process. *Acinetobacter* sp. B2 was isolated from industrial wastewater enriched by benzene vapor. The degradation test in defined liquid medium supplemented with 100 mg l^{-1} showed that it could degrade benzene at 17.0 ± 0.2 mg l^{-1} hr^{-1}, and total benzene removal was more than 95 % in 6 hours. Compatibility tests of strain B2 with indigenous species in wastewater (with and without benzene) using PCR-DGGE showed that the strain was active, and was stable to some extent with the indigenous wastewater species in both conditions. These results suggested that *Acinetobacter* sp. strain B2 has a potential application as a bioaugmented culture agent for benzene waste treatment.

Keywords: benzene-degrading bacteria, *Acinetobacter* sp., benzene, PCR-DGGE, bioaugmentation

Introduction

Benzene is a volatile organic hydrocarbon with water solubility of 1.84 g l^{-1} at 30 °C. Although it can be released into the environment from natural sources, such as crude oil seeps, forest fires, and plant volatiles, the major source of benzene release is from human activities including automobile refueling operation, petrochemical, pharmaceutical and chemical industrial production processes and emission, etc. (Edgerton and Shah, 1992). Due to its intensive uses and chemical properties, benzene ubiquitously contaminates not only air, but wastewater as well. Because it is a known human carcinogenic agent, benzene contamination in industrial waste/wastewater and environment required extensive research.

Biological treatments, which rely on microbial activity to mineralize the target pollutant, are an effective, economical, and environmental-friendly process for pollutant remediation. Accordingly, several benzene-degrading bacteria were previously reported, for example *Pseudomonas* sp. (Kim et al., 2005); (Bertoni et al., 1996), *Burkholderia* sp. (Johnson and Olsen, 1997); (Leahy et al., 2003) *Pseudoxanthomonas* sp. (Kim et al., 2008)and

Acinetobacter (Kim and Jeon, 2009) Although these bacteria have been shown in the laboratory to effectively degrade benzene and have the potential use as bioaugmented bacterial culture for benzene treatment, when applied to benzene-contaminated the bioaugmented bacterial culture generally suffers from activity inhibition and microbial stability in the existing wastewater treatment environment. Thus, this study aimed to isolate benzene-degrading bacteria, which can be utilized as bioaugmenting agents and be stably maintained in the treatment process.

Methodology

Media

Bacterial cultivation was carried out in a modified minimal medium (T-medium) (Mathur et al. 2007); (Sompornpailin et al., 2014)The medium composition comprised (per liter): K_2HPO_4 4.56 g, KH_2PO_4 3.4 g, $Na_2HPO_4 \cdot 12H_2O$ 5.37g, NH_4Cl 0.8 g, $NaNO_3$ 2.49 g, and trace element, $MgSO_4 \cdot 7H_2O$ 0.1 g, $FeCl_3 \cdot 6H_2O$ 0.02 g, $MnSO_4 \cdot H_2O$ 0.88 mg, $Na_2MoO_4 \cdot 2H_2O$ 1 mg, $CaCl_2 \cdot 2H_2O$ 3.9 mg, $ZnSO4 \cdot 7H_2O$ 0.04 mg, $CoCl_2 \cdot 6H_2O$ 0.04 mg, and 2% (w/v) agar powder was added to prepare T-medium agar.

Screening and isolation of benzene-degrading bacteria

Industrial wastewater was collected and incubated with under agitation at 120 rpm in the presence of benzene vapor for 48 hrs at room temperature (28-33 °C). The suspension was then plated onto T-medium agar and incubated with benzene vapor for 48 hrs. Each colony was then picked and cultured in 100-ml T-medium supplemented with benzene vapor with agitation at 120 rpm, room temperature, for 24 hrs. For benzene biodegradation test, 24-h grown cultures were used as an inoculum at 1% (v/v) in 30-ml T-medium in a 120-ml gas-tight glass vial with 100 mg l^{-1} benzene supplied in a liquid phase. The test vial was then capped and incubated at 30 °C. At specified time intervals, the liquid sample was taken, extracted and analyzed for residual benzene (as described below).

Benzene extraction and detection by GC-FID

Benzene was extracted from the liquid sample by adding one to one volume ratio of dichloromethane. The mixture was then vigorously mixed for 30 min and centrifuged at 10,000 rpm for 10 min. The solvent phase was taken to determine the residual benzene by gas chromatography (GC2014, Shimadzu, Japan) equipped with an DB-5 capillary column (0.25 mm × 30 m × 0.25 m) coupled with a flame ionization detector (FID). The GC conditions were with a 10% split ratio and gas flow rate of 450 ml min^{-1}. The GC temperature was programmed at constant temperatures as followed: an injection port at 260°C, an oven at 60°C, and the detector at 300°C. Benzene peak retention time was at 2.051 min.

Bacterial identification

The selected benzene-degrading bacterium was identified by 16s rRNA gene sequence analysis. Total DNA extraction method was modified from Burgmann (Bürgmann et al., 2001) and Miller (Miller, Bryant et al. 1999) using a SDS-lysis method followed by phenol-chloroform-isoamyl alcohol extraction. The genomic DNA was used as a template for polymerase chain reaction (PCR) amplification using 63f and 1387r primers (5'- CAGGCCT AACACATGCAAGTC-3' and 5'-GGGCGGWGTGTACAAGGC-3', respectively) (Marchesi et al., 1998). The PCR product of about 1,300 bp was sequenced (Macrogen, Korea) and the corresponding sequence was analyzed with BLASTn program (NCBI).

Benzene biodegradation and stability tests of the selected benzene-degrading bacterium in wastewater

The selected benzene-degrading isolate was cultured as described above. Then, it was used as an inoculum at 1% (v/v) for two sets of filtered, non-sterile wastewater test systems; one was supplemented with benzene vapor and the other with no supplementation. The test was carried out for 7 days at room temperature. After defined time intervals, the following parameters were determined: 1) residual benzene was analyzed using GC-FID; 2) bacterial cell number was analyzed to represent cells survival in the test systems as colony-forming unit (CFU) ml^{-1}; and 3) the stability of the selected bacterium in the wastewater bacterial community was determined using Denaturing Gradient Gel Electrophoresis (DGGE). The total DNA in the test system was extracted as previously described (Bürgmann et al., 2001; Miller, Bryant et al. 1999) and used as a template for PCR amplification with 341f-GC and 520r primers (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC CTACGGGAGGCAGCAG-3', and 5'- ACCGCGGCTGCTGGC-3', respectively). DGGE was performed on 8% polyacrylamide gel with a denaturing gradient of 30–60%; where 100% denaturant gels contain 7M urea and 40% formamide. Electrophoresis was performed at a constant voltage of 150 V for 6 h in 1x TAE running-buffer at 60^{o}C. The gel was stained with 1x GelStar™ Nucleic Acid Gel Stain (Lonza Rockland, Inc., USA) and photographed under UV light (Muyzer et al., 1993). The DNA control band of the selected bacterium was identified and used to monitor its stability in the wastewater bacterial community in the test system.

Results and discussion

Identification of benzene-degradation bacterium

Among 39 bacterial isolates from the benzene-enriched conditions, the isolate B2 was selected based upon the fast-growing characteristic. Identification based on 16s rRNA gene sequence analysis indicated that the isolate B2 was a member of genus *Acinetobacter* sp. (Table 1). A benzene, toluene and ethylbenzene (BTE) degrading *Acinetobacter* sp. has previously been reported (Kim and Jeon 2009). However, the 16s rRNA gene sequences of *Acinetobacter* sp. B113 and *Acinetobacter* sp. B2 (this study) do not show similarity.

Table 1: Identification of isolated B2 based on 16s rRNA gene sequencing

Organism	Accession number	Identity (%)	E-value	Strain characteristic
Acinetobacter sp. MemClNew	KJ920201.1	100	0.0	An organophosphate-degrading bacterium
Acinetobacter sp. Bap30	JF682491.1	100	0.0	Benzo[a]pyrene-degrading bacterium
Acinetobacter calcoaceticus LCR102	FJ976611.1	100	0.0	Phosphate solubilizing-bacterium
Acinetobacter sp. 75	AB638862.1	100	0.0	Long chain alkane-degrading bacteria
Acinetobacter sp. B113	EU883929.1	98	0.0	BTE-degrading bacteria

Benzene degradation by *Acinetobacter* sp. B2

Benzene utilization and growth of *Acinetobacter* sp. B2 was investigated. More than 95% of benzene was utilized by *Acinetobacter* sp. B2 within the first 6 hrs of incubation corresponding to an increase of bacterial growth (Figure 1). The degradation rate was 17 ± 0.2 mg l^{-1} hr^{-1}.

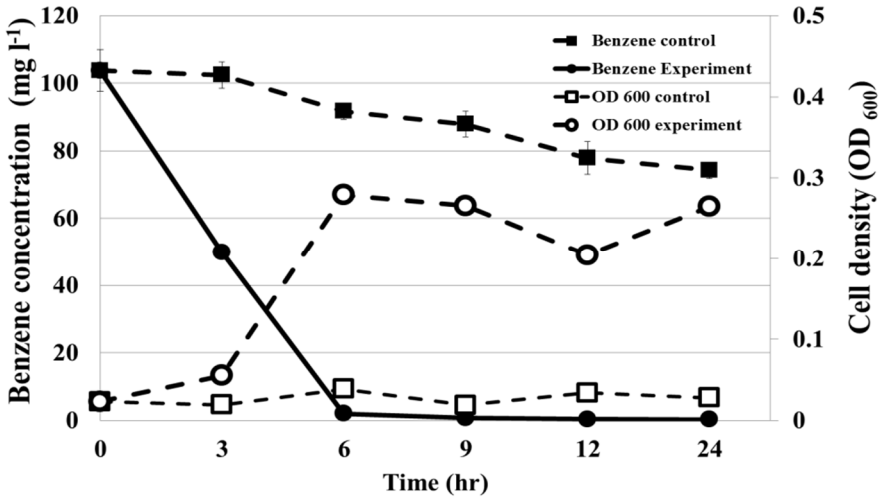

Figure 1: Benzene utilization and growth of *Acinetobacter* sp. B2 supplement with100 mg l^{-1} benzene in T-medium.

Benzene biodegradation and stability tests of *Acinetobacter* sp. B2 in wastewater

Further investigation was conducted to determine benzene biodegradation and stability of *Acinetobacter* sp. B2 in non-sterile wastewater to represent a contaminated site with indigenous microbes. Two test sets (with and without benzene) were carried out and the following results of benzene residual, cell survival numbers, and cell stability were determined.

Under the test conditions in non-sterile wastewater, more than 98 % of the benzene was degraded within the first 4 days only when *Acinetobacter* sp. B2 was added. This suggests that *Acinetobacter* sp. B2 was mainly responsible for benzene degradation (Figure 2A). Nevertheless, B2 cell numbers were observed at a reasonable level up to 4 days of incubation and decreased afterwards (Figure 2B). This suggests that in the presence of benzene, growth of indigenous microbes in wastewater was suppressed probably due to benzene toxicity. When benzene level was decreased to a non-toxic level, indigenous species in the wastewater could outgrow B2. This result was in agreement with DGGE result (Figure 3A) in which the DNA band representing B2 was observed with comparatively high intensity prior to day-4 of incubation and became less intense thereafter. This was more obvious in the test system without benzene where B2 was outgrown by indigenous microbes from day 1 (Figure 3B). These results suggested that *Acinetobacter* sp. B2 has potential to be used for treatment of benzene-contaminated wastewater and the cells can be stably maintained in such condition as long as benzene is presented.

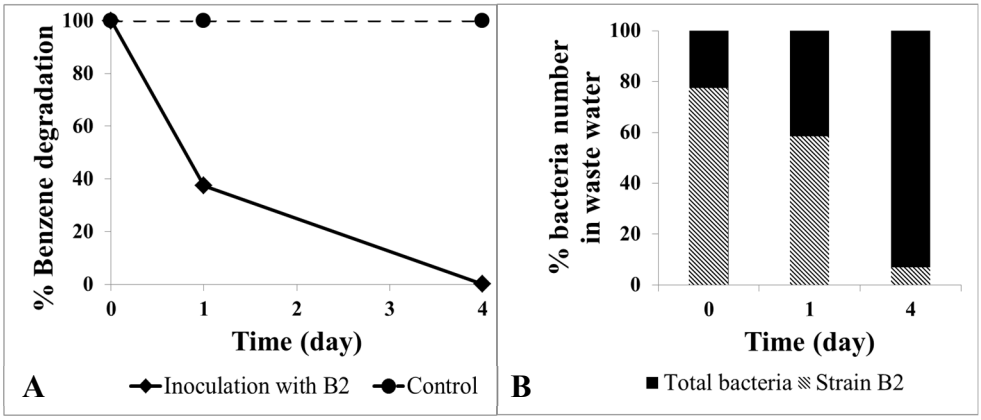

Figure 2: Benzene biodegradation and stability test of *Acinetobacter* sp. B2 in the wastewater supplement with benzene. (A) Degradation of benzene at an initial concentration of 1000 mg l^{-1} in non-sterile wastewater without (●, dashed line) and bioaugmentation with *Acinetobacter* sp. B2 (◆, solid line). (B) Cell numbers of *Acinetobacter* sp. B2 in comparison to total cell number. The number was initially obtained as CFU ml^{-1} and is expressed in percentage. The result was an average of triplicate independent experiments.

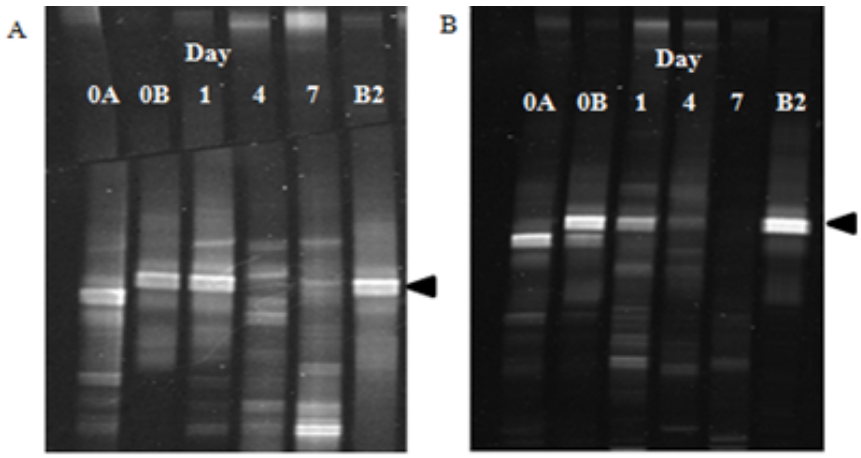

Figure 3: DGGE analysis of bacterial community in non-sterile wastewater (A) in presence of benzene at an initial concentration of 1000 mg l^{-1}, and (B) in the absence of benzene. The numbers indicate day of incubation, where 0a and 0b represent wastewater at day 0 before and after bioaugmentation with *Acinetobacter* sp. B2. The arrow symbol indicates the 16s rRNA band of *Acinetobacter* sp. B2.

Conclusion

Acinetobacter sp. B2 is an efficient benzene-degrading bacterium, as shown in both laboratory defined medium as well as in non-sterile wastewater. Cell stability can be reasonably maintained in the presence of benzene. As a consequence, *Acinetobacter* sp. B2 has potential application for treatment of benzene-contaminated wastewater.

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