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Biodegradation of di-*n*-butyl phthalate by a stable bacterial consortium, HD-1, enriched from activated sludge

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HIGHLIGHTS

- We report a stable and high efficiency DBP-degrading microbial consortium HD-1.
- Consortium HD-1 could mineralize about 90% of 1200 mg/L DBP after 48 h cultivation.
- The consortium has a broad pH value and temperature range.
- The dynamic changes of microbial structures and intermediates were investigated.

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ABSTRACT

HD-1, a stable microbial consortium capable of mineralizing di-*n*-butyl phthalate (DBP), was developed from activated sludge. The dominant microorganisms in the consortium, *Gordonia* sp., *Burkholderia* sp. and *Achromobacter* sp., were identified by denaturing gradient gel electrophoresis (DGGE). The consortium could mineralize approximately 90% of 1200 mg/L DBP after 48 h of cultivation. The optimal DBP degradation conditions were 25–30 °C and pH 8.0–9.0. The addition of yeast (0.5 g/L), sodium acetate (0.5 g/L, 1.0 g/L), Brij 35 (0.2%, 0.5%, 1.0%), or Triton X-100 (0.2%) enhanced DBP degradation. The DBP degradation rate was influenced by the presence of dimethyl phthalate (DMP) and diethyl phthalate (DEP). Only one main intermediate, phthalic acid, could be monitored by gas chromatography–mass spectrometry (GC–MS) during the degradation process. The HD-1 consortium also utilized phenol, *o*-dihydroxybenzene as the sole carbon and energy source. The results indicate the consortium may represent a promising application for DBP bioremediation.

1. Introduction

Phthalate esters (PAEs) are a prominent group of environmental pollutants and endocrine-disrupting compounds in many environmental conditions, such as marine sediments (Yuan et al., 2010), river water (Chi, 2009) and wastewater treatment plants (Dargnat et al., 2009; Huang et al., 2010). These compounds are concerning because they have been shown to interfere with the reproductive system and human and animal development (Lottrup et al., 2006). DBP, a representative PAE, is employed in widespread applications, predominantly in polymers and other various products, such as insecticides, packaging materials, cosmetics, coverings, clothes and insulators in electric disposals (Dargnat et al., 2009). DBP only physically bound to the plastic structure have been easily released into diverse environments (Fang et al., 2010). In addition, DBP can be taken up by crops and thus enter the food supply chain system (Liao et al., 2010), which may harm aquatic organisms and human health.

Previous studies have revealed that DBP can be removed by natural processes in natural environments, such as hydrolysis, photo degradation and biodegradation (Chen et al., 2009; Jonsson et al., 2006; Lau et al., 2005). Unfortunately, due to its chemical structure, two of those studies did not achieve any notable effects for removing DBP. The best mechanism for thorough DBP removal was proven to be microorganism-mediated metabolic transformation through environmental biodegradation. The DBP metabolic pathway in microorganisms involves initially converting DBP into phthalic acid (PA). Subsequently, PA is transformed by two dioxygenase attack pathways to 4,5-dihydroxyphalate in Gram-negative bacteria and to 3,4-dihydroxyphthalate in Gram-positive bacteria, forming the common intermediate protocatechuate in aerobic conditions (Wu et al., 2011b). Several studies have reported many bacterial strains with DBP-degrading ability, such as *Rhodococcus* sp. (Jin, 2010; Lu et al., 2009), *Agrobacterium* sp. (Wu et al., 2011b), *Enterobacter* sp. (Fang et al., 2010) and *Gordonia* sp. (Wu et al.,

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2011a). However, there are few observations detailing DBP degradation by a microbial consortium derived from enriched cultures. Existing evidence suggests that more biodegradation and mineralization can be expected in enriched mixed cultures than in pure cultures (Saratale et al., 2009). Because multiple metabolic capacities of mixed cultures can increase the number of catabolic pathways available for contaminant biodegradation, different strains may attack organic contaminant molecules at different positions or may use the intermediates produced by another strain for further decomposition. In addition, high diversity in a microbial consortium can expand microorganisms' survival in the environmental field. Therefore, mixed cultures are more suitable for bioremediation in natural environments and represent more realistic behaviors of environmental microorganisms in bioremediation processes than pure strains. Therefore, an enriched consortium is necessary and ideal for DBP degradation.

In the present study, an aerobic microbial consortium, designated as HD-1, was enriched from activated sludge from a wastewater treatment plant. The biodegradation potential of the consortium was investigated, and the influences of environmental factors on HD-1's degradation ability were also examined. DGGE and GC–MS were employed to analyze the microbial community structure and DBP degradation intermediates.

2. Methods

2.1. Chemicals

Dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), and di-n-octyl phthalate (DOP) were purchased from Aladdin-reagent Co., Shanghai, China at 99% purity. Ethyl acetate and methanol (HPLC grade) solvents were obtained from China National Medicine Group (Shanghai, China).

2.2. Sampling, enrichment and obtainment of DBP-degrading consortium

The consortium was enriched from activated sludge sampled from Hangzhou East China Pharmaceutical Factory, Zhejiang Province, China. Activated sludge (200 mL) was domesticated for one month under the pressure of a DBP gradient from 0.1% to 1% (v/v) with a changing period of three days in a 500 mL Erlenmeyer flask at 30 °C and 140 rpm. Sterile mineral medium (MM) was added to the domesticated liquid to a total liquid volume of 200 mL. The MM contained (L^{-1}): $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2{\cdot}2H_2O$ 0.04 g, K_2HPO_4 1.70 g, $FeSO_4{\cdot}7H_2O$ 0.05 g, and $NaNO_3$ 0.5 g, $(NH_4)_2SO_4$ 1.0 g.

After one month, 5 mL of the supernatant from the enrichment solution was inoculated into the MM supplemented with 1200 mg/L DBP in a 250 mL Erlenmeyer flask, cultured under 140 rpm and 30 °C. The consortium was serially subcultured for 20 generations for stabilization over a period of two days. The final microbial consortium was named HD-1.

2.3. Degradation experiments using the consortium

DBP degradation by the HD-1 consortium was determined in MM. Batch experiments were performed in a 250 mL Erlenmeyer flask containing 70 mL MM. The bacterial cells were harvested by centrifugation (10,000 rpm, 5 min), washed by phosphate buffer saline (PBS, pH 7.4) and then resuspended in the same buffer. Two milliliters of cell suspension (OD_{600} = 1.5) were dispensed into the culture medium. The degradation and growth conditions were monitored at 1200 mg/L DBP, 30 °C and 140 rpm, with sampling at 6 h intervals.

The following environmental factors were assayed to investigate their effects on DBP degradation within 48 h of cultivation (in MM containing 1200 mg/L DBP at a 140 rpm shaking rate): temperature (15, 20, 25, 30, 35, 40 and 45 °C); initial pH value (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0); carbon source (sodium acetate, yeast, glucose, peptone and mannite) at three concentrations (0.5 g/L, 1.5 g/L and 3.0 g/L) and surfactant (Tween 80, Brij 35, Sodium dodecyl sulfate (SDS) and Triton X-100) at three concentrations (0.2%, 0.5% and 1.0% (v/v)).

2.4. PCR–DGGE analysis of the consortium during degradation

The HD-1 consortium was cultured at the optimal DBP degradation conditions in MM. During 48 h of cultivation, 2 mL of culture medium was sampled at 6 h intervals. The samples were centrifuged (10,000 rpm, 5 min) to acquire the bacterial biomass.

At each sampling point, total bacterial genomic DNA from the HD-1 consortium was extracted using the Ultra bacterial DNA extraction kit (Sangon Corporation, Shanghai, China). The universal PCR Primers, F27 (5′-AGAGTTTGATCCTCGCTCAG-3′) and R1492 (5′-GCTACCTTGTTACGACTT-3′), were used to amplify the 16S rRNA gene. The amplification product was used as the template for the next amplification with primers F984GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC) and R1378 (CGGTGTGTACAAGGCCCGGGAACG) (Heuer et al., 1997) to amplify the V6–V7 region of 16S rRNA. The length of the expected amplified fragment was approximately 400 bp. PCR amplification was performed in a 50 μL reaction volume containing 5 μL of Taq buffer, 1 μL of template, 1 μL of each primer (20 mM), 4 μL of each dNTP (0.25 mM), and 1.5 units Taq polymerase (TaKaRa Biotechnology (Dalian) Co. Ltd., China). Reactions were performed in a MyGene Series Gradient Thermal Cycler (LongGene Scientific Instruments Co. Ltd., China). The thermal cycling conditions were: 1 cycle at 94 °C for 5 min, 35 cycles of 94 °C for 45 s, annealing at 55 °C (16S rRNA) or 58 °C (16S rRNA V6–V7) for 45 s and 72 °C for 45 s, an extension cycle at 72 °C for 10 min, and a final cycle at 4 °C for 10 min. PCR products (5 μL) were taken to evaluate the expected size and quantify on 1.0% (w/v) agarose gel electrophoresis. The remaining PCR products were purified with AxyPrep™ PCR Cleanup Kit and quantified by Smartspec3000 (Bio-Rad Laboratories, Hercules, CA, USA).

DGGE analysis was performed on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products (4 ng) were loaded onto 6% (w/v) polyacrylamide gels in a 1 × TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 7.4) using a denaturing gradient ranging from 35% to 55%. Electrophoresis was performed at 160 V for 5 h at 60 °C. Gels were silver stained after electrophoresis following the protocol described by Bassam et al. (1991). Digital images of the gel were obtained by scanning.

2.5. Degradation of other PAEs

The capability of the HD-1 consortium to degrade PAEs was examined in MM containing initial concentrations of 300 mg/L, 600 mg/L and 1200 mg/L of DMP, DEP, DBP and DOP as the sole carbon or energy sources, NaOH was used to adjust to a pH of 8.0. To investigate the degradation of the four PAEs by HD-1 in mixed PAEs, the flask contained 70 mL MM, 2 mL HD-1 culture suspension (OD_{600} = 1.5) and PAEs (a mixture of DMP, DEP, DBP and DOP [each at concentrations of 300 mg/L, 600 mg/L and 1200 mg/L]). To demonstrate the degradation influences between different pairs of PAEs (using all four PAEs that were examined), the flasks contained 600 mg/L of two different PAEs. After 48 h of cultivation, the culture medium was treated as follows to detect PAEs using HPLC.