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Kinetics of di-*n*-Butyl Phthalate Degradation by a Bacterium Isolated from Mangrove Sediment

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Abstract Biodegradation of the endocrine-disrupting chemical di-*n*-butyl phthalate (DBP) was investigated using a bacterium, *Pseudomonas fluorescens* B-1, isolated from mangrove sediment. The effects of temperature, pH, salinity, and oxygen availability on DBP degradation were studied. Degradation of DBP was monitored by solid-phase extraction using reversed-phase HPLC and UV detection. The major metabolites of DBP degradation were identified as mono-*n*-butyl phthalate and phthalic acid by gas chromatography-mass spectrometry (GC-MS) and a pathway of degradation was proposed. Degradation by *P. fluorescens* B-1 conformed to first-order kinetics. Degradation of DBP was also tested in seawater by inoculating *P. fluorescens* B-1, and complete degradation of an initial concentration of 100 μg/l was achieved in 144 h. These results suggest that DBP is readily degraded by bacteria in natural environments.

Key words: Biodegradation, phthalates, di-*n*-butyl phthalate, kinetics, mechanism

Phthalate esters (PAEs) are a class of chemical compounds primarily used as plasticizers to improve the mechanical properties of plastic resin, particularly its flexibility [11]. Plasticizers are used predominantly in building materials, home furnishings, transportation, textiles, and to a limited extent in packaging of food and medical products [17]. The United States Environmental Protection Agency (US EPA) and similar regulatory agencies in other countries have classified PAEs as top priority pollutants and endocrine disrupting compounds. They are suspected of interfering with the reproductive systems and behavior of humans and wildlife, even at very low concentrations, by disturbing the endocrine system [11].

Di-*n*-butyl phthalate (DBP) is one of the phthalates most frequently identified in diverse environments including groundwater, river water, drinking water, ocean water, soil, lake sediment, and marine sediments; it is the most widely used plasticizer worldwide, including China [24].

Studies of the biodegradation of several PAEs have been carried out, mainly focused on the degradation of dimethyl phthalate ester and related isomers [7, 10, 17, 19, 23, 27–29], diethyl phthalate [2, 6], butyl-benzyl phthalate [3, 14, 20], and di-(2-ethylhexyl) phthalate [4, 13, 16, 21]. Only a few papers have been published on the biodegradation of DBP [1, 5, 24, 26].

The coastal environments of big cities around the world have suffered oil spills, storm water runoff, and, in some cases, sewage effluents [30]. Such events lead to direct input of PAEs via sewage effluents and industrial wastewater. Mai Po Nature Reserve is the largest remaining wetland in Hong Kong; it plays a very important role in supporting a wide range of wild life. Its mangrove ecosystem, an important intertidal estuarine wetland along the coastline of land with a subtropical climate, is closely associated with human activity and is subject to PAE contamination. Thus it seemed possible that the mangrove sediment might contain PAE-degrading bacteria with special characteristics such as high salinity-tolerance.

In this paper, we detected degradation of DBP by a bacterium isolated from mangrove sediment. The kinetics and mechanism of DBP degradation were studied by solid-phase extraction using HPLC and GC-MS. We also studied the biodegradation of DBP in seawater by inoculating the bacterium.

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MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade, and we used water purified with a Milli-Q system in all experiments. Standard DBP was purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were from Aldrich (Milwaukee, WI, U.S.A.).

Isolation of Microorganisms by Enrichment Culture

Microorganisms using DBP as the sole source of carbon and energy were isolated by the enrichment-culture technique. The initial enrichment culture was established by inoculating 100 ml sterile mineral salts medium (MSM) containing DBP (1 mg/l) as the sole carbon and energy source in 250-ml Erlenmeyer flasks with 5 g of fresh mangrove sediment taken from Mai Po Nature Reserve in Hong Kong. The MSM consisted of the following chemicals (mg/l): $(NH_4)_2SO_4$, 1,000; KH_2PO_4, 800; K_2HPO_4, 200; $MgSO_4 \cdot 7H_2O$, 500; $FeSO_4$, 10; $CaCl_2$, 50; $NiSO_4$, 32; $Na_2BO_7 \cdot H_2O$, 7.2; $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 14.4; $ZnCl_2$, 23; $CoCl_2 \cdot H_2O$, 21; $CuCl_2 \cdot 2H_2O$, 10; and $MnCl_2 \cdot 4H_2O$, 30; and the pH was adjusted to 7.0±0.1 with HCl or NaOH, unless otherwise specified. The Erlenmeyer flasks were incubated in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, New Jersey, U.S.A.) at 150 rpm and 30.0±0.5°C in the dark. The DBP-degrading cultures were obtained by enrichment transfer at approximately 1-week intervals on the basis of substantial depletion of DBP, by transferring 1.0 ml of the active culture medium to a new Erlenmeyer flask containing 100 ml of freshly made MSM with gradually increasing concentrations of DBP (1 to 10 mg/l). The DBP-degrading enrichment cultures were transferred more than 10 times before bacteria were isolated from the enrichment culture.

Bacteria in enrichment cultures displaying the ability to degrade DBP were diluted in MSM and plated on Nutrient Agar (NA) plates (Difco Lab., Detroit, MI, U.S.A.). After 48 h of incubation at 30°C, a number of well-separated colonies of different morphologies were formed; these were re-streaked onto fresh NA plates for purification. Pure cultures were subsequently Gram stained and identified using the API 20 NE Multi-test System (bioMerieux, Marcy l'Etoile, France) as described elsewhere [28].

Biodegradation of DBP in Shaking Flasks and Seawater

Experiments on the kinetics of DBP degradation were conducted in 250-ml Erlenmeyer flasks containing 100 ml MSM and incubated as described above. DBP was sterilized by passage through a 0.2-μm-pore-size membrane filter (Pall Gelman Laboratory, Ann Arbor, MI, U.S.A.). All tests were conducted in triplicate.

The biodegradation of xenobiotic compounds at low substrate concentrations frequently follows pseudo first-order kinetics [18]. DBP degradation by *P. fluorescens* B-1 was assumed to fit the Monod first-order kinetic equation:

$$\ln C = -Kt + A \quad (1)$$

where C is the DBP concentration, t is time, K is the first-order rate constant, and A is a constant. The half-life of DBP biodegradation by *P. fluorescens* B-1 can then be calculated from:

$$t_{1/2} = 0.693/K \quad (2)$$

A pure culture isolated as described above was also inoculated into seawater taken from Mai Po Natural Reserve in Hong Kong. DBP was not detected in fresh seawater samples (<1 μg/l). The experiments were performed in 3-l Erlenmeyer flasks containing O_2-saturated medium in the dark, with continuous agitation by means of a magnetic stirrer. An aliquot of DBP and a bacterial inoculum (10 ml) were added to each flask, which was then filled with seawater to a final volume of 2.5 l. The flasks were covered with cotton wool plugs and incubated at 21±1°C. An abiotic control was prepared in the same way but with formaldehyde (2%) added; this permitted assessment of any reduction in DBP concentration as a result of physicochemical processes such as adsorption, photolysis, etc. If the seawater had been autoclaved, its physicochemical characteristics would have been altered by the high temperature treatment, and this would prevent accurate assessment of abiotic degradation.

Analysis and Identification of DBP Metabolites

A Waters Sep-Pak C_{18} cartridge (500 mg) was conditioned by 2 ml of methanol, followed by 5 ml of water. A 50 ml sample of the culture medium was centrifuged and the supernatant filtered through a 0.2-μm membrane prior to solid-phase extraction (SPE). The sample was acidified to pH 2 with 0.1 N HCl, and passed through the cartridge at a flow rate of 2 ml/min. The analytes retained on the SPE cartridge were eluted with methanol (1 ml×2), and the eluate purged to dryness with pure nitrogen gas. Finally, the residue was dissolved in 0.5 ml of methanol prior to assay by HPLC and detection by UV or GC-MS. Recoveries of DBP and PA after SPE were 99% and 92%, respectively.

To monitor DBP degradation we used an Agilent 1100 series HPLC system consisting of a G1322A degasser, a G1311A QuatPump, a G1316A COLCOM, and a G1315B diode array detector (DAD, Agilent, CA, U.S.A.) set at 210 nm wavelength. A personal computer equipped with an HP ChemStation (Hewlett Packard, CA, U.S.A.) was used to acquire and process chromatographic data, and an Agilent Zorbax Eclipse XDB-C_8 column (150×4.6 mm, particle size 5 μm) was used for separation. The mobile phase was a mixture of methanol, water, and 50 mM phosphoric acid (pH 2.35) (70:25:5, v/v) and the flow rate was 1.0 ml/min. Under these chromatographic conditions,

baseline separation was obtained within 30 min for DBP and its metabolites. The areas of peaks were used for quantification. All compounds studied were quantified using external standards.

Metabolites of DBP degradation were identified with a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, CA, U.S.A.) equipped with an Agilent 5973 mass selective detector (Agilent, CA, U.S.A.). The column used was an HP-624 fused-silica capillary column (25 m×0.2 mm i.d., 1.12 μm film thickness), and the temperature program was a 5-min hold at 50°C, an increase to 280°C at 10°C/min, and a 15-min hold at 280°C. The injection volume was 1 μl and the carrier gas was helium (1.0 ml/min). The mass spectrometer was operated at an electron ionization energy of 70 eV. Instrumental library searches, comparison with available authentic compounds, and mass fragmentation patterns were used to identify suspected metabolites.

Results and Discussion

Isolation and Characterization of a DBP-Degrading Strain

A bacterial strain that degraded DBP efficiently was isolated from the mangrove sediment of Mai Po Nature Reserve after several enrichment transfers and cloning on agar plates containing DBP as the sole carbon source (see Materials and Methods). The isolate was preliminarily identified as *Pseudomonas fluorescens* B-1, with 94.9% similarity, using the API 20NE biochemical test system. It was a Gram negative, obligate aerobic, noncapsulated, nonsporulating rod, forming light yellow colonies. The growth of *P. fluorescens* B-1 with DBP as the sole carbon and energy source is shown in Fig. 1. Bacterial biomass increased over the entire incubation period as DBP was depleted.

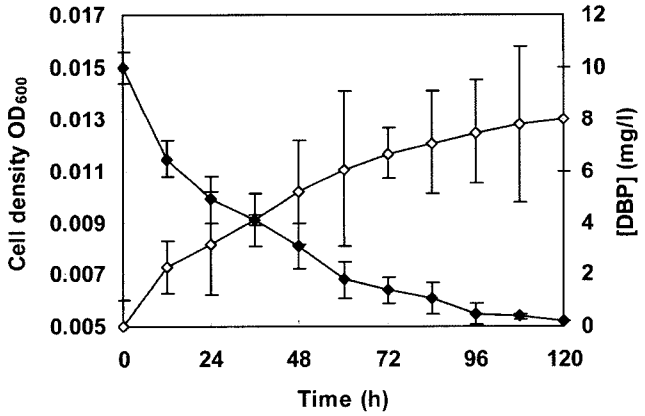

Fig. 1. Growth of *Pseudomonas fluorescens* B-1. $[DBP]_0$=10 mg/l, T=30°C.
Data are averages of triplicate determinations, and vertical bars represent standard deviations. Cell density (◇), DBP concentration (◆).

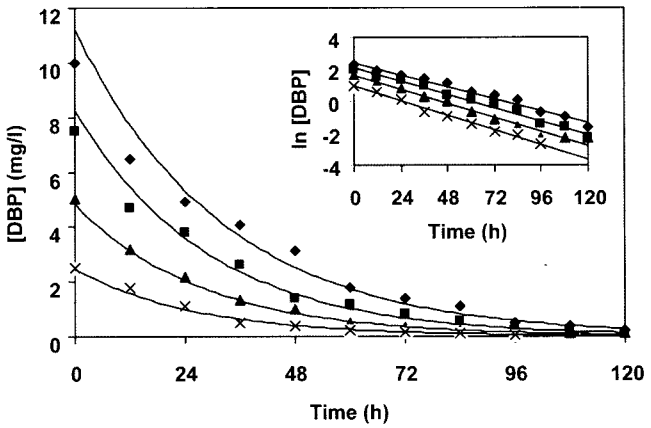

Fig. 2. DBP biodegradation by *Pseudomonas fluorescens* B-1 at different initial concentrations at 30°C.
Initial concentrations of DBP (mg/l) were: 10 (◆), 7.5 (■), 5 (▲), 2.5 (×). The lines drawn are fits obtained with the exponential model.

Effects of pH, Temperature, Salinity, and Oxygen Availability on DBP Degradation

Degradation of DBP by *P. fluorescens* B-1 was optimal between pH 7.0 and pH 8.0 and the optimum temperature was 37°C. Degradation was roughly constant between 15 and 25‰ salinity, and the optimum rate of agitation was 150 rpm.

Kinetics of DBP Degradation

The kinetics of DBP degradation by *P. fluorescens* B-1 at initial DBP concentrations from 2.5 to 10 mg/l was investigated, and the results are shown in Fig. 2. Exponential regression was applied to the data in Fig. 2 according to Eq. (1), and simulation indicated that the experimental data fitted the Monod first-order model very well; plots of ln [DBP] versus time were linear ($0.978<r^2<0.990$) (see the insert of Fig. 2), indicating first-order kinetics. This result agrees well with an earlier study [25]. The rate constant K obtained was 0.0349/h. Therefore, the first-order equation can be expressed as: ln C=−0.0349t+A, and the half-life of DBP degradation by *P. fluorescens* B-1 was 19.9 h.

Degradation of DBP and Its Degradation Intermediates

Selected reversed-phase HPLC chromatograms obtained during the course of DBP biodegradation experiments are shown in Fig. 3. In addition to the peak of the parent compound, two major transient metabolites were observed at HPLC retention times of 2.19 and 6.32 min. Other metabolites forming very small peaks could not be further characterized. The peak at retention time 2.19 min was identified as PA by comparison with the retention time and spectrum of authentic PA obtained with a diode array detector. The second peak at 6.32 min was estimated to be MBP by a similar approach. This was confirmed by comparing its GC-MS mass spectrum with the published mass spectrum of MBP in the database at NIST (National

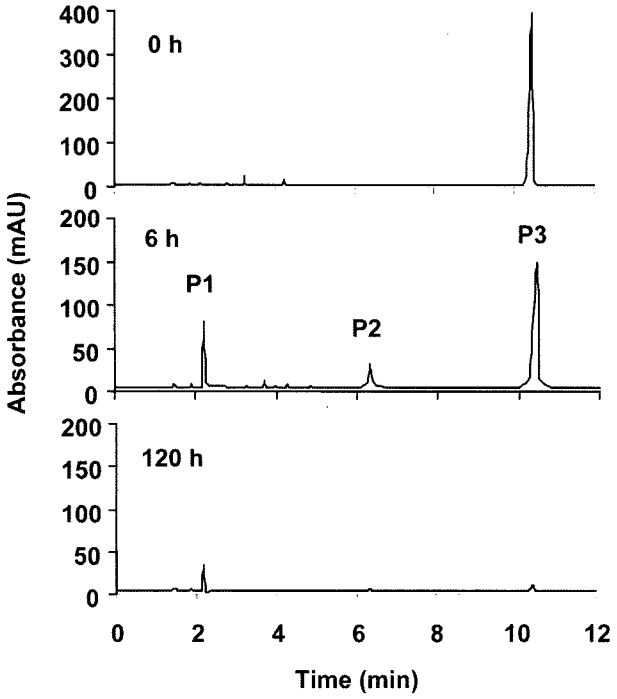

Fig. 3. Reverse-phase HPLC chromatograms at different times during DBP degradation. $[DBP]_0$=10 mg/l, T=30°C.
P1, phthalic acid (PA); P2, mono-*n*-butyl phthalate (MBP); and P3, di-*n*-butyl phthalate (DBP).

Institute of Standards and Technology). When the DBP degradation products were analyzed directly by GC-MS, the three main compounds were again identified as DBP, MBP, and PA (Table 1).

Decreasing concentrations of DBP and the transient appearance of the two intermediates were studied over a period of 120 h (Fig. 3). MBP was the major intermediate produced during the initial 6 h; its concentration remained relatively low and then fell below the level of detection. A small amount of PA was also produced during the first 6 h of degradation and it was also degraded over time. The detection of PA and MBP as intermediates in this study is consistent with previous reports under anaerobic [1] and aerobic [25] conditions. Based on the above results and the information available in the literature [5, 22], a biochemical pathway for the metabolism of DBP by *P. fluorescens* B-1 may be proposed (Fig. 4).

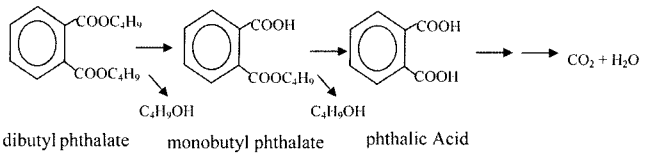

Fig. 4. Proposed pathway of DBP degradation by *Pseudomonas fluorescens* B-1.

Microbial metabolism is the principal route of removal of phthalate isomers and their esters from aquatic and terrestrial systems such as sewage, soils, sediments, and surface waters [22]. Biodegradation involves a series of reactions common to all phthalate carboxylic esters [1, 5, 27, 28] with hydrolysis of the ester linkage in the key initial step. Phthalate esters have the basic structure of esterified benzene-dicarboxylic acids with two alkyl chains, and primary biodegradation involves the sequential hydrolysis of the ester linkages between the alkyl chains and the aromatic ring, first forming the monoester and then PA [2, 12, 27–29]. Thus, the proposed degradative pathway for DBP involves sequential cleavage of the ester bonds to yield the phthalate monoester and then PA, which can be further metabolized to carbon dioxide and water [5, 10, 15, 27].

Biodegradation of DBP in the Seawater Microcosm by *P. fluorescens* B-1

The natural seawater taken from Mai Po Nature Reserve had the following characteristics: salinity, 22.21‰; pH, 6.8; PO_4^{3-}, 0.179 mg/l; NH_4^+, 5.54 mg/l; NO_3^-, 0.263 mg/l; NO_2^-, 0.221 mg/l; and SiO_2, 2.38 mg/l. In order to investigate the effectiveness of *P. fluorescens* B-1 in bioremediation of DBP-contaminated waters, we spiked this natural seawater with DBP at a concentration of the same order as that detected in coastal waters [8, 9] and inoculated it with *P. fluorescens* B-1. Figure 5 shows the resulting changes of DBP concentration. Ninety % of the DBP was eliminated in the first 96 h, compared with a decrease of less than 10% in the controls. The DBP was completely degraded in 144 h. At the same time, the change of concentration in seawater not inoculated with *P. fluorescens* B-1 was almost identical to that in the abiotic control (data not shown). From these results, it can be concluded that DBP was removed from the inoculated system by biodegradation.

Table 1. Identification of di-*n*-butyl phthalate and its two metabolites by gas chromatography-mass spectrometry.

Compound	Molecular ion *m/z*	Base peak *m/z*	Characteristic peak *m/z*	Retention time (min)
PA[a]	166	104	76, 104,	8.51
MBP[b]	222	149	41, 65, 105, 127, 149, 167	15.45
DBP[c]	278	149	41, 76,104, 149, 205, 223	28.61

[a]Phthalic acid.
[b]Mono-*n*-butyl phthalate.
[c]Di-*n*-butyl phthalate.

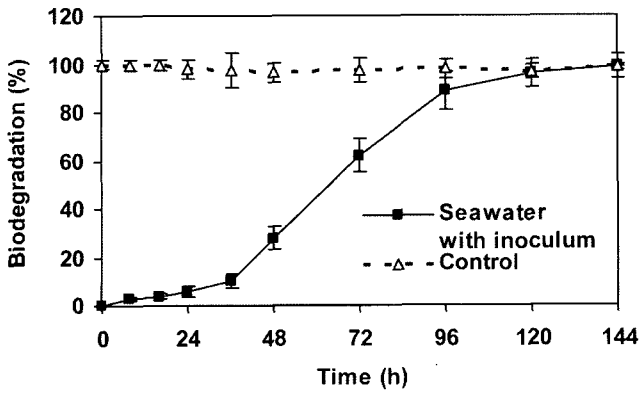

Fig. 5. Percentage of DBP degraded in seawater inoculated with *P. fluorescens* B-1. $[DBP]_0$=100 mg/l, T=21°C.
Data are averages of triplicate determinations, and vertical bars are standard deviations.

The degradation of DBP in natural seawater by *P. fluorescens* B-1 did not satisfactorily fit the first-order kinetic model. However, we obtained a high correlation coefficient (0.95) by fitting the experimental data to the integrated expression of a second-degree polynomial. The sigmoid form allowed three phases of degradation to be clearly defined: an initial phase of adaptation of the bacteria to the medium during which the concentration of DBP did not change appreciably, followed by a sharp and rapid decrease of the substrate, and finally a phase of slower degradation until the compound was completely eliminated.

In conclusion, a bacterium capable of utilizing DBP as a sole source of carbon and energy was isolated from the mangrove sediment and identified as *Pseudomonas fluorescens* B-1. Optimal degradation was observed when temperature, pH, salinity, and oxygen availability were 37°C, 7.0–8.0, 15–25%, and 150 rpm, respectively. Aerobic degradation of DBP by *P. fluorescens* B-1 conformed to the first-order kinetic model and the major metabolites were mono-*n*-butyl phthalate and phthalic acid. *P. fluorescens* B-1 also degraded DBP in natural seawater, indicating that this strain could be used in bioremediation of PAEs-contaminated environments.

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