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Short Communication

Aerobic degradation of diethyl phthalate by *Sphingomonas* sp.

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Abstract

An aerobic diethyl phthalate (DEP) degrading bacterium, DEP-AD1, was isolated from activated sludge. Based on its 16S rDNA sequence, this isolate was identified belonging to *Sphingomonas* genus with 99% similarity to *Sphingomonas* sp. strain C28242 and 98% similarity to *S. capsulate*. The specific degradation rate of DEP was concentration dependent with a maximum of 14 mg-DEP/(L h). Results of degradation tests showed that DEP-AD1 could also degrade monoethyl phthalate (MEP), dimethyl phthalate (DMP), dibutyl phthalate (DBP), and diethylhexyl phthalate (DEHP), but not phthalate and benzoate.

Keywords: 16S rDNA; Degradation; Diethyl phthalate; Endocrine disruptor; *Sphingomonas*

1. Introduction

Phthalates are a group of refractory organics used as additives in the manufacturing of plastics, polyvinyl acetates, celluloses, polyurethanes and polyvinyl chloride. They are also widely used in paints, adhesives, cardboard, lubricants and fragrances (Staples et al., 1997). Since phthalates are not chemically bound to the host polymers, they may leach from the host, and eventually migrate into the environment. They have been detected in landfill leachate, surface water, sewage sludge, and sediment. Phthalates not only are toxic and mutagenic, but may also have endocrine disrupting effects on aquatic species (Knudsen and Potinger, 1999).

Biodegradation is the dominant mechanism of phthalates degradation in surface waters, soils and sediments (Staples et al., 1997). Many studies have been reported on the biodegradation of dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) under either aerobic or anaerobic condition. Several aerobic pure cultures have been isolated for the degradation of butylbenzyl phthalate (BBP) (Chatterjee and Dutta, 2003), DEP (Chang et al., 2004) and DBP (Xu et al., 2005); however, many characteristics of these isolates remain unclear. In this study, an aerobic DEP-degrading culture was isolated from activated sludge. Its phylogeny and morphology were characterized by 16S rDNA-based and electron microscopic means. Its DEP-degrading kinetics and capability of degrading other phthalates were also investigated.

2. Methods

2.1. Culture enrichment and isolation

The DEP-degrading bacterium was first enriched from the activated sludge sampled from a local municipal wastewater treatment plant. Enrichment was carried out in a 2-L reactor operated in a sequencing batch mode, starting with 1 L of seed sludge and 1 L of feed solution containing 100 mg/L of DEP as the sole carbon source. Each batch cycle lasted 24 h, including 22 h of aeration, 1 h of settling and 1 h of drawing and refilling. At the end of each cycle, 1 L of supernatant was drawn and replaced with an equal volume of feed solution. DEP concentration in the feed solution was increased from 100 to 500 mg/L with 100 mg/L increments. Four cycles were operated for each DEP concentration. After a total of 20 cycles, the diluted solutions were spread onto the nutrient agar plates

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containing DEP as the sole carbon source. Colonies were picked and streaked onto individual agar plates for further purification. Only one isolate, DEP-AD1, was able to degrade DEP in the solution.

2.2. DNA extraction, PCR, sequencing, phylogenetic analysis

The DNA of the DEP-AD1 culture was extracted using a commercial extraction kit (Bacterial Xpress, CHEMICON®, CA). The extracted DNA was amplified by polymerase chain reaction (PCR), and then sequenced. The 16S rDNA sequences of DEP-AD1 and its closet references retrieved from GenBank were aligned and checked manually using BioEdit. Phylogenetic tree was then constructed using the neighbor-joining method with MEGA 2.1 (Liu et al., 2005).

2.3. Growth condition of DEP-AD1

In this study, biomass concentrations in the mixed liquor (expressed in mg-protein/L) were measured by the optical density (OD) at 600 nm using a UV spectrometer (Shimadzu UV-160A, Japan). The OD measurements were then converted to protein concentrations using an established calibration curve, for which protein concentrations were measured by the modified Lowry method. Experiments on effects of pH and temperature for the growth of DEP-AD1 were conducted in 50 mL tubes. Each tube was added with 30 mL of feed solution containing 200 mg/L of DEP and 1 mL of enriched culture with a protein content of 31.5 ± 3.8 mg/L. Tests on the effect of temperature were first examined at four levels, i.e. 20, 25, 35 and 45 °C, at pH 7. These were followed by tests on the effect of pH at seven levels from pH 3 to 9 at the identified optimal temperature.

2.4. Degradation of DEP and others

Afterwards, experiments on DEP degradation were carried at the identified growth pH and temperature in 250 mL shaking flasks. Each flask contained 2 mL of enrichment culture (31.5 ± 3.8 mg-protein/L) and 100 mL of fresh feed solution containing DEP at a concentration that varied from 40 to 79, 152, 312, 456 and 612 mg/L. Mixed liquor samples were sampled every 6–12 h for DEP and biomass concentration analyses. Experiments were also conducted to incubate DEP-AD1 in feed solutions containing individually other dialkyl-phthalates, including DMP, DBP and DEHP, plus three possible intermediates of DEP degradation, i.e. monoethyl phthalate (MEP), phthalate and benzoate, to see whether DEP-AD1 could degrade these compounds.

2.5. Chemical analysis

DEP was quantified using a HPLC (Shimadzu, Japan) with a UV detector (SPD-10AV, Shimadzu) at 254 nm. After centrifugation at 12,000*g* for 5 min, sample was separated using an Allsphere ODS-2 3u column (4.6×150 mm, particle size 3 μm) (Alltech, US). The mobile phase was a mixture of acetonitrile and water at the ratio of 48/52 (v/v) for DEP quantification, and 25/75 (v/v) for quantification of MEP and phthalate, the two degradation intermediates. The mobile phase was adjusted to pH 2.5 using H_3PO_4 and was delivered at a flow rate of 1 mL/min.

3. Results and discussion

3.1. Microbial identification and phylogenetic analysis

Based on BLAST analysis of its 16S rDNA sequence (DQ010645), DEP-AD1 seemed to be a species in the *Sphingomonas* genus. Recently, Chang et al. (2004) also reported the isolation of a strain of *Sphingomonas* from river sediment that was capable of degrading eight phthalic esters, including DEP. The genus *Sphingomonas* forms a phylogenetically tight group in the α-4 subclass of the *Proteobacteria* (Takeuchi et al., 1994). It exists widely in various environments including soil (Xia et al., 2005), sediment (Fredrickson et al., 1995), natural water (Tabata et al., 1999), and wastewater (Neef et al., 1999). *Sphingomonas* may also degrade aromatic chemicals (Borde et al., 2003) and adsorb cadmium (Tangaromsuk et al., 2002). Fig. 1 illustrates the phylogenetic relationship of DEP-AD1 with species of close similarity. Among them, *Sphingomonas* sp. strain C28242 (99% of similarity based on 1258 bps) was isolated from paper-making chemicals. *S. capsulata* and *Novosphingobium* sp. K39 have the ability to degrade toluene, naphthalene (Fredrickson et al., 1995) and chlorophenol (Mannisto et al., 1999).

3.2. Growth conditions of DEP-AD1 and its degradation of DEP

DEP-AD1 grew well at pH 7 and temperatures ranging from 25 to 35 °C. A prolonged lag phase was needed for DEP degradation at 20 °C, and little DEP was degraded at 45 °C. On the other hand, DEP-AD1 grew well at 25 °C at the slight acidic condition of pH 4–7, but could hardly grow at pH 3 or pH 8 and 9. Degradation experiments were conducted for DEP at 25 °C and pH 6.0 with initial concentrations varying from 39 to 610 mg/L. Results in Fig. 2 shows that the degradation of DEP followed the

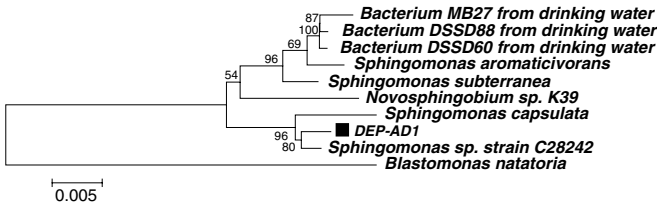

Fig. 1. Phylogenetic relationship of the DEP-AD1 to others.

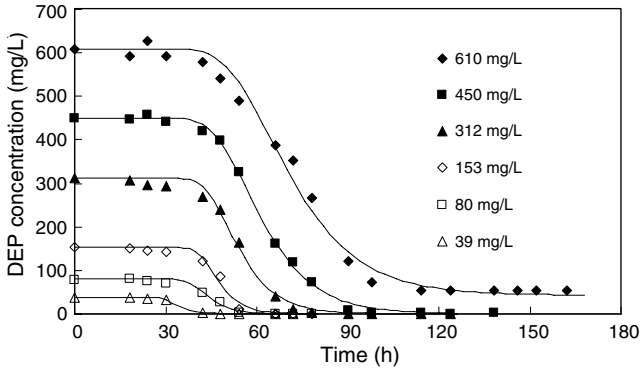

Fig. 2. Best-fitted curves for DEP degradation by the modified Gompertz model.

Table 1
Kinetic parameters of aerobic DEP degradation at 25 °C and pH 6.0

Initial concentration (mg/L)	λ (h)	μ_m (mg-DEP/(L h))	A (mg/L)	R^2
39	28	3.6	39	0.995
80	37	5.6	80	0.991
153	40	10.6	153	0.986
312	42	13.7	312	0.996
450	45	14.0	450	0.998
610	49	13.5	567	0.991

modified Gompertz model (Zwietering et al., 1990), which could be expressed as follows:

$$S = S_0 - A \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A} \cdot (\lambda - t) + 1\right]\right\} \quad (1)$$

where S was substrate concentration at time t, S_0 the initial concentration, A the degradation potential, μ_m the maximum degradation rate, and λ lag phase. The best-fit kinetic parameters compiled in Table 1 shows that degradation of DEP required a lag time of 30–50 h and the degradation rate was concentration dependent with a maximum of 14 mg-DEP/(L h). The cell yield was estimated as 0.21 ± 0.02 mg-protein/mg-DEP, determined from the ratio between the protein concentration increase and the DEP concentration decrease in the mixed liquor.

For comparison, degradation of DEP at low concentrations was found following the first-order kinetics by a strain of *Sphingomonas* (Chang et al., 2004), and by mixed cultures in sediment (Yuan et al., 2002) and in activated sludge (O'Grady et al., 1985). On the other hand, degradation of DEP at moderate concentrations of 50–400 mg/L by an isolate from sewage sludge followed the Monod kinetics (Reardon and Zhang, 1992) with a maximum specific rate of 0.4 h^{-1} for DEP utilization and a half rate constant of 28 mg-DEP/L.

3.3. Degradation of dialkyl-phthalates by DEP-AD1

In all DEP degradation tests, the mixed liquor contained residual DEP, plus MEP and phthalate, without any fatty acids or other organic intermediates. This indicated that DEP was degraded by DEP-AD1 by de-esterification, as suggested by Staples et al. (1997). Results of 20-day incubation tests showed that DEP-AD1 was able to grow in solutions containing DMP (initial concentration 194 mg/L), DBP (139 mg/L), MEP (194 mg/L) or DEHP (78 mg/L), but not in phthalate (160 mg/L) and benzoate (144 mg/L). This showed that DEP-AD1 was able to degrade some other dialkyl-phthalic esters, probably by de-esterification, but was unable to de-aromatize the phthalate.

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