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Microbial Degradation of the Endocrine-Disrupting Chemicals Phthalic Acid and Dimethyl Phthalate Ester Under Aerobic Conditions

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Synthetic chemicals may be a threat to ecological processes due to persistence and bioaccumulation, and recently they have been found to disrupt the normal development of organisms. Phthalates are widely used as additives in plastic manufacturing and to improve mechanical properties of the plastic resin, particularly flexibility (ECETOC 1988; Giam et al. 1984; Nilsson 1994). However, in order to provide the required flexibility, the phthalate plasticizer is not bound covalently to the resin and is able to migrate into the environment (Nilsson 1994). Dimethyl phthalate ester is typically used in cellulose ester-based plastics, such as cellulose acetate and cellulose butyrate (Staples et al. 1997). Plasticizers are also widely used in building materials, home furnishings, transportation, clothing, and to a limited extent in food and medical products. Because of the global utilization of plasticized polymers at large quantities, phthalates and their esters have been detected in every environment in which they have been sought for (Giam et al. 1984). Some of phthalates and their degradation intermediates are suspected to cause cancer and kidney damage and have been added to the list of priority pollutants by the US Environmental Protection Agency (US EPA 1992). Known as endocrine-disrupting chemicals, selective phthalates may also interfere with the reproductive system and normal development of animals and humans (Allsopp et al. 1997; Gray et al. 1999; Jobling et al. 1995).

Microbial degradation is believed to be the principal route for complete destruction of phthalate in the environments (Staples et al. 1997). Considerable research has been conducted on the molecular biology and organization of degradative genes in selective bacteria over the last decade (Chang & Zylstra 1998; Kleerebezem et al. 1999; Roslev et al. 1998; Stahl & Pessen 1953; Sugatt et al. 1984; Wang et al. 1996). However, few studies have been focused on the pathway description of phthalates degradation, and fewer on the degradation ability and processes of bacteria. Therefore, the objectives of this study were: 1) to isolate and identify bacteria in pure culture capable of phthalate degradation from an activated sludge; and 2) to assess the ability of the bacteria in degrading PA and DMP.

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MATERIALS AND METHODS

The initial bacterial culture was established by incubating 10 ml activated sludge from the wastewater treatment facility at the Swire Institute of Marine Science, 200 ml of a mineral salt medium with phthalic acid (400 mg/l) or dimethyl phthalate (100 mg/l) in a 250 ml Erlenmeyer flask as the sole source of carbon and energy. The mineral salt medium 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 initial pH of the culture medium was adjusted with HCl or NaOH to 7.0 ± 0.1. The flasks were incubated in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, New Jersey, USA) kept at 150 rpm and 30.0 ± 0.5°C in the dark. The initial PA or DMP-degrading enrichment was transferred once a week (approximately) on the basis of depletion (more than 85%) of the substrate compound by transferring 40 ml of the culture to a new flask containing 160 ml of freshly made mineral salt medium with gradually increasing concentrations of PA (from 400 to 1000 mg/l) or DMP (from 100 to 500 mg/l). Each of the cultures was transferred more than 20 times prior to be used in the isolation of bacteria.

In order to achieve well-separated individual colonies, it was necessary to lower the density of bacteria in mineral salt medium before transferring 100µl onto the nutrient agar plates (Difco Lab., Detroit, Michigan). After 48 hr of incubation at 30°C, a number of individual colonies of different bacterial types were visible. Colonies of different morphological appearance were streaked onto fresh nutrient agar plates and incubated at 30°C for 48 hr. Newly developed colonies on the streaked plates were assessed based on colony morphology and microscopic observation for purity. When purified, bacteria were Gram stained and identified using API 20NE Multitest System following the instructions of the manufacture (bioMerieux, Marcy l'Etoile, France).

Each isolated bacterial species and their different combinations were then used and tested for their degradation ability on PA or DMP. One bacteria species was identified and found capable of mineralizing PA, while two reconstituted consortia showed the ability to degrade DMP. The species capable of degrading PA was exposed to a series of PA concentrations, including 300, 600, 900, 1200 mg/l. The two consortia were subjected to DMP concentrations of 400 and 600 mg/l respectively.

All biodegradation experiments were carried out at 30°C in the dark. Experiments on PA degradation were conducted in 250ml Erlenmeyer flasks with 200ml mineral salt medium (as described above) and different concentrations of PA. The initial pH value was adjusted to neutral pH 7.0 $\pm$ 0.1. DMP biodegradation experiments were carried out in 100ml flasks with 100 ml mineral salt medium (as described above) and different concentrations of DMP. Both tests for PA and

DMP were conducted in triplicate. Periodically culture aliquot (2 ml) from each flask was withdrawn by syringe aseptically and stored frozen (-20°C) in a glass vial until analyzed. Sterile controls were prepared by autoclaving for 20 min on three successive days before introduction of the substrate using 0.2-μm-pore-size membrane filter on a syringe (Pall Gelman Laboratory, Ann Arbor, Michigan).

In preparation for HPLC analysis, thawed culture samples from PA and DMP degradation experiments were centrifuged and filtered through PVDF or Nylon Acrodisc Minispike syringe filters (0.2-μm-pore-size). The first five drops were discarded to avoid the influence of phthalates or metabolites adsorption onto membranes. Samples were separated and quantified on an Agilent 1100 series HPLC system (Agilent Technologies, Hewlett-Packard, California) consisting of a quaternary low-pressure degasser, a quaternary high-pressure pump, a model 7725*i* manual sample injector with a 20 μl sample loop, and diode array and multiple wavelength detectors. Separation of parent compounds and metabolites was accomplished by using a 4.6×150 mm Eclipse 5-μm XDB-C8 reversed-phase liquid chromatography column (Agilent Technologies). Methanol-water (1:1, v/v) delivered at a flow rate of 1.0 ml/min was used as the mobile phase in the HPLC analysis of DMP and mono-methyl phthalate (MMP). The mobile phase for separation of PA consisted of methanol–0.02 mol/l H_3PO_4 (pH 3.0) (1:3, v/v). PA, MMP and DMP were quantified by the external standards method at wavelength of 280 nm. The calibration curves were linear for these compounds in the range from 10 to 1000 mg/l. The UV-visible spectra were recorded at identical retention times to confirm the identification of these compounds.

The microbial biomass was determined by optical density measurements at 600 nm spectrophotometrically using an UV 1201 (Shimadzu Co., Kyoto, Japan). Only the upper suspended aliquot was measured if flocs were observed in the culture flask.

RESULTS AND DISCUSSION

A total of five bacteria were isolated from the sewage sludge during incubation with PA and DMP. One bacterium capable of utilizing PA as a sole carbon and energy source was identified as *Comamonas acidovorans* strain Fy-1 (id% = 99.5%). None of the other four bacteria were capable of degrading PA and their combinations were tested for the capability of degrading DMP. Two consortia showed the ability to use DMP as the sole carbon and energy source. Consortium I was consisted of *Pseudomonas fluorescens* (id% = 95.5%), *Pseudomonas aureofaciens* (id% = 90.0%), and *Sphingomonas paucimobilis* (id% = 92.9%), while Consortium II consisted of two bacteria species, namely *Xanthomonas maltophilia* (id% = 96%) and *Sphingomonas paucimobilis.*

The time course of PA degradation at different concentrations (i.e. 300, 600, 900, 1200mg/l) and the growth of bacterial biomass are shown in Fig. 1. It can be seen

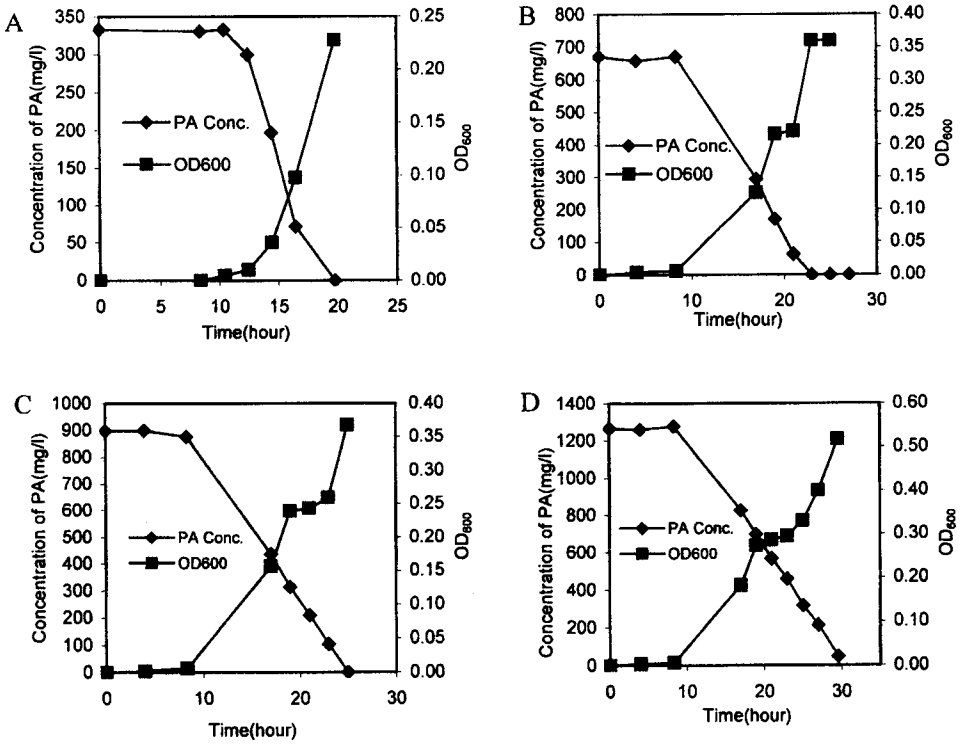

Figure 1. Degradation of PA by *Comamonas acidovorans* strain Fy-1 and the bacterial growth at different PA concentrations. (**A** to **D** represent PA concentrations from 350 to 1200 mg/l).

that the degradation of PA by *Comamonas acidovorans* strain Fy-1 generally had a lag time around 0.5 day depending on the initial concentrations. As the PA concentrations increased, the time needed to complete degradation also increased, ranging from 0.8 to 1.9 days. At each PA concentration, the biomass of bacterial cells increased as PA was degraded, which demonstrated the ability of *C. acidovorans* strain Fy-1 to use PA as the sole carbon and energy source effectively.

Comamonas acidovorans strain Fy-1, which was isolated from the sewage sludge, grew well in a mineral salt medium supplemented with different concentrations of PA. As shown in Fig. 1, the biomass of *C. acidovorans* strain Fy-1 increased accordingly with the depletion of PA. Thus, it is evident that PA was used by *C. acidovorans* strain Fy-1 as the sole carbon and energy source. *C. acidovorans* strain Fy-1 was previously reported to be able to degrade polyurethane, which is a class of plastics (Allen et al. 1999). This bacteria species, together with other species such as *Acinetobacter calcoaceticus*, *Achromobacter* sp., *Pseudomonas* sp., *Flavobacterium devorans*, *Bacillus lentus*, *Bacillus mascerans* and *Bacillus*

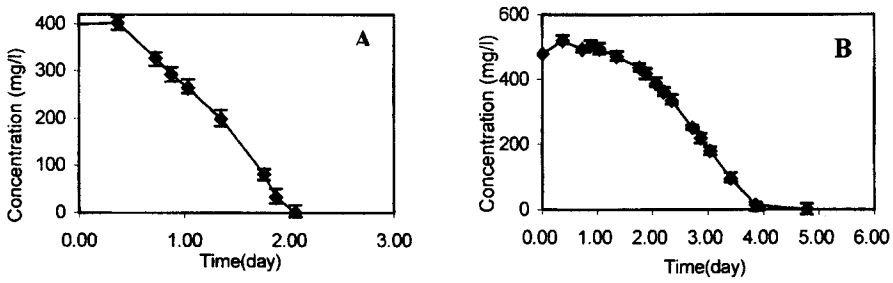

Figure 2. DMP degradation by Consortium I (A) and Consortium II (B). Error bars in both diagrams represent standard deviation.

thuringiensis, has also been tested for the ability to remove 75% of polychlorinated biphenyls (PCBs, mostly penta to heptachlorinated isomers) (88% w/v in the transformer oil) from transformer oil (Rojas-Avelizapa et al. 1999). However, there are few studies so far reported the capability of *C. acidovorans* to use PA as the sole carbon and energy source. Our results provide evidence that a naturally occurring aerobic microorganism in the sludge has the potential to completely mineralize high concentration of PA (1200 mg/l) within a very short period of time (1.3 days). Despite the difference in lag time when C. acidovorans strain Fy-1 was fed at various concentrations of PA, the later degradation phase for each degradation curve was almost linear. This phenomenon suggests that once the corresponding enzyme system for PA degradation is induced and activated, they act fast and the concentrations of substrate play a relatively minor role.

Fig. 2a shows that 400 mg/l DMP was completely degraded by the Consortium I within 2 days. Similarly, the Consortium II was found to completely degrade 500 mg/l DMP in less than 5 days (Fig. 2b). The concentrations of both PA and DMP in the sterilized controls were constant during the experiments, which indicated that the effects of natural hydrolysis and photolysis on degradation of PA and DMP were negligible.

Although the wastewater incubation was capable of utilizing DMP as the sole carbon and energy source, there was no single bacterial species isolated from it for such capability. The reason for this phenomenon may due to the fact that DMP degradation requires the cooperation among various enzymes that belongs to different bacterial species. This hypothesis was partially supported by the later part of the results in this study, that two consortia of isolated bacteria species showed the ability to use DMP as the sole carbon and energy source and completely mineralization was achieved within a short period of time (Fig. 2). The compositions of those two consortia were *Pseudomonas fluorescens, Pseudomonas aureofaciens,* and *Sphingomonas paucimobilis* for Consortium I and *Xanthomonas maltophilia* and *Sphingomonas paucimobilis* for Consortium II.

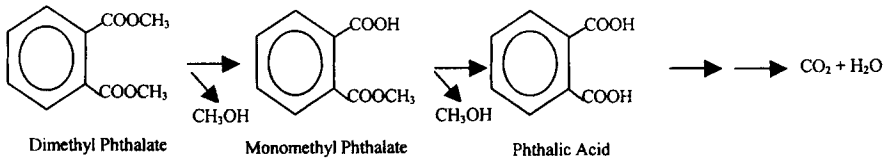

Figure 3. Proposed metabolic pathway of dimethyl phthalate (DMP) degradation by bacterial consortia isolated from an activated sludge of wastewater treatment plant

Both of them consisted the bacteria species *Sphingomonas paucimobilis*, which suggests that the enzyme system in *Sphingomonas paucimobilis* should have an important role in the course of DMP degradation, especially the degradation of PA. Surprisingly, *Comamonas acidovorans* strain Fy-1 was not required in either of the consortia.

A UV-visible spectral library of DMP and its possible metabolite standards were created using the Agilent diode array and multiple wavelength detector systems. Subsequently, the metabolites during degradation of DMP were identified when both the retention time on HPLC and UV-visible spectrum of a metabolite matched with the standard. In doing so, two intermediates were identified as mono-methyl phthalate (MMP) and PA during DMP degradation by both consortia from an activated sludge under aerobic condition. The proposed metabolic pathway is constructed and shown in Fig. 3. Degradation of DMP followed two steps of demethylation resulting in monomethyl phthalate and PA before the cleavage of aromatic ring.

The aerobic biodegradation of DMP involves several steps of transformation before the substrate becomes fully mineralized. The microbial metabolism of DMP by the acclimated consortia in our investigation was initiated by an initial ester hydrolysis to form MMP and methanol, and followed by a further hydrolysis of MMP to PA presumably by the same hydrolytic enzyme (Fig. 3). The two steps are acid-producing processes and the pH value of the culture medium is expected to show a decrease unless the acidic organic intermediates, i.e. MMP and PA, can be removed quickly from the medium. Degradation of PA is an acid-consuming process in which organic acid, PA, is mineralized to CO_2 and water. There is one recent report from this laboratory (Fan et al, 2001) stating that DMP was only partially (<40%) degraded under mono-substrate condition, although PA was degraded completely at both single and dual (with DMP) substrates conditions using an acclimated culture. That fact can be attributed to the formation of MMP and PA as intermediates resulting in a decrease of the pH value to approximately 3 in the culture medium. Such low pH was shown to be inhibitory to the microorganisms in the culture for any further degradation of DMP to occur (Fan et al. 2001). At the same time, accumulation of high concentrations of degradation intermediates also made the metabolic processes

from DMP to MMP and then to PA unfavorable thermodynamically due to concentration effects. DMP-degrading enzymes including esterase may also be sensitive to the low pH in the culture medium. As a result, the degradation process was almost halted completely after the first 4 days when DMP was the sole source of carbon and energy in the culture medium in the previous study (Fan et al. 2001).

However, there is no apparent decrease of pH value during the degradation of DMP by the two reconstituted consortia in this study. Therefore, the intermediates MMP and PA should be removed quickly from the system. This result provided the evidence that once the enzyme system of bacteria within both consortia were activated, they were capable to remove the intermediates fast enough so that the pH value would not drop to their sensitive level during the degradation of DMP. This characteristic of the consortium in this study is highly desirable because the mineralization of DMP could be achieved smoothly within very short period of time.

Although the only bacterial species demonstrated the ability to degrade PA in this study was not included in either consortium and none of the bacteria in the consortia was capable to degrade PA by themselves, the DMP degradation processes were not affected by the accumulation of PA, one of the intermediates. The reason for this may be explained by the activation of the enzyme system for DMP, the induced enzymes in the consortia can further degrade intermediates produced in the former reactions. Those enzymes cannot be activated and induced by PA alone. As shown in Fig. 3, it is apparent that hydrolysis of ester bond is the initial step in the degradation of DMP producing sequentially MMP and PA before the cleavage of the aromatic ring. Dioxygenases have been known for their ability to cleavage the aromatic ring (Carwrithgt et al. 2000) and it is possible that the site of cleavage is more likely between the two carboxyl groups. The metabolic pathway proposed in this study is identical with previous studies (Nomura et al. 1989; Staples 1997; Wang et al. 1997).

In summary, degradation of PA has been achieved at a concentration as high as 1200 mg/l in less than 2 days using a single bacterial species, *Comamonas acidovorans* strain Fy-1, which was isolated from wastewater. Two consortia showed capability of degrading DMP to MMP and PA as the intermediates before the cleavage of the aromatic ring. It is clear that a highly efficient system can be constructed for degradation of PA and DMP using natural microorganisms.

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