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Biodegradation of an endocrine-disrupting chemical di-*n*-butyl phthalate ester by *Pseudomonas fluorescens* B-1

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Abstract

Di-*n*-butyl phthalate ester (DBP) is known as an endocrine-disrupting chemical. A pure culture capable of using DBP as the sole source of carbon and energy from mangrove sediment was identified as *Pseudomonas fluorescens* B-1. Microbial degradation of DBP was studied in batch experiments for several environmental factors. The effect of initial DBP concentrations on the degradation was investigated between 2.5 and 10.0 mg l^{-1}, and the results showed that the biodegradation process conformed to the first-order kinetic model. The pH value of the culture medium also played an important role in the biodegradation of DBP, the optimum pH being 7.0. The effects of temperature and oxygen availability on the kinetics of DBP biodegradation were also determined. Degradation of DBP by *P. fluorescens* B-1 was quantified by reversed-phase high-performance liquid chromatography after solid-phase extraction. Two metabolites of DBP degradation were identified as mono-butyl phthalate and phthalic acid by gas chromatography-mass spectrometry. The results suggest that DBP can be degraded by indigenous microorganisms from the mangrove environment.

Keywords: Di-*n*-butyl phthalate; Biodegradation; Kinetics; Metabolites; Endocrine-disruptor; Plasticizer

1. Introduction

In recent years, phthalate esters have attracted increasing attention owing to their widespread use, ubiquity in the environment, and endocrine-disrupting activity (Fatoki and Ogunfowokan, 1993; Wang et al., 2004). Phthalate esters are synthetic compounds used as plasticizers to improve mechanical properties of the plastic resin, particularly flexibility (Cartwright et al., 2000). Plasticizers are used predominantly in building materials, home furnishings, transportation, clothing, and to a limited extent in food and medical product packaging (Niazi et al., 2001). Release of phthalates into the ecosystem or wastewater effluent occurs during the production phase and via leaching and volatilization from plastic products during their usage and/or after disposal (Psillakis et al., 2004). Even at very low concentration they are suspected of interfering with reproductive systems and behaviour in humans and wildlife, through disturbance of the endocrine system (Jobling et al., 1995; Colon et al., 2000; Petrović et al., 2001). As a result, several regulatory bodies, such as the US Environmental Protection Agency (US EPA, 1991), the European Union (1993) and China National Environmental Monitoring Center (Wang et al., 1995) classified phthalate esters as a top priority pollutant for risk assessment, mandating the reduction and control of phthalate pollution.

Recently, many studies on phthalate and esters mainly focused on the biodegradability of isomers of dimethyl phthalate ester (Patel et al., 1998; Niazi et al., 2001; Vega and Bastide, 2003; Wang et al., 2003a,b; Fan et al., 2004; Gu et al., 2004; Wang et al., 2004), butyl-benzyl phthalate by pure cultures of microorganisms (Kim et al., 2002; Chatterjee and Dutta, 2003), and the biodegradability and removal of refractory di-(2-ethylhexyl) phthalate in wastewater treatment systems (Ejlertsson et al., 1996; Roslev et al., 1998; Cheng et al., 2000; Juneson et al., 2001; Marttinen et al., 2003). Only a few papers on the biodegradability of DBP by pure culture have been published (Benckiser and Ottow, 1982; Eaton and Ribbons, 1982; Wang et al., 1995, 1999). However, DBP is one of the most frequently identified phthalates in diverse environmental samples including

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groundwater, river water, drinking water, ocean water, soil, lake sediment and marine sediments and it is the most widely used plasticizer worldwide including China (Wang et al., 1995).

The objectives of this investigation were to isolate microorganisms capable of degrading DBP, characterize the biochemical degradation pathway, and elucidate the environmental factors influencing the degradation process.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical-reagent grade, and purified water by the Milli-Q system was used throughout the experiments. Di-*n*-butyl phthalate was from ACROS Organics (Geel, Belgium), HPLC-grade methanol from TEDIA Co. (Fairfield, Ohio, USA) and all other reagents from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Enrichment culture and culture medium

The initial enrichment culture was established by inoculating a 250-ml Erlenmeyer flask containing 100 ml mineral salts medium (MSM) supplemented with DBP (1 mg l^{-1}) as the sole carbon and energy source with 5 g fresh sediment from Mai Po Nature Reserve in Hong Kong. The MSM comprised (mg l^{-1}): $(NH_4)_2SO_4$ 1000; 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. The pH of the culture medium was adjusted with HCl or NaOH to 7.0 ± 0.1 or as otherwise specified. The flasks were incubated in the dark in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, New Jersey, USA) operating at 150 rpm and 30.0 ± 0.5°C. The DBP-degrading cultures were obtained through enrichment transfer at approximately 1-week intervals on the basis of depletion of DBP, by transferring 1.0 ml of the active culture to a new Erlenmeyer flask containing 100 ml of freshly made MSM with gradually increasing concentrations of DBP (1–10 mg l^{-1}). The DBP-degrading enrichment cultures were transferred more than 10 times prior to the isolation of bacteria from the enrichment cultures.

2.3. Isolation of microorganism

Bacteria in the enrichment culture showing ability in degrading DBP were diluted in MSM before plating on the nutrient agar (NA) plates (Difco Lab., Detroit, Michigan). After 48 h of incubation at 30°C, a number of well-separated individual colonies of different morphological types appeared and were further streaked onto fresh NA plates (Difco Lab., Detroit, Michigan) to purify the cultured organisms. Pure cultures were used subsequently for Gram-staining and then identification using API 20 NE Multi-test System (bioMerieux, Marcy 1'Etoile, France) as described elsewhere (Wang et al., 2003b).

2.4. Biodegradation of DBP

All flasks were incubated at 30°C on a rotary shaker operated at 150 rpm in the dark or otherwise specified. Experiments on DBP degradation were conducted in 100-ml Erlenmeyer flasks with 50 ml MSM. All tests were conducted in triplicate. Sterile controls were prepared by autoclaving before introduction of DBP, which passed through a membrane filter 0.2-μm pore size (Pall Gelman Laboratory, Ann Arbor, Michigan). Culture medium (15 ml) was centrifuged and the supernatant was passed through a 0.2-μm membrane filter prior to solid-phase extraction (SPE).

2.5. Solid-phase extraction procedure

A Waters Sep-Pak C_{18} cartridge (500 mg, polypropylene) was conditioned by 2 ml of methanol, and then 5 ml of water. Ten millilitres of filtrate was acidified to pH 2 with 0.01 N HCl, and then passed through the cartridge at a flow rate of 2 ml min^{-1}. The analytes retained on the SPE cartridge were eluted with 2 ml of methanol, and eluate was evaporated under pure nitrogen gas to dryness. Finally, the residue was dissolved in 0.5 ml methanol prior to quantification by high-performance liquid chromatography (HPLC) with an ultra-violet detector or gas chromatography-mass spectrometry (GC-MS). The recoveries of DBP and PA after SPE were 99% and 92%, respectively.

2.6. Analysis of DBP and its metabolites

The Agilent 1100 series HPLC consisting of a G1322A degasser, a G1311A QuatPump, a G1316A COLCOM and a G1315B diode array detector (DAD, Agilent, USA) set at 210 nm, was used for the quantification of DBP concentration. A personal computer equipped with HP ChemStation (Hewlett Packard, USA) was used to acquire and process chromatographic data. 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 methanol:water:50 mM phosphoric acid mixture (70:25:5, v/v) at pH 2.35 and the flow rate was 1 ml min^{-1}. Under these chromatographic conditions, baseline separation could be obtained within 12 min for DBP and its metabolites. The peak area was integrated and used as the analytical signal for quantification. All compounds studied were quantified using external standards.

DBP degradation metabolites were identified using a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, USA) equipped with an Agilent 5973 mass selective detector (Agilent, USA). The column used was a HP-624 silicone-coated, fused-silica capillary column

(25.0 m × 0.2 mm i.d., 1.12 μm film thickness). The temperature program consisting of a 5-min hold at 50°C, an increase to 280°C at 10°C min^{-1}, and a 15 min hold at 280°C. The injection volume was 1 μl and the carrier gas was helium (1.0 ml min^{-1}). The mass spectrometer was operated at an electron ionization energy of 70 eV. Instrumental library searches, comparison with available authentic compounds, and mass fragmentation pattern were used to identify the suspected metabolites.

3. Results and discussion

3.1. Biodegradation of DBP by Pseudomonas fluorescens B-1

DBP was degraded quickly during the enrichment process without an apparent lag period at the beginning of degradation. One bacterial species capable of utilizing DBP as the sole source of carbon and energy was isolated from the culture medium and was identified as *P. fluorescens* B-1 with 94.9% similarity by API 20NE system.

In order to determine the effect of initial DBP concentrations on degrading efficiency, *P. fluorescens* B-1 was inoculated and cultured at a range of DBP concentrations. During incubation, DBP was utilized for microbial growth and cell maintenance. The biodegradation of DBP by *P. fluorescens* B-1 at initial concentrations of 2.5–10 mg l^{-1} is shown in Fig. 1. A number of models have been applied to describe the biodegradation of organic pollutants, and the exponential functions (pseudo first-order kinetics) are frequently used as a convenient way to describe the biodegradation progress for xenobiotic compounds at low substrate concentrations (Nyholm et al., 1996). The DBP biodegradation by *P. fluorescens* B-1 is assumed to fit to the Monod first-order kinetic equation, which has the following form:

$$\ln C = -Kt + \mathrm{A}, \tag{1}$$

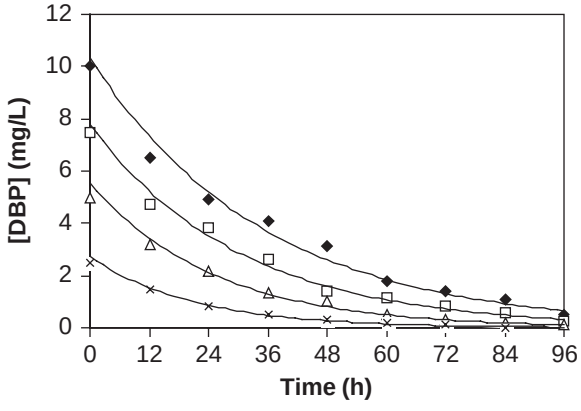

Fig. 1. Effect of initial concentration of di-*n*-butyl phthalate (DBP) on degradation by *P. fluorescens* B-1 at 30°C and pH 7.0. Initial concentrations of DBP used (mg l^{-1}) were: 10(♦), 7.5(□), 5(△), 2.5(×).

Table 1
Kinetic equations of di-*n*-butyl phthalate biodegradation by *P. fluorescens* B-1

Initial concentration (mg l^{-1})	Kinetic equations	Rate constants (h^{-1})	$t_{1/2}$ (h)	r^2
2.5	$\ln C = 1.005 - 0.0482t$	0.0482	14.38	0.9733
5.0	$\ln C = 1.712 - 0.0401t$	0.0401	17.28	0.9934
7.5	$\ln C = 2.052 - 0.0330t$	0.0330	21.00	0.9829
10.0	$\ln C = 2.335 - 0.0287t$	0.0287	24.15	0.9767

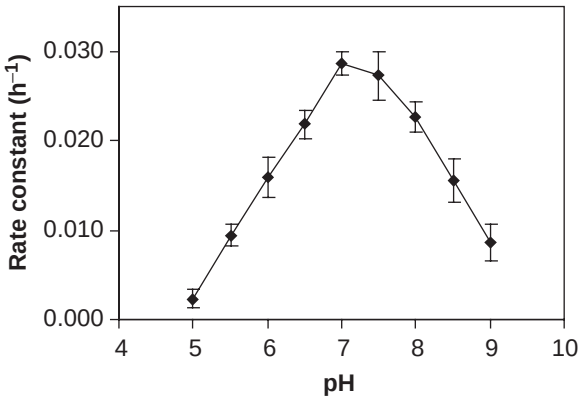

Fig. 2. Effect of pH on di-*n*-butyl phthalate degradation rate constant by *P. fluorescens* B-1 at 30°C and the initial concentration $[DBP]_0$ 10 mg l^{-1} calculated using the first-order kinetic equation.

where C is the initial concentration of DBP; t is time; K is the first-order rate constant and A is a constant.

Then the half-life of the DBP biodegradation by *P. fluorescens* B-1 can be expressed as

$$t_{1/2} = \ln 2/K. \tag{2}$$

The kinetic equations for the DBP biodegradation by *P. fluorescens* B-1 are listed in Table 1. The relationship between the different initial substrate concentrations and the initial degradation rates was linear ($r^2 > 0.97$). As substrate concentrations increased from 2.5 to 10.0 mg l^{-1} in the culture, the half-life value also showed an increase from approx. 14 to 24 h, respectively.

3.2. Effect of pH

The hydrogen ion concentration in the culture medium greatly influences the bacterial growth since pH value limits activity of enzymes. Degradation of *ortho*-dimethyl phthalate (*o*-DMP) was particularly sensitive to low pH (Wang et al., 2003a; Fan et al., 2004; Gu et al., 2004). Accumulation of degradative intermediates such as phthalic acid also acidifies the culture medium, thereby inhibiting the further bacterial degradation of intermediates (Wang et al., 2003b). The relationship between the degradation rate constants and pH for *P. fluorescens* B-1 is shown in Fig. 2. The rate constants of DBP degradation increased quickly when pH value

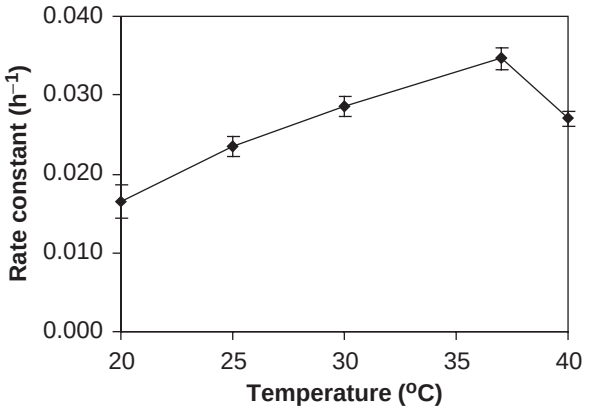

Fig. 3. Effect of temperature on di-*n*-butyl phthalate degradation by *P. fluorescens* B-1 at pH 7.0 and an initial concentration $[DBP]_0$ 10 mg l^{-1}.

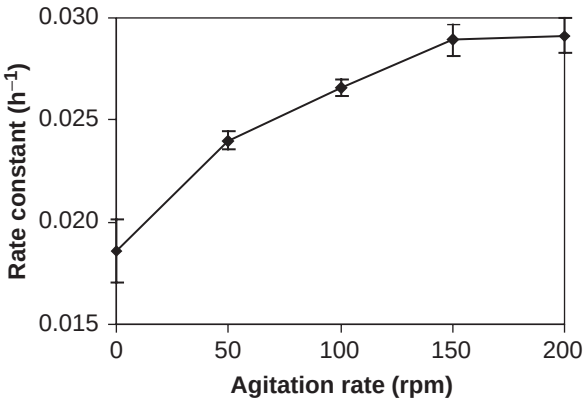

Fig. 4. Effect of agitation rate on di-*n*-butyl phthalate degradation by *P. fluorescens* B-1 at initial concentration $[DBP]_0$ 10 mg l^{-1}, pH 7.0 and 30°C.

of the culture was increased from 5.0 to 7.0. A high rate constant was achieved for *P. fluorescens* B-1 at pH 7.0. When the pH was 7.5, its rate constant was 0.0272 h^{-1}, a slight decrease from the peak rate constant of 0.0286 h^{-1}. However, at pH > 7.5 the rate constants decreased sharply. De-esterification of phthalate esters generates acidic intermediates, which may accumulate, especially when the complete degradation cannot be achieved by the microorganism (Eaton and Ribbons, 1982; Sivamurthy et al., 1991; Kurane, 1997; Wang et al., 2003b). The pH selected for degradation by *P. fluorescens* B-1 for subsequent experiments was pH 7.0.

3.3. Effect of temperature

When the effect of temperature on DBP degradation by *P. fluorescens* B-1 was assessed (Fig. 3), the rate constants increased with the increase of temperature between 20°C and 37°C. Higher temperature resulted in the lowering of the degradation rate constant; the optimum was 37°C, at which temperature the degradative enzyme reached the highest activity for the temperature tested in this study.

3.4. Effect of oxygen availability

When the effect of dissolved oxygen content on DBP degradation by *P. fluorescens* B-1 was determined by comparing the kinetics under different agitation conditions (Fig. 4), increase in agitation rate up to 150 rpm resulted in an increase in rate constants calculated from the degradation data. As *P. fluorescens* B-1 is an aerobic microorganism, dissolved oxygen serves as an essential electron acceptor, plays an important role in the growth physiology of the microorganism, and furthermore is important in the degradation of DBP. Better mass transfer efficiency, resulting from a higher mixing rate, may lead to higher dissolved oxygen content in the culture medium. However, further increasing the agitation rate to 200 rpm did not result in further improvement of the rate constant. Therefore, a rate of 150 rpm was selected for the experiment.

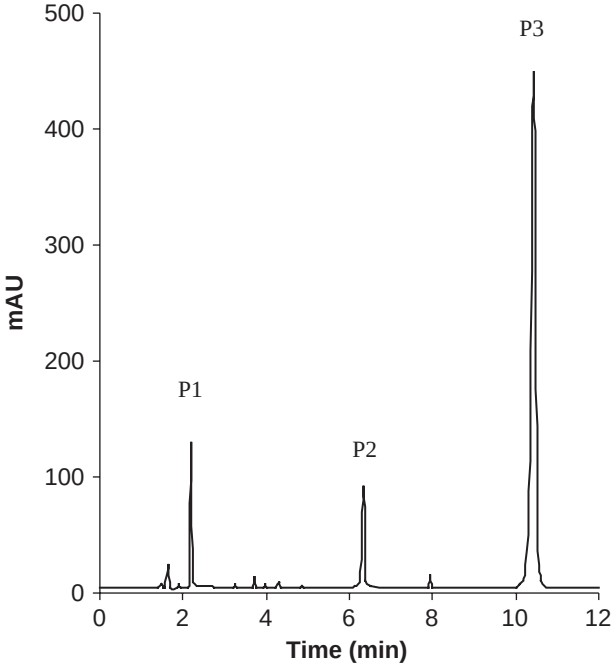

Fig. 5. A representative HPLC chromatogram of the culture filtrate for di-*n*-butyl phthalate degradation by *P. fluorescens* B-1 at $[DBP]_0$ 10 mg l^{-1}, pH 7.0 and 30°C. P1, P2 and P3 are abbreviations of phthalate acid (PA), monobutyl phthalate (MBP), and di-*n*-butyl phthalate (DBP), respectively.

3.5. Identification of DBP degradation metabolites of by P. fluorescens *B-1*

DBP was rapidly transformed in the culture medium inoculated with *P. fluorescens* B-1 when DBP served as the sole source of carbon and energy. A representative HPLC chromatogram of the culture filtrate from DBP degradation experiment is presented in Fig. 5. In addition to the parent compound peak of DBP, two major transient metabolites

Table 2
Identification of di-*n*-butyl phthalate and its two metabolites by gas chromatography-mass spectrometry

Compound	Molecular ion (*m*/*z*)	Base peak (*m*/*z*)	Ions for SIM	Retention time (min)
PA	166	104	18,50,76,104,148	8.51
MBP	222	149	41,65,105,149,167	15.45
DBP	278	149	41,76,104,205,223	28.61

DBP: Di-*n*-butyl phthalate; MBP: Mono-butyl phthalate; PA: Phthalic acid.

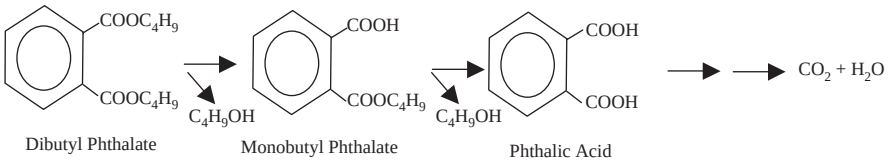

Fig. 6. A proposed biochemical degradation pathway for di-*n*-butyl phthalate by *P. fluorescens* B-1 isolated from mangrove sediment.

were observed, with HPLC retention times (r.t.) at 2.19 and 6.32 min, respectively. Other metabolites with very small peaks were also detected in the samples, but could not be isolated for further characterization. The peak at 2.19 min was identified as phthalic acid (PA) by comparing the retention time and the UV-visible spectrum (obtained using the diode array detector) of the concentrate from the degradation experiment with authentic PA. Using similar approaches, the second peak at 6.32 min was seen to be identical to MBP. In order to positively confirm the identity of this intermediate, GC-MS was also used. The unknown peak on HPLC was identified as MBP by comparing the mass spectrum at particular retention time with the published mass spectrum at NIST (National Institute of Standards and Technology) Database. DBP degradation culture by *P. fluorescens* B-1 was also analyzed by GC-MS, three main compounds were also identified as DBP, MBP and PA (Table 2). The result obtained by GC-MS was consistent with that by HPLC, confirming the identity of these degradation intermediates.

3.6. Metabolic pathway of DBP degradation by P. fluorescens B-1

Decreasing concentrations of DBP and transient appearance of two intermediates were observed over 96 h. MBP was produced during the initial 6 h as a major intermediate and then was degraded, and could not be detected during further incubation. A small amount of PA was also concomitantly produced during the first 6 h of degradation, and the PA produced was also degraded over the time of incubation. In sterile mineral medium, without the inoculation of *P. fluorescens* B-1, DBP and PA concentrations did not show appreciable change during the whole experimental period (data not shown). Detection of PA and MBP as intermediates of DBP degradation in this study was consistent with previous reports (Benckiser and Ottow, 1982; Wang et al., 1999). Based on the above results, a biochemical pathway for metabolism of DBP by *P. fluorescens* B-1 may be proposed (Fig. 6).

Microbial degradation of phthalate esters is believed to be the principal process responsible for removal of phthalate and esters from aquatic and terrestrial systems, such as sewage, soils, sediments, and surface waters. The biodegradation process follows a series of reactions common to all phthalate esters (Benckiser and Ottow, 1982; Eaton and Ribbons, 1982; Wang et al., 1995; Wang et al., 2003a,b), hydrolysis of the ester linkage is the key initial step during degradation. Phthalate esters have the basic structure of an esterified benzene-dicarboxylic acid with two alkyl chains, and primary biodegradation involves the sequential hydrolysis of the ester linkage between each alkyl chain and the aromatic ring, forming first the monoester and subsequently PA (Johnson et al., 1984; Cartwright et al., 2000). The proposed degradative biochemical pathway involves sequential cleavage of the ester bond to yield the phthalate monoester and then PA which can be further metabolized to produce carbon dioxide and water (Eaton and Ribbons, 1982; Kurane, 1997; Wang et al., 2003b; Gu et al., 2004). The two ester linkages frequently require the cleavage by two different bacteria suggesting the highly specificity of the esterases involved for each of the individual ester bonds (Gu et al., 2004). Dimethyl phthate (DMI), an isomer of *o*-DMP and dimethyl terephthalate (DMT), was mineralized only in the presence of both *Klebsiella oxytoca* Sc and *Methylobacterium mesophilium* sr (Gu et al., 2004) and each of the bacteria was responsible for cleavage of one specific ester bond only. However, DMT was mineralized by *Pasteurella multocida* Sa or *Sphingomonas paucimoblis* SY isolated from the same mangrove sediment at different rates (Gu et al., 2004).

In this study, *P. fluorescens* B-1 was capable of carrying out the complete degradation of DBP by itself. In contrast, degradation of both *o*-DMP and DMI requires at least two bacteria to complete the hydrolysis of the two ester bonds,

suggesting that the structural and conformational requirements by the esterase enzymes involved are highly specific for the substrate and degradation intermediates in the degradation process of selective phthalate esters (Wang et al., 2003b; Gu et al., 2004; Wang et al., 2004). Further investigation using molecular and proteomic approaches may elucidate the biological basis for such selectivity observed between substrate and the enzyme.

4. Conclusions

The results obtained in the present study showed that DBP could be rapidly degraded by *P. fluorescens* B-1 isolated from mangrove sediment. The optimum pH for the degradation was 7.0. The biodegradation kinetics could be described using a first-order kinetic model. Degradation of DBP by *P. fluorescens* B-1 proceeded through MBP and PA before cleavage of the aromatic ring. The results suggest that DBP can be degraded by natural bacteria indigenous to the mangrove environment.

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