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Kinetics of *n*-butyl benzyl phthalate degradation by a pure bacterial culture from the mangrove sediment

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Abstract

n-Butyl benzyl phthalate (BBP) is an endocrine-disrupting chemical. Biodegradation of BBP by a bacterium, *Pseudomonas fluorescens* B-1, isolated from the mangrove sediment, was investigated. The microorganism can utilize BBP as the sole carbon and energy source, where concentrations of BBP disappeared within 6 days under shake culture conditions. Effects of BBP concentration, pH, temperature, and salinity on BBP biodegradation were studied, respectively. The process of BBP biodegradation was monitored by reversed-phase high-performance liquid chromatography with ultra-violet detection after solid-phase extraction. The biodegradation of BBP could be fitted to a first-order kinetic model. The major metabolites of BBP degradation were identified as mono-butyl phthalate, mono-benzyl phthalate, phthalic acid and benzoic acid by gas chromatography–mass spectrometry, and a preliminary metabolic pathway of BBP was proposed.

Keywords: *n*-Butyl benzyl phthalate; Biodegradation; Kinetics; Endocrine-disruptor; *Pseudomonas fluorescens* B-1

1. Introduction

Phthalate esters are widely used plasticizers in building materials, home furnishings, transportation, clothing, paints, lacquers, cosmetics, and food and medical products packaging [1–3]. Release of phthalate esters into the environment during the manufacturing, use and disposal has caused serious concerns, since some of them are suspected to be mutagens, hepatotoxic agents and carcinogens [4–6]. In recent years, phthalate esters have attracted increasing attention because they are considered as endocrine-disrupting chemicals [1,7,8].

Since the rates of photolysis and chemical hydrolysis of phthalate esters are very slow, metabolic breakdown by microorganisms is considered to be one of the major routes for the environmental degradation of these pollutants in aquatic and terrestrial systems, such as sewage, soils, sediments, and surface waters [9]. In the literature, a number of papers have been published for the degradation of phthalate esters by microorganisms [10–16]. However, most of them are on the biodegradation of dialkyl phthalates, and less attention was paid to microbial degradation of alkyl aryl phthalate, such as *n*-butyl benzyl phthalate (BBP) by pure culture.

BBP is a plasticizer in papers and paperboards used as packaging materials for aqueous, fatty and dry foods [17], and it exerted estrogenic activities in several tests [1,18,19]. Up to now, only two papers [20,21] described the biodegradation of BBP using *Fusarium oxysporum* f. sp. and *Gordonia* sp., respectively. It will be meaningful to find more bacterium species with some advantages, such as wide availability, high environmental endurance, or strong degradation capacity. The mangrove sediment subjected to phthalate esters contamination might contain BBP-degrading bacteria, which may have some special characteristics, for example, high salinity-tolerance.

In this article, a pure culture capable of using BBP as the sole carbon and energy source was isolated from the mangrove sediment. The kinetics of BBP biodegradation was studied in shake culture conditions. Effects of BBP concentration, pH, salinity and temperature on the BBP biodegradation were studied, respectively. A preliminary metabolic pathway of

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BBP was proposed based on the metabolites identified by gas chromatography–mass spectrometry (GC–MS).

2. Materials and methods

2.1. Chemicals

All reagents were of analytical-reagent grade and purified water by Milli-Q system was used throughout the experiments. *n*-Butyl benzyl phthalate and phthalic acid (PA) were all purchased from ACROS Organic Inc. (New Jersey, USA). HPLC-grade methanol was purchased from TEDIA (Ohio, USA). Other reagents were purchased from Sigma–Aldrich (Missouri, USA).

2.2. Enrichment culture and culture medium

The initial enrichment culture was established by inoculating 100 ml sterile mineral salt medium (MSM) with 5 g fresh mangrove sediment taken from Mai Po Natural Reserve in Hong Kong with BBP (1 mg l^{-1}) in a 250 ml Erlenmeyer flask. The MSM consisted of the following chemicals (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; $Na_2B_4O_7{\cdot}H_2O$: 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 value of the culture medium was adjusted with HCl or NaOH to 7.0 ± 0.1 or otherwise specified. The Erlenmeyer 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 BBP-degrading cultures were obtained through enrichment transfer at approximately 1 week intervals on the basis of substantial depletion of BBP that was monitored by an HPLC method described later, 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 BBP. The BBP-degrading enrichment cultures were transferred more than 10 times prior to the isolation of bacteria from the enrichment culture.

2.3. Isolation of microorganisms

Bacteria in the enrichment culture showing ability in degrading BBP were diluted in MSM before plating on the Nutrient Agar (NA) plates (Difco Lab., Detroit, Michigan). After 48 h of incubation at 30 °C, several well-separated, individual colonies of different morphological types appeared and were further streaked onto fresh NA plates (Difco Lab., Detroit, Michigan) for purification. 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 [16].

2.4. Biodegradation of BBP by the isolated strain

Experiments on the kinetics of BBP degradation was conducted in 250 ml Erlenmeyer flasks with 100 ml of MSM. Flasks in experiments were incubated at the same conditions as described above. In addition, several sets of the experiments were conducted to determine the effects of pH, temperature and salinity on the biodegradation of BBP. Sterile controls were prepared by autoclaving before introduction of BBP, which passed through 0.2-μm-pore-size membrane filter (Pall Gelman Laboratory, Ann Arbor, Michigan). The experiments were carried out in triplicates, and the mean values (or mean values ± standard deviation) were used in the later text.

2.5. Solid-phase extraction procedure

A Waters Sep-Pak C_{18} cartridge (500 mg) was conditioned by 2 ml of methanol, and then 5 ml of water. The culture medium (15 ml) was centrifuged and the supernatant was passed through a 0.2-μm membrane filter. The filtrate (10 ml) was acidified to pH 2 with 0.1N HCl, and then passed through the cartridge at a flow-rate of 2 ml min^{-1}. The analytes retained on the SPE cartridge was eluted with methanol (1 ml × 2), and the eluate was purged to dryness with pure nitrogen gas. Finally, the residue was dissolved in 0.5 ml of methanol prior to determination by high-performance liquid chromatography with ultra-violet detection or GC–MS. The recoveries of BBP and PA after SPE were 96% and 93%, respectively.

2.6. Analysis of BBP and its metabolites

The Agilent 1100 series HPLC system consisting of a G1322A degasser, a G1311A QuatPump, a G1316A COLCOM and a G1315B diode array detector (DAD, Agilent, USA) set at 254 nm wavelength, was used for the detection and quantification of BBP and its metabolites concentrations. A personal computer equipped with a HP ChemStation (HP, 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 as separation column. A gradient elution mode was carried out in order to obtain well-separated intermediates and parent compound. The mobile phase was a binary gradient mixture of 50 mM phosphoric acid and methanol. The gradient started at 35% (v/v) methanol maintained isocratically for the first 10 min, thereafter the methanol content was raised linearly to 65% within 2 min, and held for in 7 min, finally being returned to the original condition (35% methanol) in 1 min. The total run time was 20 min. The flow-rate was maintained at 1.00 ml min^{-1}. Under these chromatographic conditions, baseline separation was achieved for BBP and its metabolites. Peak area was used as the analytical signal for quantification. All compounds studied were quantified using external standards.

Identification of metabolites of BBP was performed 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-5MS 5% phenyl methyl siloxane-coated capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The operative gas chromatographic conditions were the following: the column temperature during injection was 50 °C, then after 2 min, it was linearly programmed to increase to a final temperature of 300 °C at 6 °C min^{-1}, and then isothermally held for 16 min. The injection volume was

1 μl and injection temperature was 220 °C. The carrier gas was helium at a flow-rate of 1.5 ml min^{-1}. A mass range of 30–550 amu was scanned in all electron ionization mass spectroscopy studies where the electron energy was 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. Isolation and characterization of a BBP-degrading strain

Three BBP-degrading strains were isolated from the mangrove sediment. These strains were assigned as strain A, B and C, respectively. Each strain was separately cultured in a 250 ml flask containing 10 mg l^{-1} BBP, and their degradation abilities were compared by determining the change of BBP concentrations using the HPLC method described above. Strain A, B and C could degrade BBP by 35 ± 1.6%, 28 ± 1.3% and 46 ± 1.7% in 72 h, respectively. Therefore, strain C was chosen for further study.

Strain C was a Gram negative, obligate aerobic, non-capsulated, non-sporulated rod and formed light-yellow colonies on solid medium. It was identified as *Pseudomonas fluorescens* B-1 with 94.9% similarity using API 20NE biochemical test system.

3.2. Effect of the initial BBP concentration

Microbial degradation of BBP by *P. fluorescens* B-1 at different initial concentrations ranged from 2.5 to 20 mg l^{-1} was investigated. BBP biodegradation by *P. fluorescens* B-1 is assumed to fit to the Monod first-order kinetic equation as follows:

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

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

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

Exponential regression was applied to the experimental data according to Eq. (1). The plots of ln[BBP] versus time shown in Fig. 1 are linear ($0.9495 < r^2 < 0.9905$) under the BBP concentrations tested, suggesting the first-order kinetic. The rate constant K obtained from our experimental results was 0.031 h^{-1}. Therefore, the first-order equation can be expressed as: $\ln C = -0.031t + A$, and the half-life of BBP biodegradation by *P. fluorescens* B-1 was 22.35 h.

3.3. Effect of pH

The relationship between the rate constant and pH value is shown in Fig. 2. The rate constant of BBP degradation increased quickly when pH value increased from 5.0 to 7.0.

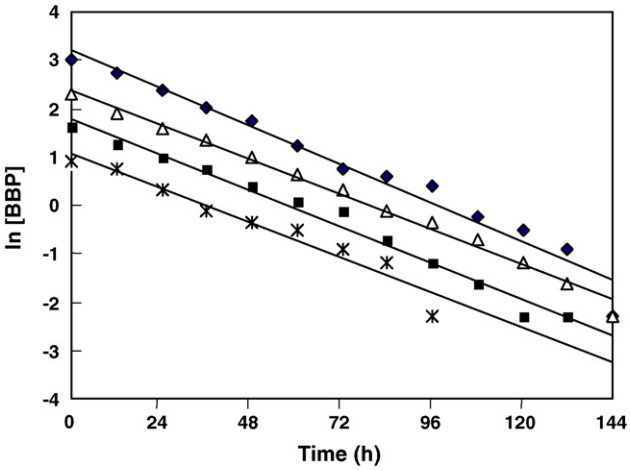

Fig. 1. Pseudo-first-order rate plots of ln[BBP] vs. time for the degradation of BBP by *P. fluorescens* B-1 at 30 °C and pH 7.0. Initial BBP concentration used (in mg l^{-1}) were 20 (♦), 10 (▲), 5 (■) and 2.5 (*).

The highest rate constant was achieved at pH 7.0. When pH was above 7.0, the rate constant decreased. That is, the hydrogen ion concentration in the culture medium could greatly influence the degradation of BBP. Since acids, such as phthalic acid, were generated during the process of de-esterification of phthalate esters, the pH value of culture medium after BBP biodegradation is usually lower than that of the non-inoculated MSM, especially when mineralization cannot be achieved. The pH value of culture medium needs be controlled. The optimum pH for the degradation activity of *P. fluorescens* B-1 was 7.0.

3.4. Effect of temperature

The temperature dependence of BBP biodegradation by *P. fluorescens* B-1 was studied in the range of 20–40 °C. A plot of rate constant as a function of temperature shows that increases in temperature greatly enhance the rate of BBP biodegradation (Fig. 3). However, temperature at 40 °C led to decrease in the rate constants. The optimum temperature was 37 °C.

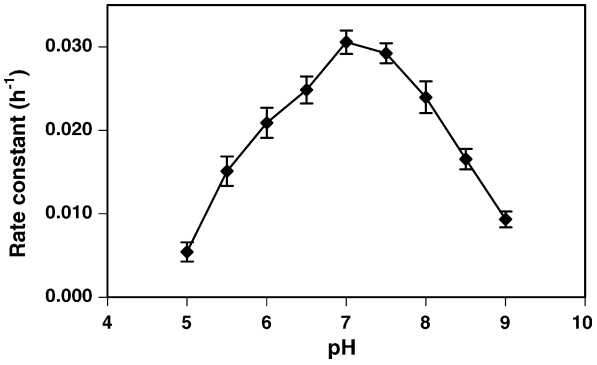

Fig. 2. Effect of pH on BBP degradation by *P. fluorescens* B-1. $[BBP]_0 = 10$ mg l^{-1}, $T = 30$ °C. The error bar represents standard deviation of the mean values.

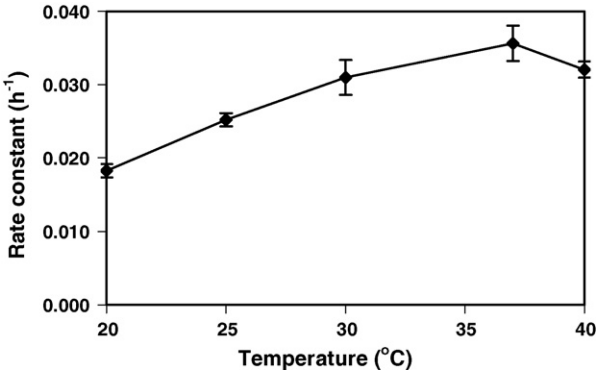

Fig. 3. Effect of temperature on BBP degradation by *P. fluorescens* B-1. $[BBP]_0 = 10\,mg\,l^{-1}$, pH 7.0. The error bar represents standard deviation of the mean values.

3.5. Effect of salinity

Different salinities of the culture medium ranged from 0 to 35‰ were tested and the results are presented in Fig. 4. With the increase of salinity from 0 to 15‰, the rate constants increased greatly. The fastest BBP degradation was found at 15‰. Salinity above 15‰ resulted in a decrease of degradation rate mainly due to the inhibition to the growth of *P. fluorescens* B-1.

3.6. Identification of BBP metabolites

To explore the metabolic pathways of BBP by *P. fluorescens* B-1, metabolites of BBP were identified. Besides the parent compound BBP (M5), four major metabolites (M1–M4) were observed (Fig. 5). The peaks M1 and M2 were identified as phthalic acid (PA) and benzoic acid (BA) by comparing their retention time and the spectra with those of the authentic standards. The peaks M3 and M4 were identified as mono-butyl phthalate (MBuP) and mono-benzyl phthalate (MBeP) using GC–MS by comparing their mass spectra with the published mass spectra from NIST (National Institute of Standards and Technology) database. NIST library records showed a strong similarity between the profiles for each compounds. The MS spectra of the metabolites M3 and M4 are shown in Fig. 6. Except the molecular ion peak at *m/z* at 222, i.e., at 90 mass units less

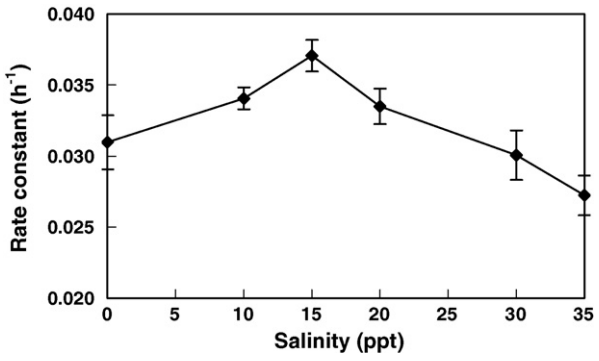

Fig. 4. Effect of salinity on BBP degradation by *P. fluorescens* B-1. $[BBP]_0 = 10\,mg\,l^{-1}$, pH 7.0, $T = 30\,^{\circ}C$. The error bar represents standard deviation of the mean values.

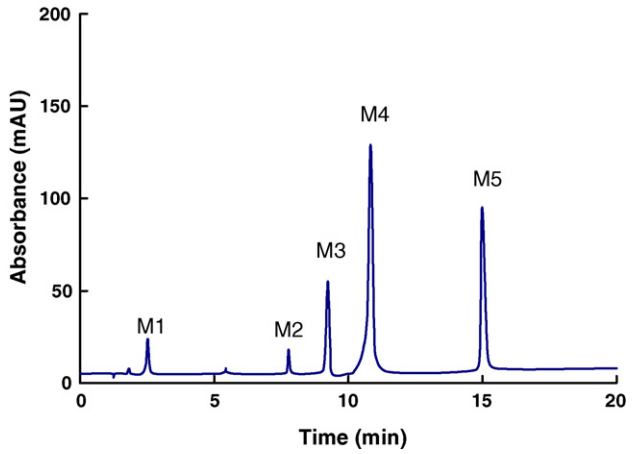

Fig. 5. A representative HPLC chromatogram of BBP and its metabolites. Column: Agilent Zorbax Eclipse XDB-C_8 column (150 mm × 4.6 mm); mobile-phase: 50 mM phosphoric acid and methanol in a gradient elution mode; flow-rate: 1.00 ml min^{-1}; wavelength of detection: 254 nm.

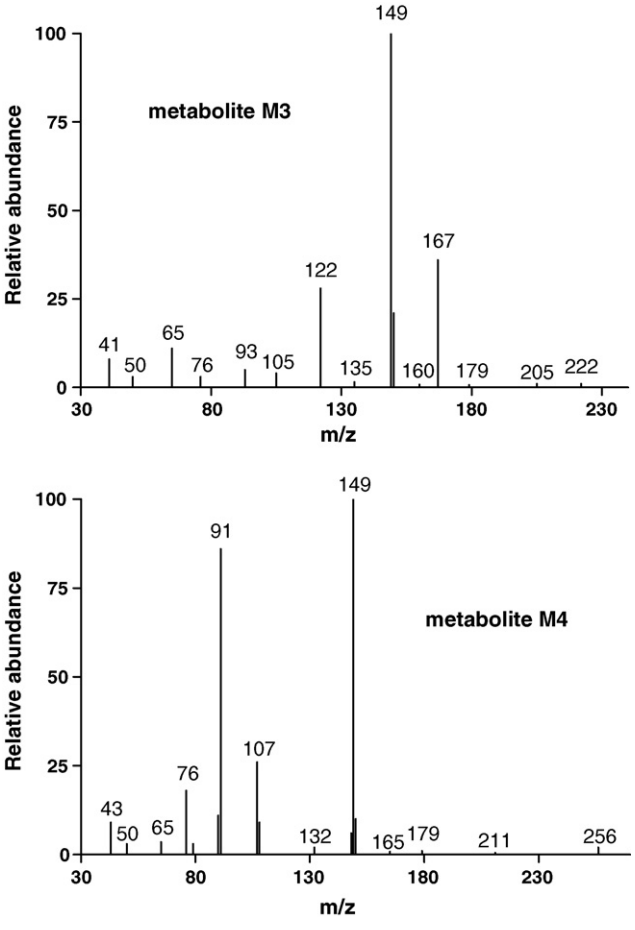

Fig. 6. MS spectra of the two metabolites. Column: HP-5MS 5% phenyl methyl siloxane-coated capillary column (30 m × 0.25 mm); column temperature: 50–300 °C in programmed mode; carrier gas: helium at a flow-rate of 1.5 ml min^{-1}; electron energy: 70 eV.

than that of the parent compound BBP, other discernible peaks in the mass spectrum of M3 are at 167, 149, 122, 65 and 41. While the metabolite M4 showed some large peaks in the mass spectrum at 149, 107, 91 and 76 in addition to its molecular ion peak at *m/z* at 256, i.e., at 56 mass units less than that of the parent compound BBP.

3.7. Metabolic pathway of BBP by *P. fluorescens* B-1

Analysis of culture medium extracts, by GC–MS and HPLC, indicated that BBP and four metabolites were present. Decreasing concentrations of BBP were observed over a 144 h time course, and the appearance of intermediates. The major metabolites were the BBP monoesters (MBuP and MBeP). The amount of MBuP was always less than that of MBeP, indicating that MBeP is easier to be accumulated than MBuP. The result also meant that during the biodegradation of BBP, the moiety of butyl group is easier to be utilized than benzyl group by *P. fluorescens* B-1. Small amounts of PA and BA were also produced; BA could be degraded in the following process. In addition, in sterile mineral medium, without the inoculation of *P. fluorescens* B-1, BBP concentration did not show apparent change during the experiment.

BBP was first metabolized to MBuP and MBeP and their quantities increased with incubation time. Our result is consistent with previous work [3]. Chatterjee and Dutta [21] found that MBuP, MBeP and PA were the degradation intermediates during BBP biodegradation, without BA. In this study BA was detected during the BBP biodegradation by *P. fluorescens* B-1 in addition to MBuP, MBeP and PA. The different results between this study and the work by Chatterjee and Dutta could be because two bacteria were very different. Based on the above results, a tentative metabolic pathway for the biodegradation of BBP by *P. fluorescens* B-1 may be proposed (Fig. 7). The proposed metabolic pathway involves sequential cleavage of the ester bond to yield the phthalate monoester and PA and then BA.

P. fluorescens B-1 isolated in this study was another BBP-degrading bacterium, and very different from the bacteria in the literature [20,21]. Therefore, the metabolic pathway of BBP by this bacterium was very different from those reported in previous studies [20,21]. In addition, *P. fluorescens* B-1 degraded BBP far more efficiently than *Gordonia* sp. [21], but its BBP-degrading capability was inferior to the cutinase from *F. oxysporum* f. sp. [20].

Microbial degradation of phthalate is believed to be the principal sink for phthalates in aquatic and terrestrial systems, such as sewage, soils, sediments, and surface waters, with the biodegradation process reported to follow a series of stages common to all phthalate esters [22]. Phthalate has 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 [12,13,23,24]. In the bacterial degradation of phthalate esters, some organism can selectively hydrolyze only one ester bond to yield monoalkyl phthalate and an alcohol, and the latter compound is then used for

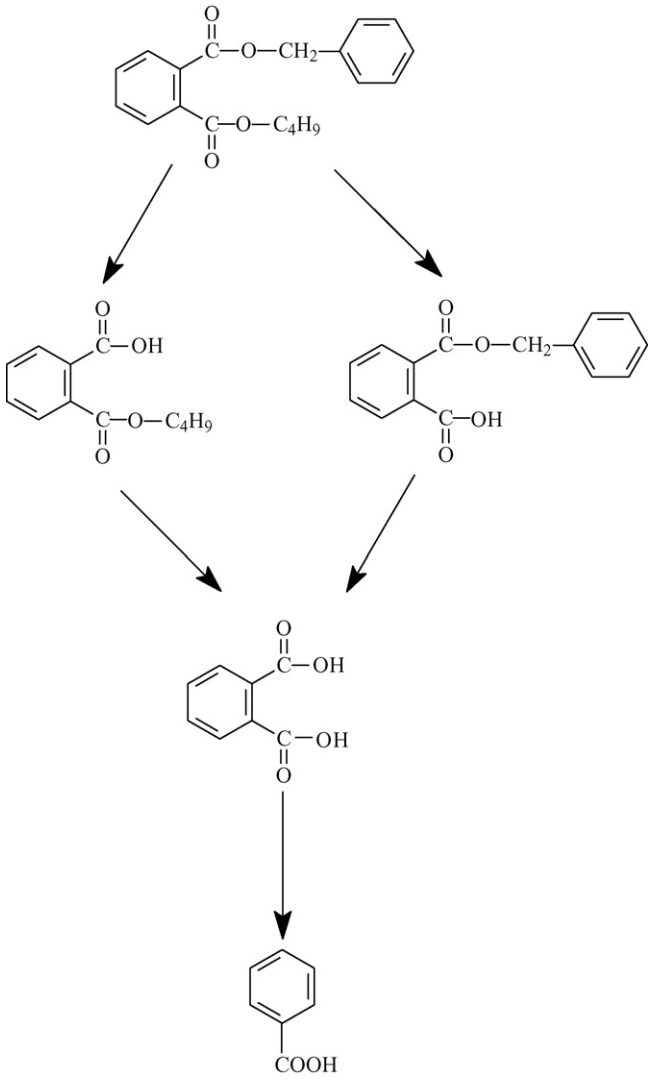

Fig. 7. A proposed metabolic pathway for BBP biodegradation by *P. fluorescens* B-1.

growth, while other organisms are capable of complete mineralization of either the monoalkyl or dialkyl phthalates [25]. The metabolic pathway of alkyl aryl phthalate was usually similar to that of dialkyl phthalates [9].

4. Conclusions

BBP could be rapidly degraded by *P. fluorescens* B-1 isolated from the mangrove sediment. The optimum pH, temperature and salinity for BBP degradation by *P. fluorescens* B-1 were 7.0, 37 °C and 15‰, respectively. The kinetics of BBP biodegradation could be described using a first-order model. Degradation of BBP by *P. fluorescens* B-1 proceeded through MBuP, MBeP, PA and BA before cleavage of the aromatic ring.

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