

Biodegradation of di-*n*-butylphthalate and phthalic acid by a novel *Providencia* sp. 2D and its stimulation in a compost-amended soil

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Abstract A novel 2D strain that could effectively degrade di-*n*-butylphthalate (DBP) and its major metabolite, phthalic acid (PA), was isolated from compost and identified as *Providencia* sp. 2D. A complete degradation of DBP (200 mg L⁻¹) was observed within 3 days under optimal conditions obtained by response surface methodology. This strain 2D could utilize various phthalic acid esters (PAEs) as substrates for growth, and could co-metabolize DBP in the presence of extra C sources. A novel combination of two common pathways in PA degradation was proposed for DBP degradation pathway, representing the first report of two ring cleavage pathways of the intermediate PA in a microbial species. Strain 2D efficiently enhanced the removal rate of DBP in contaminated soil with a sharp decrease of the DBP half-life compared to non-bioaugmentation treatments. Moreover, the addition of compost improved the degradation rate of DBP in soil by stimulating microbial activity. The results support the feasibility of

remediating DBP-contaminated soils inoculated with strain 2D and treated with compost.

Keywords Di-*n*-butylphthalate · Phthalic acid · *Providencia* sp. 2D · Biodegradation · Compost addition · Soil pollution bioremediation

Introduction

Phthalic acid esters (PAEs) are a class of refractory organic compounds that are widely used as additives or plasticizers in the manufacture of plastics (Chatterjee and Dutta 2008; He et al. 2014). PAEs can pollute various environments such as soil, air, water, and sediments because they are not chemically bound to the polymeric matrix and can be released from plastic products (Liang et al. 2008; Rivera-Utrilla et al. 2012; Niu et al. 2014). Recent studies have shown that PAEs and their metabolites can be toxic for humans and the environment due to xenoestrogenic and endocrine-disrupting effects (Chatterjee and Dutta 2003; Niu et al. 2014). Specifically, di-*n*-butylphthalate (DBP), one of the most widely used and frequently identified PAE compound in different environments, can exhibit hepatotoxicity, teratogenicity, and carcinogenicity (Matsumoto et al. 2008). Because of the risks of PAEs to human health and the environment, both the United States Environmental Protection Agency and the China National Environmental Monitoring Center have listed DBP as an environmental priority pollutant (Fang et al. 2014). In addition, phthalic acid (PA), the major intermediate degradation product of most PAEs (including DBP), is suspected to cause cancer and kidney damage (Matsumoto et al. 2008; Carlstedt et al. 2013) and is a recalcitrant compound (Liang et al. 2008). Therefore, it is necessary to develop remediation strategies to eliminate DBP and its metabolite PA from the environment.

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Microbial degradation is believed to be one of the major processes that remediate soil contaminated by organic pollutants. Many bacterial strains with DBP-degrading abilities, including strains from various genera such as *Agrobacterium* (Wu et al. 2011), *Corynebacterium* (Yu et al. 2012), *Deinococcus* (Liao et al. 2010), *Enterobacter* (Fang et al. 2014), *Gordonia* (Jin et al. 2012), *Pseudomonas* (Liao et al. 2010), *Rhodococcus* (He et al. 2014), and *Sphingomonas* (Fang et al. 2007), have been isolated from activated sludge, mangrove sediment, wastewater, and municipal solid waste. The use of compost in agricultural soils may be risky when they have high concentrations of PAEs (Cai et al. 2007). However, the addition of compost has been demonstrated to be effective in biodegrading PAEs in soil (Chang et al. 2009), but the direct effect of compost on PAE-degrading bacteria remains unclear. No studies have examined the degradation of PAEs by strains isolated from compost. Thus, understanding the interaction mechanism of compost with PAE-degrading bacteria is important to remediate PAE-contaminated soil treated by compost. In most cases to date, the PAE-degrading bacteria hydrolyze PAEs to PA, which can accumulate in soil if not degraded (Liang et al. 2008). The degradation of PAEs or PA has generally been studied in batch cultures, but the degradation activity of such bacteria in PAE-contaminated soil as well as the effect of compost addition on the degrading strain is poorly known.

In the present study, we aimed to isolate a bacterial strain from manure compost that can degrade both DBP and PA. The optimum conditions and pathway of DBP biodegradation were investigated. To assess the effect of the compost addition on the isolated bacterial strain for bioremediation of DBP-contaminated soil, we compared DBP degradation in unamended and compost-amended soils inoculated with the degrader.

Materials and methods

Chemicals and media

DBP (98.7 %), dimethyl phthalate (DMP, 99.0 %), diethyl phthalate (DEP, 99.6 %), di-*n*-octylo-phthalate (DnOP, 98.0 %), di-(2-ethyl hexyl) phthalate (DEHP, 99.0 %), mono-butyl phthalate (MBP, 98.0 %), PA (99.5 %), protocatechuic acid (PCA, 97.0 %), benzoic acid (BA, 99.5 %), and catechol (99.0 %) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). A composite stock standard solution (1000 $\mu\text{g mL}^{-1}$ in dichloromethane) was purchased from Sigma-Aldrich, USA. Other chemicals were obtained from Damao Chemical Reagent Co. (Tianjin, China). No plastic equipment was used during sampling or processing. All glassware was washed with a K_2CrO_4 -

H_2SO_4 solution and double-distilled water and then baked at 400 °C for 6 h before use.

Luria-Bertani medium (LB) containing (g L^{-1}) tryptone (10), yeast extract (5), and NaCl (10) and a mineral salt medium (MSM) containing (g L^{-1}) K_2HPO_4 (5.8), KH_2PO_4 (4.5), $(\text{NH}_4)_2\text{SO}_4$ (2.0), MgCl_2 (0.16), CaCl_2 (0.02), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.0024), KNO_3 (0.0012), FeCl_3 (0.0018), and $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ (0.0015) were used in this study. The final pH was adjusted to 7.0 using 0.1 M HCl.

Culture enrichment and isolation

The microorganisms using DBP and PA as their sole C and energy sources were isolated using an enrichment culture technique. Compost samples were collected as inocula from a farm in Guangzhou, China. The initial enrichment culture was established in a 250-mL Erlenmeyer flask containing 100 mL MSM supplemented with DBP (50 mg L^{-1}) and PA (25 mg L^{-1}). The flasks were incubated in the dark at 30 °C under shaking for 1 week. Then, 1.0-mL aliquots of the active culture were transferred to new Erlenmeyer flasks containing 100 mL of freshly made MSM with gradually increasing concentrations of DBP (50–1200 mg L^{-1}) and PA (25–500 mg L^{-1}) as the sole C and energy sources. The DBP-degrading enrichment cultures were transferred more than ten times serially into fresh medium before the isolation of DBP-degrading strains. The 2D strain (hereafter, strain 2D) that could utilize DBP and PA as the sole C and energy sources for growth on MSM was selected for further DBP degradation studies.

Identification and characterization of strain 2D

Strain 2D was identified using morphology, physio-biochemical characteristics, and 16S rDNA sequence analysis. Cell morphology was observed under a scanning electron microscope (SEM; Philips XL30, Netherlands; Pigeot-Rémy et al. 2012). Physio-biochemical tests were performed with reference to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). Strain 2D culture DNA was extracted using a bacterial genomic DNA extraction kit (Omega Bio-Tek, USA), and the 16S rDNA gene of the strain was amplified from the genomic DNA using PCR. The universal primers F27 and R1492 used for amplifying the full length of 16S rDNA gene fragments have been described elsewhere (Chen et al. 2012). The purification and sequencing of 1492 amplifying products were conducted by Sangon Corporation (Shanghai, China). The resulting sequence was compared with known gene sequences in the GenBank database. Sequence data of the closest relatives were retrieved from NCBI GenBank and aligned using ClustalW with all parameters set at their default values. A phylogenetic tree was

then constructed using the neighbor-joining method with MEGA 5.05.

Preparation of the bacterial suspension

Strain 2D growing in LB medium for 16 h was harvested by centrifugation at $4600\times g$ for 5 min and washed three times with 0.9 % sterile saline. The washed bacteria were then re-suspended in the saline. For all experiments, an $OD_{600\text{ nm}}$ (optical density measurements at 600 nm) of 0.6 was used as inoculum, unless otherwise stated. Colony forming units (CFU mL^{-1}) of this suspension were quantified by the dilution plate count technique.

Optimization of DBP degradation conditions

Response surface methodology (RSM) was applied to optimize the key factors and their interactions that significantly affected DBP degradation by strain 2D. Based on the results of preliminary single-factor experiments, three key factors were selected as independent variables: pH, temperature, and inoculum size. A five-level (−1.682, −1, 0, 1, 1.682) central composite rotatable design (CCRD) consisting of 23 experimental runs with three replicates at the center point was generated using SAS ver. 9.2 (SAS Institute, Cary, NC, USA). The symbols and levels of the three independent variables are presented in Table S1. The dependent variable was the degradation of 200 mg L^{-1} of DBP in MSM at hour 72. Data were analyzed using the response surface regression procedure of SAS to fit the following quadratic polynomial equation (Chen et al. 2013):

$$Y_i = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2,$$

where Y_i is the predicted response, X_i and X_j are the variables, b_0 is a constant, b_i is the linear coefficient, b_{ij} is the interaction coefficient, and b_{ii} is the quadratic coefficient.

Substrate range tests

To examine its ability to utilize various PAEs, strain 2D was cultured in 250-mL Erlenmeyer flasks with liquid MSM and containing one (100 mg L^{-1}) of the following substrates as the sole sources of C and energy: DMP, DEP, DBP, DnOP, DEHP, MBP, PA, BA, PCA, or catechol. Cultures were replicated three times and incubated at the optimum culture conditions for 5 days. Non-inoculated cultures served as controls. The bacterial growth in Erlenmeyer flasks was determined by OD_{600} using a spectrophotometer (UV-2450, Shimadzu, Japan) and light microscopic observations.

Degradation kinetics of DBP and PA by strain 2D

Degradation tests using different initial concentrations of DBP (50–1000 mg L^{-1}) and its metabolic product PA (25–500 mg L^{-1}) were conducted in MSM under the optimal culture conditions. Non-inoculated samples were used as controls. Samples were periodically analyzed for both DBP and PA. All treatments were performed in triplicate.

Effects of extra C sources on growth and degradation ability of strain 2D

A mixed medium containing extra C sources (i.e., 10 % LB [v/v] in MSM) was used to study the effects of additional C sources on the degradation ability and growth of the bacteria. Strain 2D was incubated in the mixed medium containing 200 mg L^{-1} of DBP, and the cultures (in triplicate) were incubated under the optimum culture conditions for 3 days. Triplicate samples for MSM+DBP+bacteria were used as positive controls. Non-inoculated cultures of mixed medium or MSM served as negative control. The samples were collected at 12-h intervals for the analysis of DBP and bacterial growth.

Analysis of chemicals and metabolites

The residual DBP or PA and its metabolic products in MSM containing 100 mg L^{-1} of DBP or 50 mg L^{-1} PA were analyzed using GC/MS (QP2010 Plus, Shimadzu, Japan). Culture filtrates were collected at different intervals, and the filtrate extraction and cleanup procedures were conducted as previously reported (Jin et al. 2012). Briefly, the liquid cultures were mixed with 20 mL ethyl acetate by vibrating, and then the aqueous and organic phases were separated by centrifugation at $7200\times g$ for 5 min. This process was then repeated twice and the supernatants were combined. The extract was evaporated to near dryness, and the residue was redissolved in 10 mL methanol. All tests were conducted in triplicate. Finally, the samples were filtered (<0.22 μm), and 1.0- μL filtrate was injected into GC/MS. The following detection conditions of GC/MS were employed: an HP-5 column (0.25 $\mu\text{m}\times 0.25\text{ mm}\times 30\text{ m}$) with helium as carrier gas at a flow rate of 1.0 mL min^{-1} , an injection temperature of 250 $^\circ\text{C}$, and an ion source of 220 $^\circ\text{C}$. The GC oven temperature was programmed as follows: 100 $^\circ\text{C}$ for 2 min, raised at 15 $^\circ\text{C min}^{-1}$ to 129 $^\circ\text{C}$, then at 40 $^\circ\text{C min}^{-1}$ to 280 $^\circ\text{C}$ (hold for 5 min). Mass spectra were acquired in the electron ionization (EI) mode using an electron impact ionization of 70 eV and scanning at 50–600 amu. The scans collected for the metabolites were identified by comparing the results with both standard solution and the mass spectra library in the MS system. The detection limits of DBP and PA were 0.045 and 0.082 mg L^{-1} , respectively. The recoveries of DBP and PA

ranged from 95.1 to 102.1 % and from 92.0 to 97.4 %, respectively.

Plate assay for visual detection of phthalate 4,5-dioxygenase activity

Using the methods described elsewhere (Nomura et al. 1989; Iwaki et al. 2012), an agar plate containing MSM supplemented with 0.4 % disodium phthalate and 0.1 % quinolinic acid was used to identify the 4,5-dihydroxyphthalate pathway. Strain 2D was inoculated onto the plates and incubated for 3 days at 25 °C. The colonies were then immediately dyed red by spraying them with freshly prepared diazotized *p*-nitroaniline reagent.

Cloning of protocatechuate dioxygenase gene

The protocatechuate dioxygenase gene was amplified using two degenerate primers (forward primer 5'-RGRGAGRATCGACGTTYACGC-3', reverse primer 5'-AYTTCSTGCTCGTYCTCGCTG-3'; *Y* represents A or C, *S* represents G or C, *R* represents A or G), which were designed based on the conserved sequences of the protocatechuate dioxygenase reported by NCBI. The PCR mixture consisted of 5.0 µL 10×PCR buffer, 5.0 mM MgCl₂, 10 mM dNTP, 2.5 U Taq DNA polymerase, 1.0 µL of each primer, and 3.0 µL DNA template. The reaction mixture was added to a final volume of 50 µL with deionized water. The PCR program was a denaturing step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min, plus final extension at 72 °C for 10 min. The purified PCR product was cloned into pGEM-T vector, and the plasmid was then transformed into *Escherichia coli* DH5α. After blue-white screening, the white colonies were randomly selected and reamplified by PCR to identify the positive clones. Representative positive clones were selected for sequencing (by Sangon, Shanghai, China).

Anaerobic experiments

Strain 2D was cultured in 150-mL serum bottles with liquid MSM (50 mL) and containing one (25 mg L⁻¹) of the following compounds as the sole sources of C and energy: DBP, PA, BA, PCA, or catechol. Serum bottles were sealed with butyl rubber stoppers, and the headspace was flushed with nitrogen according to Grishchenkov et al. (2000). Anaerobic condition was kept by adding 1.0 mM Na₂S·9H₂O to the medium in the bottles. The same treatment without inoculation of strain 2D was set as negative control. As positive control, an aerobic experiment was carried out in the same serum bottles without degassing and fitted with foam stoppers. Cultures (in triplicate) were incubated on a rotary at 200 rpm at 30 °C. Samples were periodically analyzed for these compounds.

Biodegradation of DBP by strain 2D in soil and compost-amended soil

The degradation of DBP by strain 2D was examined in soil and compost-amended soil. Soil was collected from an agricultural field of South China Agricultural University (Guangzhou, China), and mature compost was collected from the farm mentioned above. Based on mixture commonly used at the farm, the ratio of the soil to compost was set at 15:1. Detailed characteristics of the soil, compost, and their mixture (compost-amended soil) are presented in Table 1.

Soil or compost-amended soil (200-g aliquots) was placed in 500-mL Erlenmeyer flasks and treated with DBP (100 mg kg⁻¹ soil) in an acetone solution. After thorough mixing and solvent evaporation, the bacterial suspension was inoculated into soil or compost-amended soil (in triplicate) using drip irrigation to achieve a final concentration of approximately 1×10⁷ cells g⁻¹. Meanwhile, the same treatments (in triplicate) without inoculation were used as negative controls. Additionally, to investigate the contribution of microbial consortia from soil or compost, the compost-amended soils were sterilized by autoclaving at 121 °C for 1 h and then were inoculated with strain 2D. The non-inoculated sterile soil treated with compost was used as positive control. All treatments were adjusted by adding sterile water to 40 % of water-holding capacity and then incubated at 30±1 °C. Residual DBP was periodically measured in soil samples (20 g). Soil extraction and cleanup procedures were conducted as previously reported (Cai et al. 2007) with slight modifications. One gram of soil sample (DW) was supersonically extracted in 20 mL of dichloromethane for 10 min and then centrifuged at 2500×g for 5 min to obtain supernatants. This process was then repeated twice for a total of three extractions. The supernatants were pooled and loaded onto a glass chromatography column (35-cm length×1 cm i.d., pre-eluted with 20 mL of dichloromethane) packed bottom-up with anhydrous sodium sulfate, silica gel, and alumina, and then eluted three times

Table 1 Main physicochemical properties of soil, manure compost, and a mixture of both used in this study

Characteristics	Soil	Compost	Mixture
Sand (%)	35.67	–	–
Silt (%)	52.34	–	–
Clay (%)	11.99	–	–
pH	6.67	8.85	7.54
Moisture (%)	38.1	31.7	35.4
OM (%)	1.5	68.9	5.7
TOC (g kg ⁻¹)	7.9	391.7	31.2
Total N (g kg ⁻¹)	1.32	22.4	2.9
Total P (g kg ⁻¹)	1.57	18.6	2.8

OM organic matter, TOC total organic carbon

with 10 mL of dichloromethane. The eluate was evaporated to near dryness, and the residue was redissolved in 1 mL dichloromethane for GC/MS analysis mentioned above. The recoveries of DBP in soil samples ranged from 92.0 to 96.5 %.

Kinetics and statistical analysis

The degradation rate constant (k) (h^{-1}) was determined using an equation based on a first-order model: $\ln C = -kt + A$, where C is the concentration of chemicals, k is the first-order kinetic constant, t is degradation in hours, and A is the constant. The biodegradation half-lives ($t_{1/2}$) of different chemicals were determined using the algorithm $t_{1/2} = \ln 2/k$. Statistical analyses, including one-way analysis of variance (ANOVA) followed by Duncan tests, were performed using Microsoft Excel 2003 and SPSS 17.0.

Results and discussion

Identification and characterization of the degrading strain

A bacterial strain capable of degrading DBP or PA was isolated from compost and was termed '2D'. This strain is a facultative anaerobic, gram-negative, and rod-shaped bacterium 1.5 to 3.0- μm long and 0.5 to 0.8- μm wide (Fig. 1). The results of physio-biochemical tests for strain 2D are described in Table S2. According to BLAST analysis of its 16S rDNA gene sequence, strain 2D belongs to the genus *Providencia* (99 % similarity to both *P. stuartii* NBRC12930 and *P. rettgeri* NBRC13501). A phylogenetic tree was constructed based on the 16S rDNA gene sequence of strain 2D and related strains using MEGA 5.05 (Fig. S1). Based on the above morphology, physio-biochemical characteristics, and 16S rDNA gene analysis, strain 2D was identified as *Providencia*

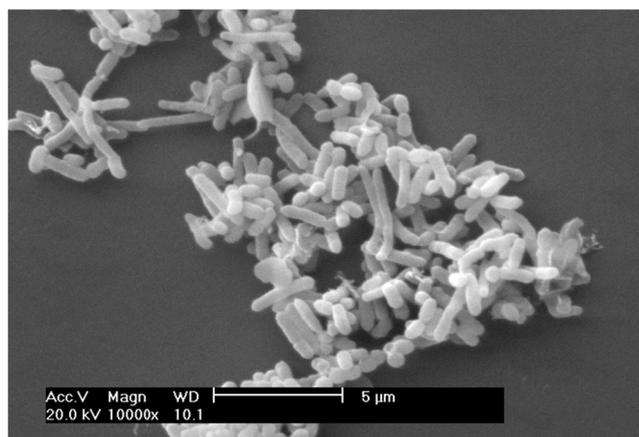


Fig. 1 Morphological characteristics of *Providencia* sp. 2D under scanning electron microscopy ($\times 10,000$)

sp. 2D. The 16S rDNA gene sequence of 1445 bp was deposited in GenBank with accession no. KP120889.

Providencia sp. is a member of the *Enterobacteriaceae* family, which can inhabit manure (Chander et al. 2006). Although previous reports have demonstrated that many bacteria from various genera can degrade PAEs, no evidence has been presented regarding bacteria of the *Providencia* genus that can degrade aromatic compounds. Furthermore, to our knowledge, the present study is the first report of the degradation of PAEs or PA by a strain isolated from compost, which provides the first evidence that *Providencia* bacteria perform efficient degradation of aromatic compounds. Previous work has shown that this genus can reduce chromate and is tolerant to the presence of various heavy metals (Thacker et al. 2006).

Optimization of culture conditions for DBP degradation using RSM

Three independent variables including pH (X_1), temperature (X_2), and inoculum size (X_3) were optimized to enhance DBP degradation using a CCRD. The effects of these three factors as well as the experimental responses (Y) are presented in Table S3. These were calculated using the response surface regression procedure of SAS, and the results of the quadratic polynomial model fitting ANOVA were evaluated using F -tests (Table 2) and t -tests (Table S4). Only statistically significant terms ($P < 0.05$) were included in the model. The first-order effect (X_3), three interaction effects (X_1X_2 , X_1X_3 , and X_2X_3), and a second-order effect (X_3^2) were not significant ($P > 0.05$) and were removed from the model. Therefore, the following fitted regression model (equations in terms of coded

Table 2 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for DBP degradation

Source	df	SS	MS	F-value	P-value*
X_1	1	38.48486	38.48486	8.712725	0.0112
X_2	1	13.04216	13.04216	2.952661	0.0494
X_3	1	7.35348	7.35348	1.664781	0.2194
$X_1 X_1$	1	6879.852	6879.852	1557.554	<0.0001
$X_1 X_2$	1	2.587813	2.587813	0.585864	0.4577
$X_1 X_3$	1	0.049612	0.049612	0.011232	0.9172
$X_2 X_2$	1	1857.305	1857.305	420.4819	<0.0001
$X_2 X_3$	1	0.070312	0.070312	0.015918	0.9015
$X_3 X_3$	1	6.189595	6.189595	1.401285	0.2577
Model	9	8760.982	973.4424	220.3811	<0.0001
Error	13	57.42212	4.417086		
Total	22	8818.404			

$R^2 = 0.9935$ (adjusted $R^2 = 0.9890$), coefficient of variation (CV) = 2.7 %
df degrees of freedom, SS sum of sequences, MS mean square

* $P < 0.05$ indicates that the model terms are significant

values for the regressor) was used to investigate the effects of pH (X_1) and temperature (X_2) on the degradation rate of DBP:

$$Y = 96.50111 - 1.678687X_1 - 0.977236X_2 - 20.81262X_1^2 - 10.81588X_2^2$$

The coefficient of determination (R^2) was 0.9935 for DBP degradation, indicating that the predicted values of this model were well correlated with the experimental values. The high value of the adjusted R^2 (0.9890) further supported the accuracy of this model (Table 2). Additionally, the low coefficient of variation (CV=2.7 %) demonstrated that the experimental results were reliable (Chen et al. 2012). Thus, this model was considered to be adequate for prediction within the range of employed variables.

With the value of inoculum size (the non-significant variable, $P=0.2194$) fixed at a minimum level ($OD_{600\text{ nm}}=0.6$), the three-dimensional response surface was plotted to directly display the effects of pH and temperature on DBP degradation by strain 2D. The model predicted a maximum DBP degradation of 96.5 % within 72 h at the stationary point (Fig. 2). Hence, the optimum culture conditions for DBP degradation by strain 2D were determined to be a pH of 8.3, a temperature of 32.4 °C, and an inoculum size of $OD_{600\text{ nm}}=0.6$. In addition, our results indicated that strain 2D could degrade DBP at a wide range of pH values (6–10) and temperatures (20–40 °C) (Table S3). These values meet the key prerequisites for a microorganism to be used in the bioremediation of complex environments (Singh et al. 2006). The optimal conditions obtained in this experiment provide a foundation for further use of this strain in the bioremediation of contaminated environments. Previous studies have demonstrated that RSM is an efficient statistical model for improving and optimizing the biodegradation conditions of various microorganisms (Ghevariya et al. 2011; Chen et al. 2012, 2013). Nevertheless, the present study is the first to use RSM based

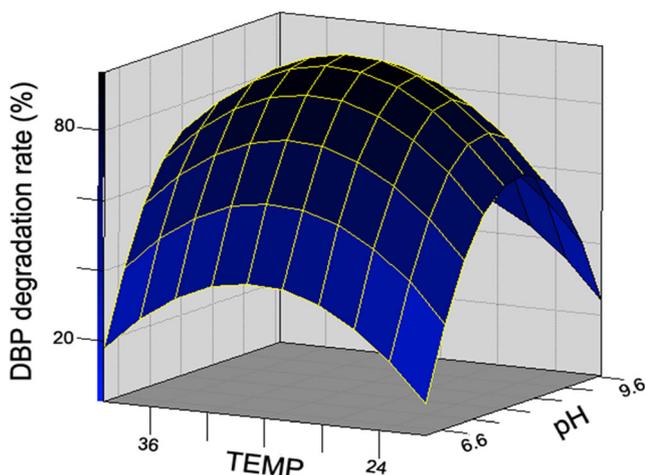


Fig. 2 Response surface plot illustrating the effects of pH and temperature on DBP degradation by *Providencia* sp. 2D. Inoculum size was set at the minimum level ($OD_{600\text{ nm}}=0.6$)

on a CCRD to optimize culture conditions for PAE degradation. Moreover, a mathematical model was successfully developed to effectively predict and optimize DBP degradation by strain 2D within the limits of selected factors.

Substrate utilization tests

Providencia sp. 2D was able to utilize all of the tested chemicals (DMP, DEP, DnOP, DEHP, MBP, PA, PCA, BA, and catechol) as growth substrates. However, the strain 2D exhibited an extraordinarily broad substrate specificity. It grew vigorously ($OD_{600} > 1.2$) in MSM containing PAEs with shorter ester chains (DMP, DEP, DBP, and MBP), PA, BA, PCA, or catechol as the sole sources of C and energy. On the contrary, it grew slowly ($OD_{600} < 0.4$) in MSM containing PAEs with longer ester chains (DnOP and DEHP). This difference in growth rate may be due to biodegradability differences in short- versus long-chain PAEs (Wu et al. 2011; He et al. 2013). For DnOP and DEHP, the steric effects of phthalate ester side chains avoid the binding of hydrolytic enzymes to phthalates, thereby inhibiting their hydrolysis (Liang et al. 2008; He et al. 2013). In any case, the ability of strain 2D to degrade various PAEs highlights that this particular bacteria has great potential and advantages in the bioremediation of PAE-polluted environments.

Biodegradation of DBP and PA at different initial concentrations

Figure S2 shows the kinetic curves of DBP and PA degradation at different initial concentrations in MSM. Strain 2D could almost completely degrade DBP within 72 h at initial concentrations of $\leq 200\text{ mg L}^{-1}$ (Fig. S2A). When the initial concentrations of DBP increased up to 500 and 1000 mg L^{-1} , the degradation rates were 89.0 and 84.9 %, respectively, within 72 h. These results indicated that the strain 2D was much more efficient to degrade DBP compared to the other strains (Liao et al. 2010; Wu et al. 2011). On the other hand, Table 3 presents the kinetic equations for DBP degradation at different initial DBP concentrations. The degradation process followed the first-order model and was characterized by values of k ranging from 0.0265 to 0.0661 day^{-1} . The coefficient of determination R^2 was 0.9564–0.9838, indicating that the experimental data were well correlated with the model. Notably, at initial concentrations of $\leq 200\text{ mg L}^{-1}$, the half-lives ($t_{1/2}$) of DBP were less than 11 h. Even with a DBP concentration as high as 1000 mg L^{-1} , the $t_{1/2}$ was only about 26 h. Generally, the half-life of DBP degradation in soil is up to several months (Amir et al. 2005). So it can be concluded that strain 2D is an ideal candidate for the efficient and rapid bioremediation of DBP-contaminated environments.

The PA can be degraded by strain 2D at all the tested concentrations (25–500 mg L^{-1}) (Fig. S2B). In particular, at

Table 3 Kinetic equations for DBP degradation by *Providencia* sp. 2D with different initial concentrations of DBP and PA

Initial concentration (mg L ⁻¹)		Kinetic equations ^a	$t_{1/2}$ (h) ^b	R^2
DBP	50	$\ln C = -0.0800t + 3.9120$	8.66	0.9838
	100	$\ln C = -0.0661t + 4.6052$	10.49	0.9805
	200	$\ln C = -0.0651t + 5.2983$	10.65	0.9721
	500	$\ln C = -0.0295t + 6.2146$	23.50	0.9594
	1000	$\ln C = -0.0265t + 6.9078$	26.16	0.9564
PA	25	$\ln C = -0.0458t + 3.2189$	15.13	0.9531
	50	$\ln C = -0.0405t + 3.9120$	17.11	0.9527
	100	$\ln C = -0.0281t + 4.6052$	24.67	0.9605
	200	$\ln C = -0.0174t + 5.2983$	39.84	0.9482
	500	$\ln C = -0.0038t + 6.2146$	182.41	0.9679

^a $\ln C$ logarithm (base e) of substrate concentration, t degradation in hours, $t_{1/2}$ biodegradation half-life, R^2 coefficient of determination

^b Each value is the mean of three replicates

concentrations of ≤ 100 mg L⁻¹, strain 2D was able to completely degrade PA within 144 h (Fig. S2B) with half-life ranging from 15.13 to 24.67 h (Table 3). However, at a higher concentration (500 mg L⁻¹), only 43.5 % of the PA was degraded within 144 h with half-life up to 182.41 h (Table 3). This difference might be attributed to low microbial activity under low pH and an acclimation period required for the bacteria before the rapid degradation at PA concentration. These results were consistent with previous findings that the lag phase increased with increasing concentration of PAEs and low pH extended the lag phase, because acids like PA were generated from the de-esterification of PAEs (Li and Gu 2007; Wang et al. 2008; Jin et al. 2012). Several studies have recently reported complete degradation of DBP by mixed cultures or by a bacterial consortium of several microbial members (Li and Gu 2007; Wu et al. 2010; He et al. 2013). However, these microorganisms were unable to degrade or only poorly degraded PA (Liang et al. 2008; Sarkar et al. 2013). Therefore, the broad catabolic capability of *Providencia* sp. 2D suggests that this bacterium may have enormous potential for the bioremediation of DBP-contaminated sites.

Effect of extra C sources on growth and degrading ability of strain 2D

The growth of strain 2D monitored by optical density (OD_{600 nm}) and the degradation curves of DBP are presented in Fig. 3. The OD_{600 nm} of MSM without the addition of LB was generally lower than 2.1. Growth of strain 2D was strongly stimulated by the presence of LB, and the OD_{600 nm} of the mixed culture significantly increased from 0.2 to 2.5 within 48 h of incubation. The degradation rates of DBP increased separately by 8.1, 15.9, and 12.6 % at 12, 24, and 36 h with the addition

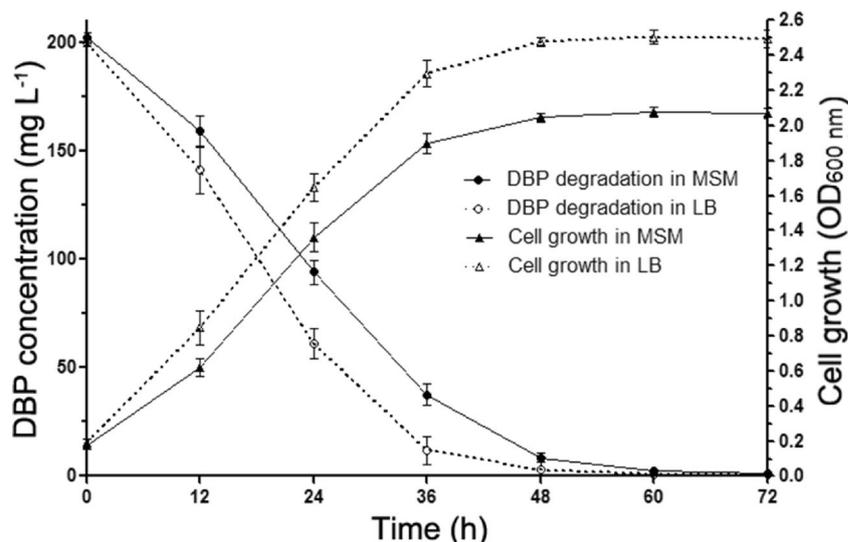
of LB, respectively. No significant changes in DBP concentration were observed in non-inoculated cultures (data not shown). These results confirm that PAE degradation is enhanced by adding the yeast extract (He et al. 2013). The increased degradation rate with the addition of LB was likely attributed to the microbial co-metabolism as a consequence of the additional C sources (Chen et al. 2012). Hence, the results suggest that the degradation of DBP in soil by *Providencia* sp. 2D can be enhanced with additional C sources.

Biodegradation pathway

To determine the pathway of DBP degradation by *Providencia* sp. 2D, the metabolites of DBP were identified using GC/MS. The intermediates of DBP degradation in culture medium were extracted and monitored at 12-h intervals for 144 h. Figure 4 shows GC/MS chromatograms and spectra of the DBP biodegradation products at 0, 24, and 48 h. At the beginning of the experiment (0 h), only an apparent peak occurred at retention time of 7.62 min. After 24 h, the peak area at 7.62 min decreased and the other new three peaks appeared at retention time of 7.33, 6.55, and 4.23 min corresponding to three new compounds, MBP, PA, and BA. All the four peaks decreased gradually before 48 h and disappeared finally after 144 h.

Generally, PAE biodegradation consists of two processes: the first one produces PA, and the second one involves ring cleavage and mineralization of PA (Liang et al. 2008). DBP was hydrolyzed by esterase firstly to MBP and then further to PA, or directly to PA, which is a major intermediate in the biodegradation of some PAEs and PAHs. The process involving ring cleavage of PA differs under aerobic and anaerobic conditions, representing two different degradation pathways (Liu and Chi 2003; Liang et al. 2008; Sarkar et al. 2013). Interestingly, both the two pathways of ring cleavage might simultaneously occur during the biodegradation of PA by *Providencia* sp. 2D (Fig. 5). The results of plate assays with a color reaction (red) confirmed that the phthalate ring was present during the 4,5-dihydroxyphthalate pathway, suggesting that PCA might have been produced in the process of PA degradation (Nomura et al. 1989). PCA was not detected in degradation metabolites of DBP (Fig. 4), probably because it was immediately degraded by strain 2D. To verify this hypothesis, we performed amplification and analysis of the potential gene encoding protocatechuate dioxygenase. The PCR amplification produced an intense band of about 500 bp DNA on an agarose gel (Fig. S3). The sequence analysis showed that the obtained fragment (488 bp, GenBank accession number KT188439) of protocatechuate dioxygenase was highly homologous to the sequence of protocatechuate 4,5-dioxygenase deposited in the GenBank, which exhibited 98, 98, 88, and 79 % similarity with protocatechuate 4,5-dioxygenase gene of *Sphingomonas paucimobilis*,

Fig. 3 Growth curve of *Providencia* sp. 2D and changes in DBP concentration in mixed and mineral salt medium (MSM) initially containing 200 mg L⁻¹ of DBP. Bars represent the standard error of three replicates



Pseudomonas paucimobilis, *Comamonas* sp. E6, and *Comamonas testosteroni*, respectively. Therefore, this result, together with those of the plate assay, suggests that PA should have been degraded via 4,5-dihydroxyphthalate to PCA, which is ultimately transformed into CO₂ and H₂O via *meta*-cleavage pathway (Liang et al. 2008). This pathway used by strain 2D is consistent with previously reported metabolic pathways of DBP by most gram-negative bacteria (Iwaki et al. 2012).

In addition, the metabolite BA was also detected, which indicated that another degradation pathway of PA might have occurred (Fig. 5). The biodegradation of PA was previously reported to produce BA through eliminating a carboxyl group under anaerobic or aerobic conditions since some enzymes can carry out this reaction from phenyl (Xu et al. 2008). Under anaerobic condition, PA might initially be metabolized to BA (Liu and Chi 2003; Liang et al. 2008). As a facultative anaerobe, *Providencia* sp. 2D is likely to preferentially metabolize PA to BA without oxygen and then BA was ring-cleaved and mineralized. To confirm this, the degradation kinetics of DBP and its possible metabolites (including PA, BA, PCA, and catechol) were investigated under aerobic and anaerobic conditions, respectively. PCA and catechol were hardly degraded by strain 2D under anaerobic condition in 144 h, while DBP, PA, and BA were degraded over 92, 83, and 50 %, respectively (Fig. S4A). All the five compounds were degraded over 90 % under aerobic condition in 60 h, especially PCA, catechol, and DBP were almost completely degraded in 24 h (Fig. S4B). These results showed that DBP, PA, and BA could be degraded by strain 2D under either aerobic or anaerobic condition, while PCA and catechol could be degraded only under aerobic condition. Hence, the oxygen level in the medium can affect the two pathways used to degrade PA. Based

on the formed metabolites and oxygen level, we proposed a pathway for complete degradation of DBP by *Providencia* sp. 2D, which was a novel combination of two pathways for PA degradation (Fig. 5). However, the exact pathway for BA degradation under aerobic condition remains unclear. Many bacteria degrading BA under aerobic conditions produce catechol (Fuchs et al. 2011), but a facultative anaerobe was also reported to degrade BA to PCA under aerobic condition (Taylor et al. 1970). In any case, strain 2D performs a complete degradation of DBP under either aerobic or anaerobic condition. Thus, the present study is the first report of two ring cleavage pathways of PA by a microbial species. Each of the two pathways for PA degradation has been reported previously in other bacterial genera (Liu and Chi 2003; Liang et al. 2008; Iwaki et al. 2012). Additionally, as a facultative anaerobe, strain 2D can achieve more stable population numbers and metabolic activity and, accordingly, presents enormous advantages to DBP degradation regardless of oxygen level in the target environment. Therefore, these findings may suggest that *Providencia* sp. 2D has evolved different catabolic pathways of DBP in soil with or without compost.

Biodegradation of DBP in soil and compost-amended soil

DBP degradation was measured in a soil subjected to different treatments to evaluate the potential of strain 2D to remediate DBP-contaminated soils. PAEs can be present in agriculture soil of China (Cai et al. 2008; Wang et al. 2013). Microbial biomass, basal respiration, and catalase activity in soil were inhibited when DBP concentration approaching to 100 mg kg⁻¹ (Gao and Chen 2008). Thus, we set 100 mg kg⁻¹ DBP in soil to investigate the efficiency of DBP degradation by strain 2D in the contaminated soil. The bioaugmentation of the three soil treatments with *Providencia*

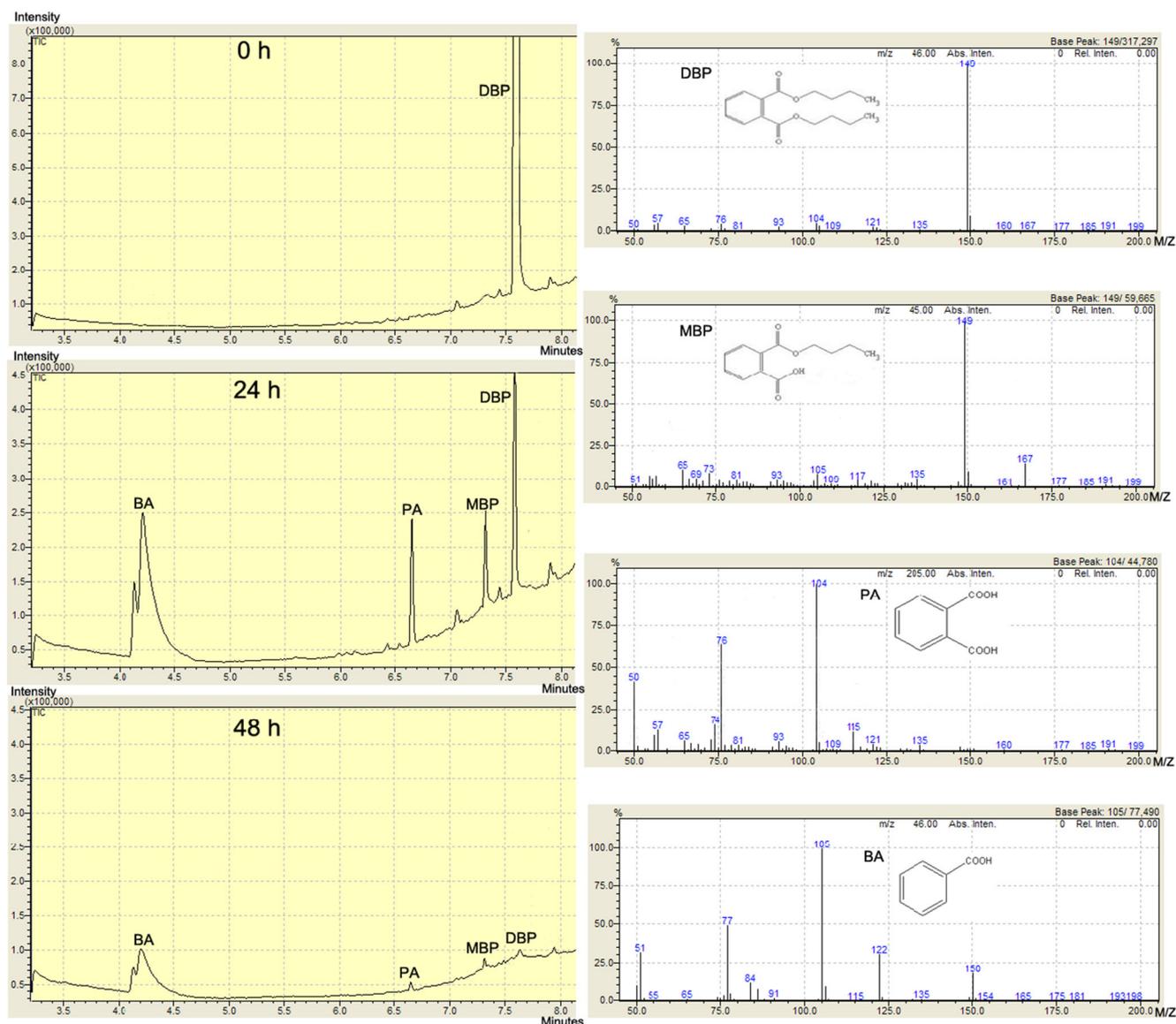


Fig. 4 GC/MS chromatograms and spectra of DBP biodegradation products at 0, 24, and 48 h in MSM. DBP di-*n*-butylphthalate, MBP mono-butylphthalate, PA phthalic acid, BA benzoic acid

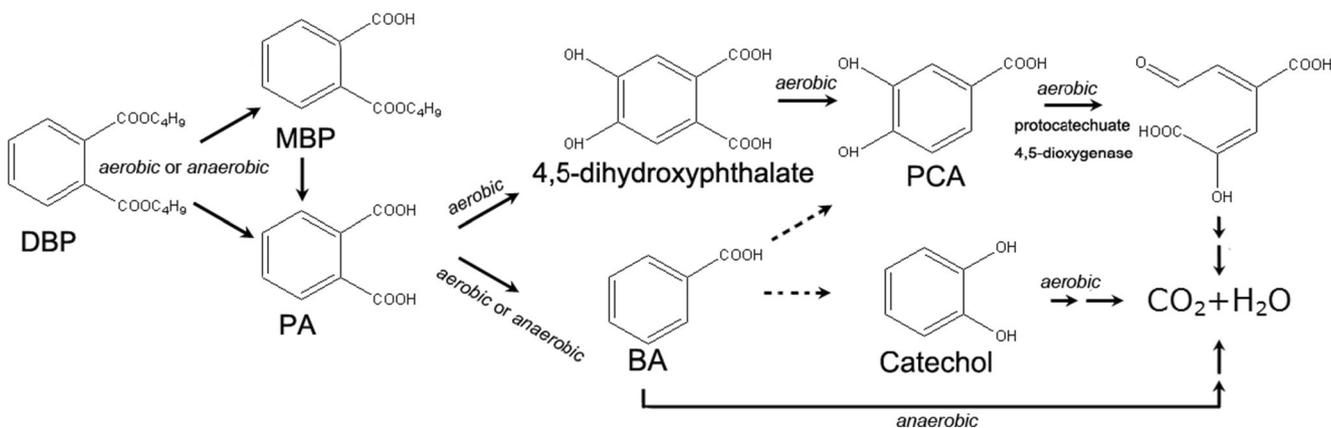
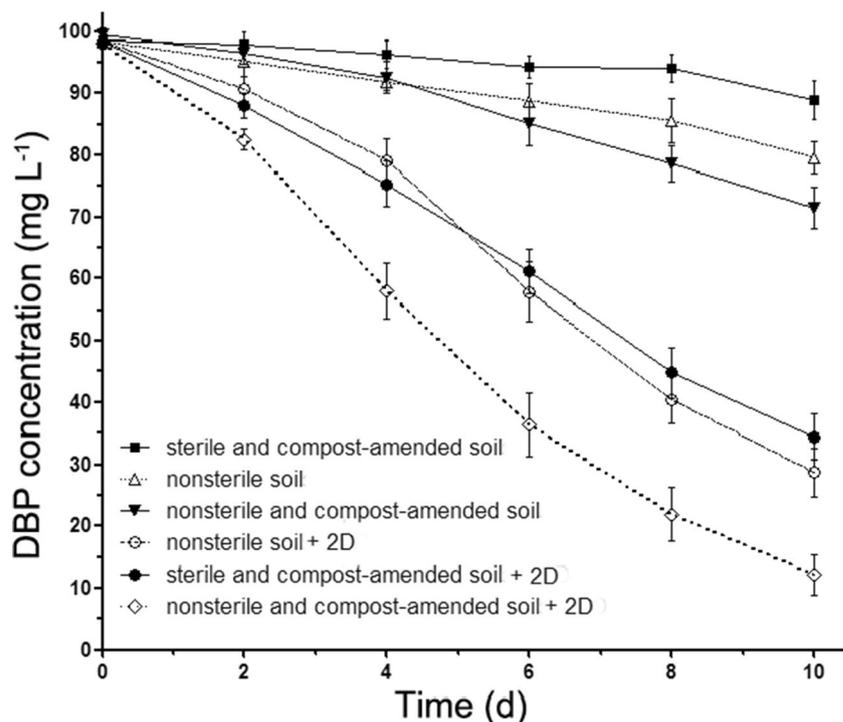


Fig. 5 A proposed pathway for degradation of DBP by *Providencia sp. 2D*. Solid arrow, based on the present experiment, dotted arrow, based on literature

Fig. 6 Degradation of DBP by *Providencia* sp. 2D in various soil treatments. Bars represent the standard error of three replicates



sp. 2D greatly enhanced DBP degradation, with 70.8 to 87.6 % of degradation compared to 9.7 to 28.1 % in non-inoculated soils (Fig. 6). The degradation kinetic equations for all treatments are presented in Table 4. The degradation process was described by the first-order model with k ranging from 0.0187 to 0.4248 day⁻¹. Compared to the non-inoculation soil treatment, the half-lives for DBP in soils inoculated with strain 2D were obviously shortened. These results suggested that strain 2D was able to efficiently degrade DBP in the contaminated soil even at high concentration (100 mg kg⁻¹). Moreover, DBP degradation in the compost-amended soil (non-sterile soil with inoculation) was improved, and the half-life was reduced by 1.2 days compared to the inoculated non-sterile soil, implying that the addition of compost indeed enhanced DBP degradation in soil. These

results were consistent with previous studies regarding the effects of compost on PAE degradation in soil (Chang et al. 2009; Yuan et al. 2011). The added compost stimulated microbial activity because of supplemental nutrients, consistent with the addition of LB into MSM stimulated the growth of strain 2D and enhanced the degradation of DBP (Fig. 3). In the negative control (non-inoculated soil), approximately 19 % of DBP in non-amended soil and 28 % of DBP in compost-amended soil were degraded. Furthermore, the half-life for DBP in the inoculated non-sterile soil treated with compost was reduced by 1.61 days compared the inoculated sterile soil treated with compost (Table 4), and the DBP degradation rate increased by 22.6 % (Fig. 6). The result suggests that the stain 2D could degrade DBP probably synergically with soil indigenous microorganisms.

Table 4 Kinetic equations for DBP degradation by *Providencia* sp. 2D in various soil treatments

Soil treatments	Kinetic equations ^a	$t_{1/2}$ (day) ^b	R^2
Sterile and compost-amended soil	$\ln C = -0.0187t + 4.6191$	37.07a	0.8829
Non-sterile soil	$\ln C = -0.0403t + 4.6374$	17.20b	0.9762
Non-sterile and compost-amended soil	$\ln C = -0.0671t + 4.6975$	10.33c	0.9647
Non-sterile soil + 2D	$\ln C = -0.2451t + 4.9867$	2.83d	0.9481
Sterile and compost-amended soil + 2D	$\ln C = -0.2137t + 4.8896$	3.24d	0.9701
Non-sterile and compost-amended soil + 2D	$\ln C = -0.4248t + 5.1945$	1.63e	0.9725

Data followed by different letters in the same column indicate significant differences at $P < 0.05$

^a $\ln C$ logarithm (base e) of DBP concentration, t degradation in hours, $t_{1/2}$ biodegradation half-life, R^2 coefficient of determination

^b Each value is the mean of three replicates

Conclusions

An efficient DBP- and PA-degrading bacterial strain, *Providencia* sp. 2D, was isolated. The complete degradation of DBP by this strain, probably a novel combination of two common pathways for PA degradation, was proposed. The process involving ring cleavage of PA represents two different degradation pathways under aerobic or anaerobic conditions, which indicated that the strain 2D presented enormous advantages to DBP degradation regardless of the oxygen level. Experimental results support the feasibility of remediation of DBP-contaminated soil inoculated with strain 2D and treated with compost.

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