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Biodegradation of di-n-butyl phthalate (DBP) by a novel endophytic *Bacillus megaterium* strain YJB3

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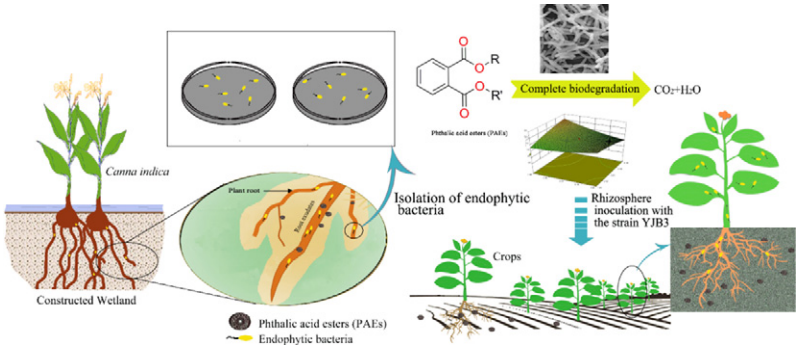
HIGHLIGHTS

- This is the first study on the biodegradation of PAEs using endophyte.
- A novel endophytic *Bacillus megaterium* YJB3 capable of degrading PAEs was isolated.
- PAEs catabolic genes in the stain YJB3 were excavated by whole genome analysis.
- The strain YJB3 is equipped with a complete degradation pathway of DBP.
- The strain YJB3 is an ideal candidate for *in situ* removal of PAEs in soil and crop.

GRAPHICAL ABSTRACT



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ABSTRACT

Phthalic acid esters (PAEs) are a group of recalcitrant and hazardous organic compounds that pose a great threat to both ecosystem and human beings. A novel endophytic strain YJB3 that could utilize a wide range of PAEs as the sole carbon and energy sources for cell growth was isolated from *Canna indica* root tissue. It was identified as *Bacillus megaterium* based on morphological characteristics and 16S rDNA sequence homology analysis. The degradation capability of the strain YJB3 was investigated by incubation in mineral salt medium containing di-n-butyl-phthalate (DBP), one of important PAEs under different environmental conditions, showing 82.5% of the DBP removal in 5 days of incubation under the optimum conditions (acetate 1.2 $g \cdot L^{-1}$, inocula 1.8%, and temperature 34.2 °C) achieved by two-step sequential optimization technologies. The DBP metabolites including monobutyl phthalate (MBP), phthalic acid (PA), protocatechuic acid (PCA), *etc.* were determined by GC–MS. The PCA catabolic genes responsible for the aromatic ring cleavage of PCA in the strain YJB3 were excavated by whole-genome sequencing. Thus, a degradation pathway of DBP by the strain YJB3 was proposed that MBP was formed, followed by PA, and then the intermediates were further utilized till complete degradation. To our knowledge, this is the first study to show the biodegradation of PAEs using endophyte. The results in the present study suggest that the strain YJB3 is greatly promising to act as a competent inoculum in removal of PAEs in both soils and crops.

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1. Introduction

DBP is one of important phthalate acid esters (PAEs) that are primarily used to improve the flexibility and workability of the products, such as polyvinyl chloride resins, lubricants, plastic packing films, adhesives, cosmetics, cellulose materials, and insecticides (Gao and Wen, 2016). PAEs are widely investigated as hazardous organic pollutants and their occurrence in soils can be of great threat to human beings and wildlife by different paths. Some of them are suspected to be mutagens, hepatotoxic agents, and carcinogens. As environmental hormone, PAEs can affect reproduction, impair development, and induce genetic aberrations in humans even at low concentrations, thereby resulting in an increasing environmental concerns (Heudorf et al., 2007). Like other PAEs, DBP is not chemically bonded to the plastic polymer and can be easily released into the environment during the manufacture, use, and disposal of plastics, leading to ubiquitous occurrence in various environmental matrices (Net et al., 2015). Thus, DBP has been listed as one of the priority pollutants by the US Environmental Protection Agency (US EPA, 2013).

PAEs in soils are dominated by DBP, as well as di-2-ethylhexyl phthalate (DEHP), which originate mainly from wide use of agricultural plastic film in facility agricultures (Li et al., 2016, Wang et al., 2015). Moreover, various fertilizers are supposed to be another main source of PAEs for contamination in agricultural soils, and the application of fertilizers contained PAEs can lead to accumulation of PAEs in both soils and crops (Mo et al., 2008). Rhizospheric bacteria can survival in rhizosphere and are more versatile for bioremediation of organic pollutants contaminated soils. However, phytoremediation is often insufficient because plants do not completely degrade these compounds through their rhizospheric microorganisms. Mobility of PAEs in the soil-plant system make themselves enter agricultural crops easily (Li et al., 2016; Zhao et al., 2015), posing a potential risk for human health owning to direct and indirect human exposure *via* the food chain (Guo et al., 2012; Mo et al., 2009). Therefore, it is necessary to find an effective way to minimize the risk of PAEs contamination in crops, ensuring the safety of agricultural products and human health.

Endophytic bacteria, a kind of microorganisms inhabit the interior of plant tissues, can be used to solve the problem mentioned above. The most important benefit of using endophytic bacteria is that toxic organic contaminants accumulated in plant tissues may be mineralized in *planta*. In particular, where use of molecular biology techniques is required to express specific pollutant-degrading genes in *planta*, endophytic bacteria are easier to manipulate than plant and have been used alternatively and preferably to improve the phytoremediation efficiency without requiring integration of foreign DNA into the plant genomes to produce transgenic plants (Barac et al., 2004; Doty, 2008). Most of the endophytes including Bacillaceae, Pseudomonaceae, Burkholderiaceae, and Enterobacteriaceae, *etc.* are facultative endophytes that can thrive inside a wide range of plant species including both monocots and dicots (Bacon and Hinton, 2006). However, all these studies are restricted to an extremely limited number of organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons (PHs), trichloroethylele (TCE), and BTEX (benzene, toluene, ethyl-benzene, and xylene) compounds (Feng et al., 2017), while degradation of PAEs using endophytic bacteria is scarcely documented.

Municipal wastewater usually contains high concentrations of phthalate esters (Gao and Wen, 2016), and constructed wetland plants are considered to be one of the major factors in PAEs removal from aquatic environments (Tang et al., 2015). Many water-borne microorganisms can colonize on the root or rhizome surface, some of which penetrate through the surface and colonize within plant tissues that constitute the reaction surfaces for endophytic microbial degradation of organic pollutants (Ijaz et al., 2015). Endophytic bacteria in plants inhabiting constructed wetlands have scarcely been reported. Shehzadi et al. (2016) isolated endophytes from the roots and shoots of *Typha domingensis*, *Pistia stratiotes*, and *Eichhornia crassipes* inhabiting a constructed wetland that treated textile effluent, in order to search for endophytic bacteria with textile effluent-degrading and plant growth promoting abilities. *Canna indica* L. is a dominant ornamental plant of tropical origin. This plant can be easily obtained and grown in many soil types including wet soils such as wetlands or river banks, allowing it to be used not only for soil remediation but also for wastewater treatment in wetland areas (Zhang et al., 2008). Boonsaner et al. (2011) found that the removal of BTEX in canna planted soil was 80% higher than that of the control soil. Moreover, the presence of *C. indica* significantly accelerated the dissipation of the pesticide triazophos (Cheng et al., 2007). However, there is scarcely study of endophytic bacteria colonizing *C. indica*. It is valuable to explore the culturable bacterial endophytes colonizing *C. indica* inhabiting a wastewater treatment constructed wetland for enhancing remediation of pollutants (Calheiros et al., 2017).

The aims of this study are: 1) to isolate and identify endophytic DBP-degrading bacteria from *C. indica*; 2) to evaluate the significant factors responsible for DBP degradation by endophyte; and 3) to provide evidences that the stain YJB3 can completely degrade DBP and to deduce the biodegradation pathway. This study is helpful to the development of endophytic resources for bioremediation of PAE-contaminated soil and water environments.

2. Materials and methods

2.1. Samples and reagents

Root samples of *C. indica* were collected from a constructed wetland for the treatment of municipal wastewater in Guangzhou, southern China.

DBP (98.7%), Dimethyl phthalate (DMP, 99.0%), Diethyl phthalate (DEP, 99.6%), di-n-octyl-phthalate (DnOP, 98.0%), Di(2-ethylhexyl) phthalate (DEHP, 99.0%), di-isononyl phthalate (DINP, 99.0%), monobutyl phthalate (MBP, 98.0%), PA, 99.5%, and PCA (97.0%) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). DBP standard (99.8%) was obtained from Sigma-Aldrich Co. (St. Louis, USA). All other chemical reagents were of analytical grade and all solvents were of HPLC grade. Ultrapure water (18.2 MΩ cm^{-1}) was prepared with a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Luria-Bertani medium (LB, pH 7.0) containing (L^{-1}) trytone 10 g, yeast extract 5 g, and NaCl 10 g and a mineral salt medium (MSM, pH 7.0) containing (L^{-1}) K_2HPO_4 5.8 g, KH_2PO_4 4.5 g, $(NH_4)_2SO_4$ 2.0 g, $MgCl_2$ 0.16 g, and 1.0 mL trace element solution were used in this study. The trace element solution contained (L^{-1}) $CaCl_2$ 20 mg, $Na_2MoO_4 \cdot 2H_2O$ 2.4 mg, $FeSO_4 \cdot 7H_2O$ 1.8 mg, and $MnCl_2 \cdot 4H_2O$ 1.5 mg.

Solid medium plates were prepared by adding 18–20 $g \cdot L^{-1}$ agar into the above mentioned liquid media.

2.2. Enrichment and separation of DBP-degrading endophytic bacteria

Healthy *C. indica* plants were uprooted from the constructed wetland mentioned above and thoroughly washed with tap water and sterilized distilled water orderly. Roots samples that were sliced into 1 cm × 1 cm pieces were sterilized by sequential immersion in 3% H_2O_2 for 3 min, 70% (v/v) ethanol for 3 min, and 5% sodium hypochlorite for 2 min, and then rinsed with sterilized distilled water to eliminate the surface sterilization agents. To verify the effectiveness of surface sterilization process, uncut root tissue and the final rinse water were spread onto LB agar plates. The absence of growth after incubation on the plates confirmed sterilization.

The sterilized root material (1.0 g fresh weight) was homogenized in a mortar and pestle containing 10 mL sterile phosphate-buffered saline (PBS, pH 7.2) to obtain a root suspension. 1 mL of the suspension was inoculated into a 250 mL sterile Erlenmeyer flask containing 100 mL MSM and 100 $mg \cdot L^{-1}$ DBP that were sterilized in an autoclave at 121 °C for 20 min and through a 0.22 μm Millipore filter, respectively.

The process was conducted in triplicate. The flasks were shaken at 150 rpm in an incubator shaker at 30 °C in the dark for 5 days. 1 mL of the enriched cultures were serially transferred into 250 mL Erlenmeyer flasks containing 100 mL sterilized MSM spiked with an increased DBP concentration gradient from 0.1% to 1% (v/v) at 100 $mg \cdot L^{-1}$ interval. The flasks were incubated under the conditions described above at 5-day intervals for a period of acclimatization of ten weeks before the isolation of DBP-degrading strains. The YJB3 strain that could efficiently utilize DBP as the sole carbon and energy source for growth on MSM was selected for further studies.

2.3. Identification of the strain YJB3

2.3.1. Morphological and physiochemical characteristics

The morphological features of the strain YJB3 were examined using scanning electron microscopy (SEM, Philips XL30, Netherlands) and transmission electronic microscopy (TEM, Philips/FEI Tecnai 10, operated at 100 kV). Samples were prepared using the methods of Prior and Perkins (1974) and Chao et al. (2010), respectively. The classical physiochemical identification of the strain YJB3 was systematically performed following the standard protocols described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1998).

2.3.2. 16S rDNA sequence analysis

Taxonomic classification was determined by sequencing the 16S rDNA that was isolated using a bacterial genomic DNA extraction kit (Omega Bio-Tek, USA) according to the protocols recommended by the manufacturer. PCR amplification of 16S rDNA was performed using primers F27 and R1492 (Table S1, Supplementary data). The 50 μL reaction mixture contained 2.5 U TaqDNA polymerase (TaKaRa, Japan), 1 × PCR Buffer (Mg^{2+} Plus), 4 μL of dNTP Mixture (0.2 mM), 20 ng of DNA templates, and 50 nM primers. The cycling conditions were 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 60 s at 56 °C, 90 s at 72 °C, and a final 10 min at 72 °C in a GeneAmp PCR system 9700 instrument (Perkin Elmer, Norwalk, CT, USA). The purification and sequencing of 1492 amplifying products were conducted by Sangon Biotech Co., Ltd. (Shanghai, China). The DNA sequence data were analyzed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular evolutionary analysis and phylogenetic tree construction of the 16S rDNA sequence data were performed using MEGA 6.0 software (www.megasoftware.net).

2.4. Inoculum preparation

The strain YJB3 was pre-cultured in LB nutrient broth medium at 30 °C with shaking at 120 rpm. After enrichment culturing for 8 h, cell pellets were harvested by centrifugation (3000 rpm for 5 min at 4 °C) and washed three times with sterile MSM to remove impurities and resuspended in MSM to obtain an OD_{600} of 1.0 (approximately 10^8 $CFU \cdot mL^{-1}$) determined by a spectrometer (UV-2450, Shimadzu Corp., Japan). The media used for DBP degradation studies contained (L^{-1}) $K_2HPO_4 \cdot 3H_2O$ 0.5 g, KH_2PO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.2–1.0 g, $CaCl_2$ 0.1–0.5 g, $FeCl_3 \cdot 6H_2O$ 0.01–0.05 g, NH_4Cl 0.5–1.5 g, $CH_3COONa \cdot 3H_2O$ 0–1.5 g, and DBP 200 mg. The pH values of the media were adjusted to 5–8 before autoclaving at 121 °C for 20 min. Batch experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL the media and 1–5% the inocula at 20–40 °C on a rotary shaker (100–250 rpm) in dark. Samples were collected after an incubation period of 5 day and GC–MS analysis was performed to detect the DBP residue and its intermediates.

2.5. Analysis of DBP and its metabolites

The residue of DBP and its metabolites were analyzed using GC–MS (QP-2010 Plus, Shimadu, Kyoto, Japan). Culture filtrates were collected at different intervals. The extraction and cleanup procedures of the filtrates were conducted as our previous report (Zhao et al., 2016). 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 5000 rpm for 5 min. This process was then repeated twice. The combined organic phases were concentrated using a rotary evaporator and evaporated to near dryness under a stream of purified nitrogen. The residue was redissolved in 10 mL HPLC grade methanol and then passed through a 0.22 μm membrane filter. Finally, an aliquot of 1.0 μL filtrates was injected into GC–MS. All the tests were conducted in triplicate. The detecting conditions of GC–MS were employed as follows: an HP-5 ms capillary column (0.25 μm × 0.25 mm × 30 m) with helium (purity, >99.999%) as carrier gas at a constant flow rate of 1.0 $mL \cdot min^{-1}$, an injection temperature of 250 °C, and an ion source temperature of 220 °C. The GC oven temperature was programmed as follows: 100 °C held for 2 min, raised at 15 °C·min^{-1} up to 129 °C, then at 40 °C·min^{-1} to 280 °C (held 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 limit of DBP was 0.041 $mg \cdot L^{-1}$. The recovery of DBP ranged from 96.6% to 103.5%.

2.6. Optimization of DBP degradation conditions

In the present study two-step sequential optimization techniques, Plackett-Burman design (PBD) and response surface methodology (RSM) based on central composite design (CCD) were adopted to optimize the DBP degradation by the strain YJB3. The PBD was employed to screen the bioprocess parameters (independent variables) that had the most significant influences on DBP degradation (Pradeep et al., 2015). The high level (+1) of each variable was set far enough from the low level (−1) to identify which parameters of the system significantly influenced degradation of DBP (Table 1). The design matrix was developed using a software Design Expert 8.0.6.1 (Stat-Ease, Inc., Minneapolis, MN, USA) as shown in Table S2.

Based on the results of PBD, the experiments were further designed through CCD with three significant variables: acetate, temperature, and inoculum size. The design plan consisted of 20 experimental runs with each key factor in the design assessed at five different levels including 2^3 factorial central composite design with six axial points ($\alpha = 1.682$) and six repetitive central points ($n_0 = 6$) (Table S3). All experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of media with 200 $mg \cdot L^{-1}$ of DBP to optimize the DBP degradation by the strain YJB3 according to the design. The degradation rate was measured after incubation for 5 days. The bacterial biomass in the fermentation broth was quantified following the method elsewhere (Patil et al., 2016). All the experiments were performed in triplicate.

The quadratic model was used to explain the behavior of the system as shown in Eq. (1):

$$\Upsilon = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} {X_i}^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where Υ represents the predicted response, χ_i and χ_j are the coded independent variables, β_0 is an interception coefficient, β_i, β_{ii}, and β_{ij} are the linear coefficients, quadratic coefficients, and interaction coefficients, respectively. Analysis of variance (ANOVA) and Fisher's test (*F*-value) were performed to check the efficiency of the statistical significance of the model. The multiple determination coefficient R^2 value was calculated to indicate the fitness of model. Three-dimensional response surface and contour plot were graphical representation of relationships between response and experimental variables that could be used for determining the optimum conditions on the DBP degradation.

Table 1
The PBD for screening of significant variables.

Independent variables	Unit	Symbol	Levels of variation of independent variable (χ_i)		Coefficient	Sum of squares	*df*	Mean square	*F* value	*p*-Value Prob > *F*
			−1	+1						
Model						1214.80	9	134.98	20.44	0.0475*
$MgSO_4 \cdot 7H_2O$	$g \cdot L^{-1}$	χ_1	0.2	1.0	1.28	19.51	1	19.51	2.95	0.2278
$CaCl_2$	$g \cdot L^{-1}$	χ_2	0.1	0.5	−0.21	0.52	1	0.52	0.079	0.8052
$FeCl_3 \cdot 6H_2O$	$g \cdot L^{-1}$	χ_3	0.01	0.05	1.16	16.10	1	16.10	2.44	0.2588
NH_4Cl	$g \cdot L^{-1}$	χ_4	0.5	1.5	0.56	3.74	1	3.74	0.57	0.5302
Acetate	$g \cdot L^{-1}$	χ_5	0	1.5	5.92	421.27	1	421.27	63.79	0.0153*
pH		χ_6	5	8	−3.98	189.61	1	189.61	28.71	0.0331*
Inoculum size	% (v/v)	χ_7	1	5	4.83	279.37	1	279.37	42.30	0.0228*
Agitation rate	rpm	χ_8	100	250	1.63	31.69	1	31.69	4.80	0.1599
Temperature	°C	χ_9	20	40	4.59	253.00	1	253.00	38.31	0.0251*

* Probability = 0.05–statistically significant at the level.

2.7. Substrates utilization tests

To examine its capacity to utilize different substrates, the strain YJB3 was cultured in liquid MSM supplemented with one of the following substrates including PA, PCA, MBP, DMP, DEP, DBP, DEHP, DnOP, and DINP (initial concentrations, 200 $mg \cdot L^{-1}$) as the sole sources of carbon and energy. Each treatment was performed in triplicate and then incubated at 30 °C, 150 rpm for 5 days. The bacterial growth was followed by measuring the increase of optical density of biomass production at 600 nm (OD_{600}) using a spectrophotometer (UV-2450, Shimadzu, Japan) and the light microscopic observation.

2.8. The whole-genome sequencing and bioinformatic analysis of the stain YJB3

The genomic DNA was extracted by Bacterial Genomic DNA Isolation Kit (OMEGA), and its concentration and purity were detected using ND-1000 spectrophotometer. The genomic DNA with concentration above 100 ng μL^{-1} and A260/A280 between 1.8 and 2.0 was subjected to sequencing by Illumina/Pacbio platform, and the generated data were submitted to Biozeron Biotechnology Co., Ltd., Shanghai for further analysis. KEGG (Kyoto Encyclopedia of Genes and Genomes) annotations for the genome of the strain YJB3 were performed for gene and protein function prediction. Moreover, we also conducted a comparative genome analysis on two other *B. megaterium* strains and eight *Bacillus* strains based on the published data from the National Center for Biotechnology Information (NCBI) database. Raw sequence data of the strain YJB3 have been deposited in the NCBI Short Read Archive with accession number SRA557441. The details of the whole-genome sequencing and bioinformatic analysis of the strain YJB3 will be published elsewhere (Feng et al., manuscript in preparation).

2.9. PCR detection of protocatechuate dioxygenase gene

To detect the presence of the gene encoding enzyme of the metabolic pathway, protocatechuate 3, 4-dioxygenase gene was determined by PCR amplification using degenerate primers as our previous description (Zhao et al., 2016). The PCR mixture consisted of 10 × PCR buffer 5.0 μL, Mg^{2+} (25 mM) 5.0 μL, 50 × dNTP (10 mM) 1.0 μL, each primer (10 pM) 1.0 μL, Taq DNA polymerase (5 U μL^{-1}) 0.5 μL, and DNA template 3.0 μL. The reaction mixture was added to a final volume of 50 μL with double-distilled water. The PCR program was an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, plus a final extension at 72 °C for 10 min. The purified PCR products were cloned into the pGME-T vectors, and the recombinant plasmids were then propagated into *Escherichia coli* DH5α competent cells. The white colonies from blue-white screening plates were randomly selected and re-amplified by PCR to identify the positive clones. Representative positive clones were selected for sequencing (by Sangon, Shanghai, China).

3. Results

3.1. Isolation of DBP-degrading endophytic strains

Isolation of DBP-degrading strains were conducted following enrichment and gradient acclimatization by exposing microorganisms to gradually increasing DBP concentrations from 0.1% to 1% at 100 $mg \cdot L^{-1}$ interval for a 10-week incubation period. Five strains capable of utilizing DBP as sole carbon and energy source were obtained from the root tissues of *C. indica*. These isolates exhibited variable degradation capacity (15.2%–71.6%) of DBP in MSM with 0.2% (v/v) DBP in 5 days of incubation (data not shown), with the strain YJB3 performing best. Thus, it was selected for further study in the present paper.

3.2. Identification of the strain YJB3

The strain YJB3 colonies growing at 30 °C for 18 h on LB nutrient broth plates were flat, faint yellow, opaque, and smooth along their edges with diameters of 0.8–2.5 mm (Fig. 1A). The SEM examination showed that its cells were long rods with 1.5–2 μm width and 5.2–7.8 μm length (Fig. 1B). The TEM micrograph showed that its cells had no flagella (Fig. 1C). These morphological characteristics of the strain YJB3 were very similar to those of the reported strains of *Bacillus megaterium*.

16S rDNA sequence of the strain YJB3 was determined and deposited in Genbank with the accession number KU291378.1. Calculation of the sequence similarity using the BLAST search and comparison to GenBank nucleotide database showed high degree of sequence similarity to *Bacillus megaterium* KU1 (JF683607.1, 99%). Phylogenetic relation of the strain YJB3 with this strain as well as other members of the genus *Bacillus* in terms of 16S rDNA sequence similarity is shown in Fig. S1. The molecular identification result further corroborated the verdict of the strain YJB3 based on its morphological and physiochemical characteristics. In conclusion, the bacterium was identified as *Bacillus megaterium* YJB3.

Besides, colonization and survival of the strain YJB3 *in planta* are very important endophytic characteristics for endophyte-assisted phytoremediation of PAE-contamination, which are necessary for effective degradation of PAEs *in planta*. Therefore, the survival rate of the strain YJB3 in plant internal tissues was assessed in our preliminary experiments by rhizosphere inoculation. Though the strain YJB3 originally isolated from the root tissues of host plant *C. indica*, it showed high levels of colonization in roots and shoots of non-host plant Chinese

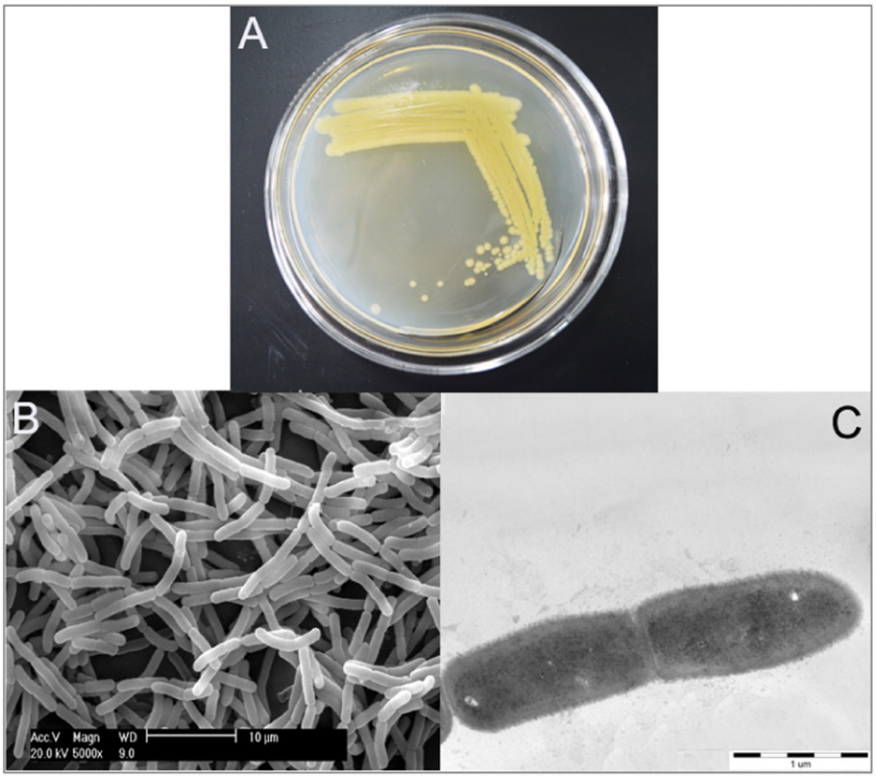

Fig. 1. Morphological properties of the colonies and cells of strain YJB3. (A) Colonies of strain YJB3 on the LB solid plate; (B) Scanning electron microscopy (SEM) photograph of strain YJB3 (5000×); and (C) Transmission electronic microscopy (TEM) photograph of strain YJB3 (7500×).

flowering cabbage (*Brassica parachinensis* L.), suggesting that the strain YJB3 was a non-host specific colonizer that could thrive inside the plant tissues and spread within the vascular system (unpublished data).

3.3. Optimisation of the DBP-degrading conditions

To obtain the optimal conditions for strain *B. megaterium* YJB3 to degrade DBP, the effects of incubation temperature, rotary speed, inoculum size, pH, and the concentrations of $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $FeCl_3 \cdot 6H_2O$, NH_4Cl, and acetate on DBP degradation were analyzed by RSM employing PBD and CCD successively. Before the optimization by PBD, the amounts of $FeCl_3$, NH_4Cl, $MgSO_4$, and $CaCl_2$ in the media were determined by single-factor tests respectively. As a more easily degradable carbon source, acetate has been used as one of the most common co-substrates for enhancing the biodegradation of recalcitrant organic contaminants (Liu et al., 2017; Zhang and Lo, 2015). In addition, the acetate existing in root exudates has been proved to be associated with enhanced phytoremediation of organic contaminants (Martin et al., 2014). Meanwhile, the result of our preliminary experiments showed that acetate was the best carbon source for enhancing the degradation of DBP as compared to other carbon sources including yeast extract, glucose, sucrose, and maltose, and the optimum concentration of acetate was 1.5 g/L (Fig. S2). Moreover, the results of single-factor tests indicated that the highest DBP degradation (79.2%) was achieved after 5 days of incubation, which was applied in the following experiments.

At the first optimisation step, a 12-run PBD was performed to identify the significant variables for DBP degradation (Table S2). The relative ranking of these variables showed that acetate ($p = 0.0153$), inoculum size ($p = 0.0228$), temperature ($p = 0.0251$), and pH ($p = 0.0331$) were the most significant factors affecting DBP degradation (Table 1). Taking into account that the physiological status of plant cells where endophytes colonized kept better when pH was set at 7.0, only the first three significant factors, acetate, inoculum size, and temperature within the tested limits were selected for further optimization by RSM based on CCD.

At the second optimization step, a 20–run CCD was used to identify the optimal levels of acetate concentration, inoculum size, and temperature. Each of these variables was assessed at five levels (Table S3). The coded ($-\alpha$, -1, 0, $+1$, $+\alpha$) and uncoded values of the variables are: acetate (χ_5, g/L): 0, 0.3, 0.75, 1.2, 1.5; inoculum size (χ_7, %): 1.0, 1.8, 3.0, 4.2, 5.0; temperature (χ_9, °C): 20, 24, 30, 36, 40. The matrix design and the observed responses are represented in Table S4.

Statistical significance and adequacy of the model were checked employing analysis of variance and were tested using Fisher's *F*–test. Applying multivariate regression analysis, a second-order polynomial expression (Eq. (2)) that predicts the effects of variables on DBP degradation is fitted as follows:

$$Y = 77.35 + 1.13\chi_5 - 0.19\chi_7 + 0.074\chi_9 - 3.06\chi_5\chi_7 - 3.76\chi_5\chi_9 - 6.06\chi_7\chi_9 + 0.9{\chi_5}^2 - 0.66{\chi_7}^2 - 1.68\chi_9 \quad (2)$$

where Y, χ_5, χ_7, and χ_9 are the coded values of DBP degradation rate, acetate, inoculum size, and temperature, respectively.

The probability value (*p*–values) of the Fisher's *F*–test for the model (<0.001) was 0.0001 for DBP degradation (Table 2), implying a high significance of the model. The adequacy of the model explained about 92.9% of the response variability based on the determination coefficient ($R^2 = 0.9286$), suggesting that the predicted values of the model were in perfect agreement with the experimental values. The adjusted determination coefficient (Adj $R^2 = 0.8672$) further demonstrated the accuracy of the model.

In an attempt to lower the cost of DBP degradation, the inoculum size of the strain YJB3 should be set at the minimum. Based on this premise, we used the model to generate a contour plot for the pair-wise combination of acetate and temperature. By setting the goals of variance analysis to maximize DBP degradation and minimize inoculum size, the

Table 2
ANOVA for response surface quadratic model of DBP degradation based on CCD.

Source	Sum of squares	*df*	Mean square	*F*-Value	p-Value Prob > *F*
Model	562.60	9	62.51	14.45	0.0001**
χ_5	17.33	1	17.33	4.01	0.0732
χ_7	0.49	1	0.49	0.11	0.7426
χ_9	0.074	1	0.074	0.017	0.8986
χ_5^2	11.61	1	11.61	2.68	0.1324
χ_7^2	6.24	1	6.24	1.44	0.2575
χ_9^2	40.83	1	40.83	9.44	0.0118*
$\chi_5\chi_7$	75.03	1	75.03	17.34	0.0019**
$\chi_5\chi_9$	113.25	1	113.25	26.18	0.0005**
$\chi_7\chi_9$	294.03	1	294.03	67.96	<0.0001**
Residual	43.26	10	4.33		
Lack of Fit	43.13	5	8.63	323.47	<0.0001**
Pure Error	0.13	5	0.027		
Cor Total	605.87	19			

$R^2 = 0.9286$
Adjusted $R^2 = 0.8672$

χ_5 acetate, χ_7 inoculum size, χ_9 temperature.
* Statistically significant at the 95% confidence level ($p < 0.05$).
** Statistically significant at the 99.95% confidence level ($p < 0.005$).

optimized parameters of acetate, inoculum size, and temperature were 1.2 g·L^{-1}, 1.8%, and 34.2 °C, respectively. Under these optimized conditions, the predicted DBP degradation was 82.8%, completely consistent with the actual detected value of 82.5%, indicating the validity and effectiveness of the model. We used 3D graphical interpretations to get a better understanding of the effects of the variables and their interactions on the response (Fig. 2). The two-factor interactions, the terms acetate × inoculum size ($\chi_5\chi_7$), acetate × temperature ($\chi_5\chi_9$), and inoculum size × temperature ($\chi_7\chi_9$), presented *p*-values of 0.0019, 0.0005, and <0.0001, respectively, showing significant interaction effects on DBP degradation (Table 2; Fig. 2).

The degradation profile of DBP was determined by the preliminary assays. DBP was degraded more and more efficiently with the growth and biomass accumulation of the strain YJB3 (Fig. S3). The analysis of relationship between the degradation rate of DBP and the biomass of the strain YJB3 displayed a linear correlation coefficient of 0.7895 ($p < 0.01$), showing that the DBP degradation rate increased positively with the biomass of the strain YJB3 (Fig. S4). Therefore, this statistical approach was successful in the present study in optimizing the DBP degradation conditions and determining the maximal degradation rate by RSM for the strain YJB3.

3.4. Substrates utilization

After 5 days of incubation in MSM containing supplemented substrates, the strain YJB3 showed a utilization capacity of various PAEs (Table S5). However, there were significant substrate specificities in terms of the types of PAEs that shorter ester chains (DMP, DEP, and DBP) were degraded more easily than the longer ones (DEHP, DnOP, and DINP). In addition, the strain YJB3 was able to utilize the common intermediates of PAE degradation, such as MBP, PA, and PCA as the sole source of carbon (Table S5).

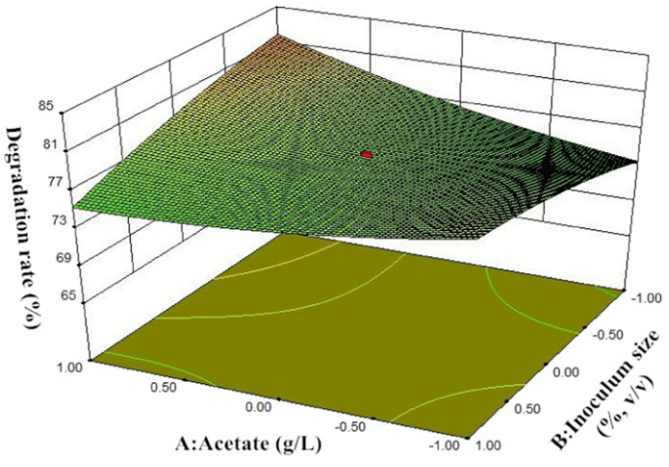

Fig. 2. Response surface graph showing the effects on DBP degradation rate.

3.5. Biodegradation pathway of DBP by B. megaterium YJB3

Metabolites formed during biodegradation were analyzed by GC–MS to elucidate the probable catabolic pathway of DBP by the strain YJB3. During the first 12 h of incubation, the peak area of DBP decreased at 6.33 min and two new peaks appeared at 6.13 min and 5.36 min, respectively, implying the formation of two new compounds (Fig. 3), which were identified as MBP and PA by comparing the mass spectra at a particular retention time with the published mass spectra from National Institute of Standards and Technology (NIST) database (Fig. 3). Moreover, the observed retention time and mass spectra of the metabolites matched precisely with those of authentic MBP and PA standards.

As the metabolization proceeded, the areas of all the three peaks mentioned before decreased rapidly and no accumulative metabolites were observed at the end of experiment, further indicating that the strain YJB3 could use these compounds as a sole carbon source, respectively. During the degradation process, the other metabolites such as PCA, β-carboxy-muconate, γ-carboxy-muconolactone, *etc.* were not detected, probably because they had disappeared owing to immediate degradation by the strain YJB3 once they were formed. Several peaks occurred in the chromatogram should be interfering peaks owing to no appreciable changes in the area and height during the whole experimental period (Fig. 3). In sterile MSM without inoculation of the strain YJB3, DBP changed little and no target metabolites were detected throughout the experiment period. These results indicated that the strain YJB3 was capable of degrading DBP completely by way of MBP, PA, PCA, *etc.* (Fig. 4) (Benjamin et al., 2015; Liang et al., 2008).

On the other hand, according to the whole-genome sequencing results, a total of 5207 predicted open reading frames (ORFs) with an average length of 810 bp were identified in the genome. Among the predicted genes, 28 ORFs were associated with PAEs degradation. The protocatechuate 3,4-dioxygenase gene contained a 756 bp ORF encoding a protein of 251 amino acids was found. The ORF encoding the putative dioxygenase was confirmed to be the target gene by successfully amplifying an expected fragment of about 750 bp DNA from the genome DNA of strain YJB3 (Fig. S5). Sequence alignment using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that the deduced amino acid sequence of the dioxygenase showed 90%, 88%, and 82% similarities to β-carboxy-cis, cis-muconolactone decarboxylase (PcaB, a key PCA-degrading enzyme) from *B. flexus* (GenBank accession no. WP_ 025749964.1), *B. megaterium* (GenBank accession no. WP_074898351.1), and *Paenibacillus* sp. DMB5 (GenBank accession no. WP_068721383.1), respectively (Lorite et al., 1998). The KEGG analysis showed that the gene encoding β-carboxy-cis, cis-muconolactone decarboxylase catalyzed β-carboxy-muconate to yield γ-carboxy-muconolactone, which was subsequently degraded *via* β-oxidation to tricarboxylic acid (TCA) (Fig. 4). In conclusion, for the first time, the DBP degradation pathway in endophytic bacterium was proposed in the present study.

4. Discussion

4.1. Isolation and identification of DBP-degrading bacterium

In the present study, an endophytic strain *B. megaterium* YJB3 was isolated from the root tissues of *C. indica*. The morphological and biochemical characteristics including color of colonies, morphology, cell size, spore formation, nitrate reduction, and Voges-Proskauer reaction of the strain YJB3 specifically differed from those of the other

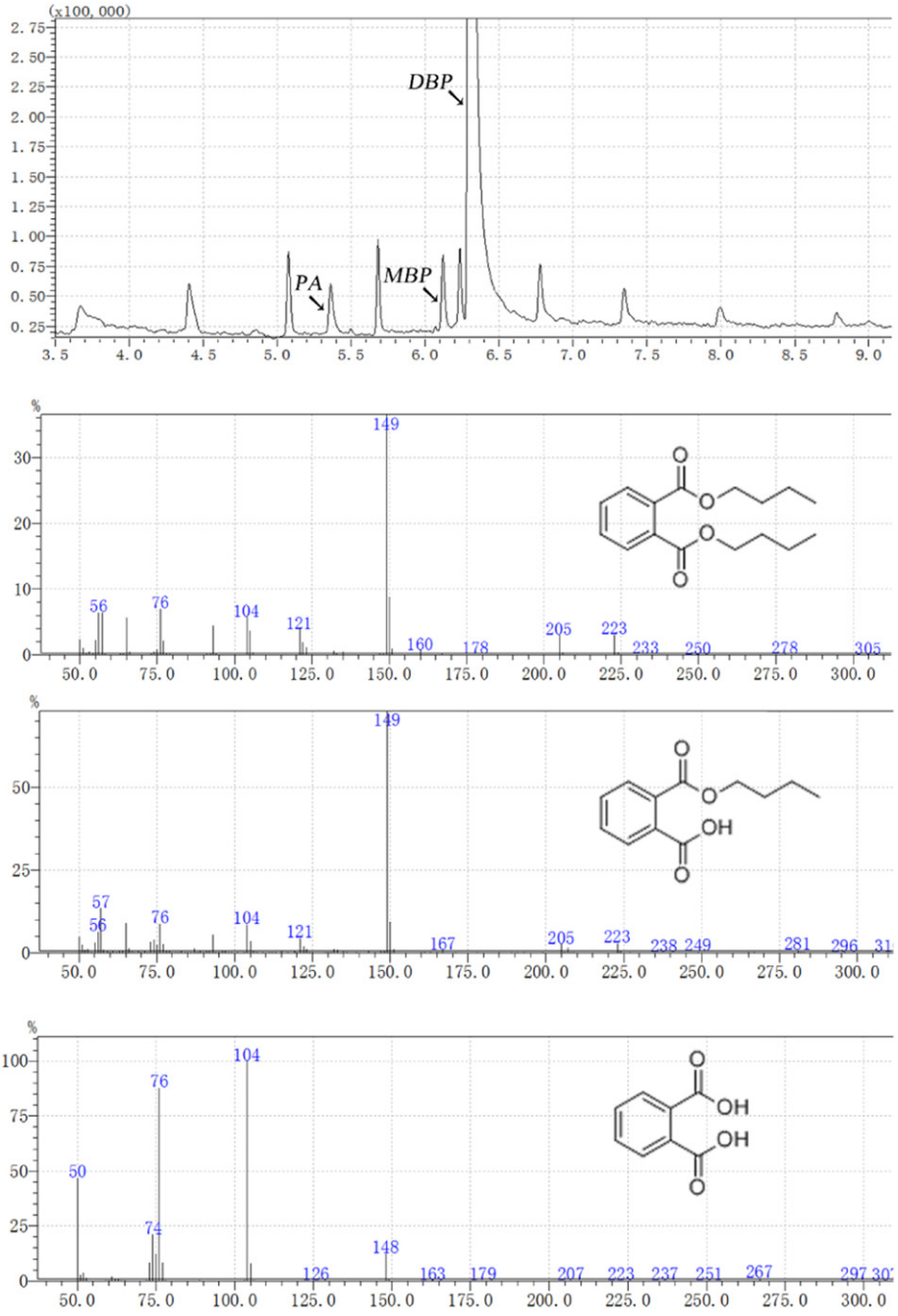

Fig. 3. GC–MS chromatograms and spectra of DBP degradation intermediates in MSM.

B. megaterium strains (Table 3). Moreover, based on comparison of complete genome sequences, different *B. megaterium* strains carry roughly 300 strain-specific chromosomal genes accounting for their differences in experimentally confirmed phenotypes (Eppinger et al., 2011). The strain YJB3 harbored 413 strain-specific chromosomal genes based on comparative genome analysis (Table S6), suggesting that it was a new strain, which would greatly promote the motivation to isolate various new *B. megaterium* strains suitable for different fields.

Many bacterial species from *Bacillus* genus have been reported to degrade various PAEs (Gao and Wen, 2016; Liang et al., 2008). For example, *Bacillus subtilis* 3C3 obtained from soil can simultaneously degrade DPP, DBP, BBP, and DEP (Navacharoen and Vangnai, 2011). While the strains *Bacillus sp.* NO11 and NO14 isolated from river sediments can completely degrade DEP, DBP, and DEHP (Chang et al., 2005). All these findings demonstrate that different species of *Bacillus* derived from external environmental sources possess desirable ability to degrade PAEs. However, at present, little information regarding PAEs degradation by endophytic *B. megaterium* strains is available. Only a few endophytic bacteria that can efficiently degrade very limited organic pollutants (such as PAHs, PHs, TCE, and BTEX) have been reported in literatures (Afzal et al., 2014; Feng et al., 2017).

The present study was a primary investigation on utilization of endophytes to degrade PAEs. The endophytic strain YJB3 capable of utilizing DBP as sole carbon source for growth and tolerating up to 1000 $mg \cdot L^{-1}$ of DBP was successfully acclimatized from PAE-contaminated *C. indica* wetland plant, indicating that PAE-degrading endophytes could reside in the internal tissues of PAE-contaminated plants and might possess specialized degradation pathways in plants. The competence of the strain YJB3 was comparable to that of the key phthalates degraders including *Arthrobacter*, *Mycobacterium*, and

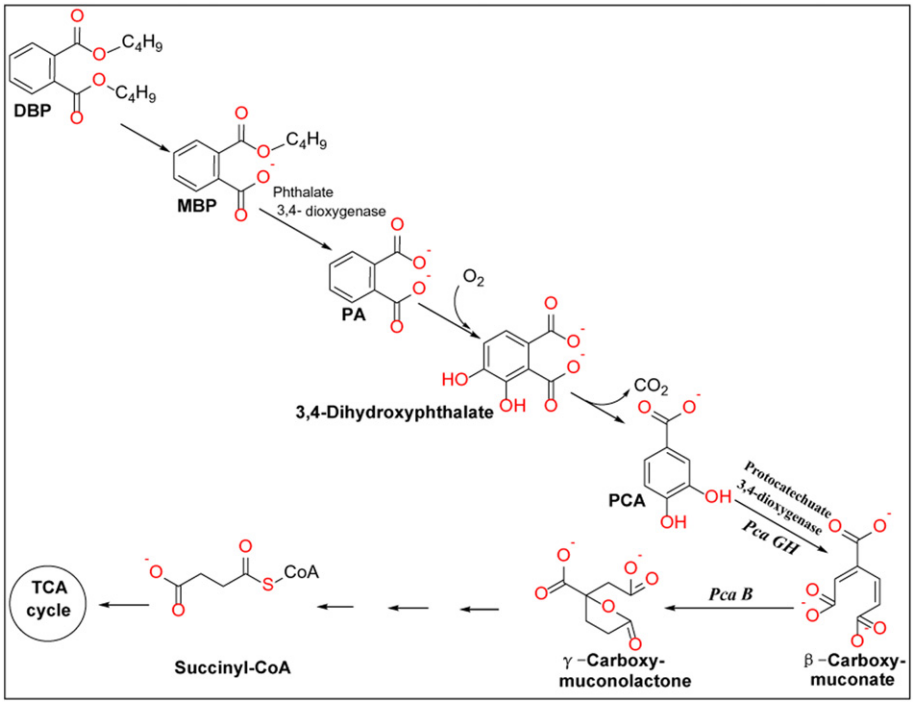

Fig. 4. A proposed pathway for degradation of DBP by the strain YJB3.

Rhodococcus that were from the external environmental sources (Benjamin et al., 2015). The concentrations of DBP and other PAEs in environment are far lower than the above mentioned tolerable value (1000 mg·L^{-1}), thus, the strain YJB3 exhibited an ability to degrade extremely high levels of DBP in environment, making it exceedingly attractive for agricultural applications in removal of PAEs in both soils and crops.

4.2. Optimization of the DBP-degrading conditions

Generally, the growth and proliferation of a microorganism and its ability to degrade toxic pollutants are strongly influenced by many experimental factors such as nutritional requirements, cell energetic status, and physico-chemical cultivation conditions (Ungureanu et al., 2015). Therefore, it is necessary to obtain desirable optimum conditions with high efficiency and low cost. In the present study, the obtained optimal conditions were successfully applied to enhance DBP degradation. The optimal conditions originated from the current experiment provide a reliable reference to effectively and rationally use this strain in bioremediation of PAE-polluted environments. In comparison with previous studies (Yang et al., 2014; Zhao et al., 2016), the time of complete biodegradation of DBP by the strain YJB3 was slightly longer, which might be due to the differences in the inoculum size, environmental conditions, bacterial characteristics, and the strain's original habitat of isolation (Badejo et al., 2013; Navacharoen and Vangnai, 2011). Nevertheless, the strain YJB3 could directly degrade DBP without accumulative metabolites under a wide range of conditions, indicating excellent environmental adaption and desirable candidate inoculant for agricultural application.

Table 3
A comparison of morphological and biochemical characteristics between the strain YJB3 and other *B. megaterium* strains.

Characteristics	*Bacillus megaterium*			
	Strain YJB3 (This paper)	Strain WS24 (Zhang et al., 2012)	Strain G3 (Liu et al., 2014)	Strain BJC3.1 (Li et al., 2009)
Color of colonies	Faint yellow	Milk white	Faint yellow	
Opacity	Opaque		Opaque	
Morphology	Long rod shape	Short rod shape	Short rod shape	
Size (μm)	1.5–2.0 in width 5.2–7.8 in length	0.3–0.5 in width 1.1–1.5 in length	1.2–1.5 in width 1.9–3.7 in length	
Motility	+	+		
Gram staining	+	+	+	+
Aeriobic growth	+			Anaerobic growth
Spore formation	+	−		+
Catalase activity	+	+		+
Glucose fermentation	Acid	Acid		Acid
Nitrate reduction	−			+
Starch hydrolysis	+	+		+
Gelatin hydrolysis	−			
Indole production	−			
Methyl red	+			
Voges-Proskauer reaction	−			+
Citrate utilization test	−			
Hydrogen sulfide test	−			
Urea test	+			

Habitat-adapted endophytic bacteria can easily colonize various plant species, distribute among plant tissues, maintain stable relationships with their host plants (Compant et al., 2010; Sun et al., 2014a), thereby improving biodegradation capabilities of organic pollutants (Feng et al., 2017). When endophytic bacteria in plants are faced with the stresses similar to those in external environments, they possess a natural capacity to cope with external stress like organic contamination *in planta* as well as *in vitro* (Siciliano et al., 2003; Sun et al., 2014b), which provides the possibility of applying endophytes to degrade PAEs in plants. The endophytic strain YJB3 has ability to recolonize the interior of plants as well, benefiting from habitat-adaptation and the hallmark of endophytism. (Naveed et al., 2014; Rosenblueth and Martínez-Romero, 2006). In this very close plant-endophyte relationship, plants provide readily co-substrates and optimal conditions for the strain YJB3, which in return can confer plants beneficial effects in removal of PAEs in soils and/or crops (Afzal et al., 2014; Reinhold-Hurek and Hurek, 2011).

4.3. Substrates utilization

Given that various PAEs co-exist in the environments and the degradation of PAEs in environments is usually incomplete (Gao and Wen, 2016; Net et al., 2015), the investigation of substrate utilization profiles is necessary. The broad range of substrates and complete degradation of PAEs are desirable characteristics for the application potential of the strain YJB3. The PAEs degradation by the strain YJB3 is comparable with that by many exogenous bacteria, such as *Providencia* sp. 2D, *Microbacterium* sp. J-1, and *Rhodococcus* sp. JDC-11 (Jin et al., 2010; Zhao et al., 2016, 2017). The increase in length of alkyl-chains exerts greater steric effects of phthalate ester side chains, which hinder the binding of hydrolytic enzymes to phthalates (Table 4S), resulting in differences in growth rate of the strain YJB3. Since some PAEs are not readily utilized as carbons sources for cell growth, their degradation are enhanced in the presence of other easily degradable substrates as electron acceptor/donors such as acetate, amino acids, and glucose (Pandey et al., 2009). Just as expected, the robust growth of the strain YJB3 occurred with the addition of acetate that served as a co-substrate for co-metabolism of PAEs (data not shown), which suggested that the addition of co-substrates could be a better option for improving the PAEs degradation by the strain YJB3. High effective and low-cost co-substrates need to be further determine in future.

4.4. Biodegradation pathway of DBP by the strain YJB3

Biodegradation of DBP by microorganisms is considered to be one of the major routes for remediation of the environments contaminated by DBP (Benjamin et al., 2015). Before application of bioremediation techniques, it is crucial to identify, at the stage of the preliminary studies, the intermediate metabolites of microbial degradation. In a desirable biodegradation process, the elimination of a parent contaminant must not be accompanied by the accumulation of toxic intermediates (Di Gennaro et al., 2005). The primary biodegradation of DBP entailed the sequential hydrolysis of the ester bonds between the alkyl chains and the aromatic ring, involving the formation of MBP followed by PA, indicating that phthalate 3, 4-dioxygenase catabolized the incorporation of two hydroxyl groups on the phthalate ring (Liang et al., 2008). Then, the aromatic ring cleavage occurred before entering the TCA cycle, finally leading to complete degradation by the strain YJB3. The results of the present study indicated that the metabolic pathway used by the strain YJB3 was in accordance with the previously reported metabolic pathways of PAEs degradation by Gram-positive bacteria (Jin et al., 2010; Liang et al., 2008). However, most of our knowledge on the aforementioned metabolic pathways of DBP degradation using pure cultures has been obtained based mainly on speculated evidences including identification of the metabolites and detection of the catabolic genes encoding key enzymes involved in PAEs degradation (Benjamin et al., 2015). In practice, deducing metabolism is an imperfect task. In contrast, the whole-genome analysis and gene annotation of the strain YJB3 enabled us to directly reveal the presence of a variety of potential genes responsible for the biodegradation of PAEs and promote the investigation of environmental bioremediation by the strain YJB3. Further studies on the genetic and biochemical characterization of dioxgenase involved in the degradation of PAEs need to be elucidated resorting to "omics" technologies, such as transcriptomics, proteomics, and metabolomics (Bouhajja et al., 2016).

Phthalate monoester and PA are the major intermediates from PAEs degradation and also have important toxicological implications (Jin et al., 2012). However, some PAE-degrading microorganisms can transform PAEs only to PA, because the latter cannot be further utilized for cell growth. For example, *Camelimonas* sp. M11 capable of degrading several PAEs into PA cannot further utilized the intermediate as sole carbon source (Chen et al., 2015). Some strains of *Gordonia* sp. cannot degrade or degrade PA slightly (Chatterjee and Dutta, 2003; Wu et al., 2010). Therefore, they need assistances of other microbes such as *Arthrobacter* sp. so as to achieve the complete degradation of PAEs. Similarly, dimethyl isophthate is completely degraded only in the presence of both *Klebsiella oxytoca* Sc and *Methylobacterium mesophilium* Sr (Li and Gu, 2007). In contrast, the strain YJB3 is capable of carrying out the complete degradation of DBP by itself without accumulation of toxic metabolites. Most importantly, the *B. megaterium* strain YJB3 is one of the most common cultivable endophytic species in Bacillaceae, which can colonize inside many plant species (Bacon and Hinton, 2006). Given this fact, it is not surprising to find that the strain YJB3 has great potential and competitive advantages over its exogenous counterparts as a bioremediation microorganism for *in planta* elimination of PAEs residues in crops.

5. Conclusions

Biotransformation and utilization of DBP and the other PAEs by *B. megaterium* strain YJB3 was demonstrated in the present study. The strain YJB3, a novel endophytic bacterium, could efficiently utilize various PAEs as sole carbon source and exhibited good adaptability to the varying environments. This strain harbored the metabolic pathway for complete degradation of DBP without accumulative toxic intermediates. The strain YJB3 has great potential and competitive advantages over its exogenous counterparts as a bioremediation organism for *in situ* elimination of DBP residues in soils and crops. This study will encourage a full use of the strain YJB3 to remove PAEs in soils and crops by employing the special partnership of plant and endophyte, thereby decreasing the accumulation of PAEs in crops and reducing risks to human health, which is of great significance to safely use the PAEs-polluted soil with agricultural production and pollution bioremediation proceeding simultaneously. To the best of our knowledge, this is the first report on the biodegradation of PAEs by endophyte strain isolated from wetland plant *C. indica*. From this study, it is confirmed that the data obtained *in vitro* will contribute to future research aimed at developing endophytic bacteria for degrading PAEs *in planta*, providing a good alternative to reduce/prevent PAEs accumulation in crops for food safety.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.298.

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