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ORIGINAL PAPER

Enhanced dissipation of DEHP in soil and simultaneously reduced bioaccumulation of DEHP in vegetable using bioaugmentation with exogenous bacteria

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Abstract The widely used plastic film containing di- (2-ethylhexyl) phthalate (DEHP) in agriculture has caused serious soil pollution and poses risks to human health through the food chain. An effective DEHP degradation bacteria, *Microbacterium* sp. J-1, was newly isolated from landfill soil. Response surface methodology was successfully employed for optimization resulting in 96% degradation of DEHP (200 mg L^{-1}) within 5 days. This strain degraded DEHP by hydrolysis of the ester bond and hydroxylation of the aromatic ring to form 2-ethyl hexanol, mono-(2-ethylhexyl) phthalate, phthalate acid, and protocatechuic acid, and subsequently transformed these compounds with a maximum specific degradation rate (q_{max}), half-saturation constant (K_s), and inhibition constant (K_i) of 1.46 day^{-1}, 180.2 mg L^{-1}, and 332.8 mg L^{-1}, respectively. Bioaugmentation of DEHP-contaminated soils with the strain J-1 greatly enhanced the DEHP dissipation rate (~88%). Moreover, this strain could efficiently colonize the rhizosphere soil of inoculated vegetables and further enhanced DEHP degradation (~97%), leading to a significant decrease (>70%) in DEHP accumulation in shoots and roots of the inoculated vegetables compared to uninoculated vegetables. The results highlighted the roles of the inoculated exogenous bacteria in simultaneously bioremediating contaminated soils and reducing bioaccumulation of DEHP in the edible part of the vegetable for food safety.

Keywords Phthalate esters (PAEs) · *Microbacterium* sp. J-1 · Degradation pathway · Soil bioremediation · Vegetable accumulation · PCR-DGGE

Introduction

Phthalate esters (PAEs), commonly used plasticizers in products such as polyvinyl chloride, are ubiquitous environmental pollutants and are recognized as environmental endocrine disruptors because of their potential to elicit reproductive and developmental toxicity (He et al. 2013; Zhao et al. 2016a,b). Among various PAEs, di-(2-ethylhexyl) phthalate (DEHP) is employed in widespread applications, such as plastic film, tubing, toys, home products, and food packaging (Guo and Kannan 2012). Studies have shown that DEHP exposure raises human health concerns for the possible dysfunctions of the endocrine, reproductive, and nervous systems, coupled with acquired diseases like cancer, allergy, and gender birth defects (Pradeep et al. 2015; Sampson and De Korte 2011). Currently, DEHP has been listed as one of the top-priority pollutants by the US Environmental Protection Agency (US EPA), China National Environmental Monitoring Center, and other international organizations (Niu et al. 2014). As a result of its low water solubility (about 0.003 mg/L) and high octanol/water partition coefficients (log*Kow* = 7.94), DEHP tends to accumulate in soil, sediment,

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and suspended solids (Chao and Cheng 2007). Its concentrations in soils of some regions in China exceed the control standard set by the New York State Department of Environmental Conservation (Niu et al. 2014; Wang et al. 2012). Our previous investigations showed that elevated DEHP concentration was frequently detected in agricultural soils, and higher DEHP concentration was observed in soils of vegetable fields due to extensive use of agricultural plastic films (Cai et al. 2008; Mo et al. 2009). Generally, diet is considered to be a key route of human exposure to PAEs through the food chain (Schecter et al. 2013). DEHP can be accumulated in vegetables grown in contaminated soil, especially in the Chinese flowering cabbage (*Brassica parachinensis*), which poses a potential threat to food safety and human health (Mo et al. 2009; Zhao et al. 2015).

The biodegradation of PAEs by microbes is considered to be a major route of degradation for these widespread pollutants in the environment, thereby ameliorating the health risks associated with them (Pradeep et al. 2015). Numerous studies have reported on the biodegradation of PAEs by different microorganisms under various environmental conditions as reviewed by Liang et al. (2008). It is generally known that PAEs with short and simple side chains can be easily biodegraded and mineralized, while PAEs with long and complex side chains are poorly biodegraded (Chang et al. 2004; Liang et al. 2008). Accordingly, the average half-life of DEHP biodegradation in a variety of environments was far longer than those of PAEs with shorter alkyl-side chains (Chen et al. 2007; Nakamiya et al. 2005). Thus, it is necessary to screen and isolate more high-efficient DEHP-degrading bacteria. Besides, the DEHP degradation efficiency of indigenous microorganisms in soil was relatively low. It was estimated that more than 41% of the DEHP added to the soil was still present after 1 year (Madsen et al. 1999). The microbial metabolisms of organic pollutants are usually not driven by energy needs, but microbes may have to suffer an energy deficit for a necessity to protect against toxicity. Thus, the processes may be helped and driven by the abundant energy that is provided by root exudates. Such stimulation of soil microbial communities by root exudates also benefits plants through increasing the availability of soil-bound nutrients and alleviating the damage of pollutants to plants (Chaudhry et al. 2005). In addition, there might be a synergistic action by both plants that increased the availability of hydrophobic compounds in soil and rhizosphere microbes that degraded pollutants. To date, many examples inoculating a specific pollutant-degrading bacterium in the rhizosphere demonstrated enhanced remediation of soils polluted with organic pollutants (Balseiro et al. 2016; Chaudhry et al. 2005; Thion et al. 2013; Yu et al. 2011). However, the interactions between PAE-degrading bacteria and plants as well as their effects on PAEs dissipation from soil are poorly studied.

In the present study, a high-efficient DEHP-degrading strain was isolated from landfill soil and characterized. Its optimum conditions, degradation kinetics, and pathway for DEHP biodegradation were investigated. To assess the interactions of DEHP-degrading bacteria and the Chinese flowering cabbage and their effects on DEHP dissipation from soils and DEHP accumulation by the Chinese flowering cabbage, the DEHP dissipation, plant accumulation, soil enzymes activities, and dissolved organic matter were studied. Cultivar *Huaguan* of the Chinese flowering cabbage demonstrated high bioaccumulation of DEHP in shoot in our previous investigation (Zhao et al. 2015). It was therefore chosen as the experimental plant. Finally, the survival condition of inoculated DEHP-degrading bacteria in the rhizosphere soil and its related microbial community composition were also analyzed by PCR-DGGE technique.

Materials and methods

Chemicals and medium

Dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), DEHP, and di-n-octyl phthalate (DnOP), and diisononyl phthalate (DINP), 2-ethyl hexanol, mono-(2-ethylhexyl) phthalate (MEHP), phthalate acid (PA), and protocatechuic acid (PCA) (all of >98% purity) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). All other chemical reagents were of analytical grade, and all solvents were HPLC grade.

Isolation and identification of the DEHP-degrading bacteria

To isolate DEHP-degrading bacteria, an enrichment culture technique with DEHP as the sole source of carbon and energy was performed according our previous method (Zhao et al. 2016a, b) with slight modifications (see supplementary M1). The strain (hereafter, strain J-1) that could utilize DEHP as the sole source of C and energy for growth on MSM was selected for further study. The isolate was characterized and identified by the morphological, physio-biochemical characteristics, and 16S rRNA gene analysis (see supplementary M1).

Optimal conditions for DEHP biodegradation

Response surface methodology (RSM) based on the Box-Behnken design was applied to determine the optimal conditions for DEHP degradation by strain J-1. In this experiment, three key factors for optimizing were temperature (20–40 °C), pH (6.0–10.0), and inoculum (OD_{600} = 0.2–1.0). The range and center point values of three independent variables

were based on the results of preceding experiments. The symbols and levels of three independent variables used in the Box-Behnken design are shown in Table S1. The dependent variable was the degradation of 200 mg L^{-1} DEHP by strain J-1 at day 5. The symbols and levels of three independent variables (temperature, pH, and inoculum size) used in the Box-Behnken design are shown in Table S1. The data were analyzed using RSM of the statistic analysis system (SAS) software (version 9.0) to fit the following quadratic polynomial equation (Eq. (1)) (Chen et al. 2012):

$$Y = b_0 + \Sigma b_i X_i + \Sigma b_{ij} X_i X_j + \Sigma b_{ii} {X_i}^2 \quad (1)$$

where Y 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.

DEHP and other PAEs degradation by strain J-1 under optimal conditions

Degradation experiments with different initial concentrations of DEHP (50–2000 mg L^{-1}) and with other PAE compounds (each 200 mg L^{-1}) including DMP, DEP, DBP, BBP, DnOP, and DINP by the strain J-1 were also conducted in MSM under the optimal culture conditions. The experiment was conducted in triplicate with the non-inoculated medium as control. Samples were withdrawn periodically from the cultures to examine growth by recording the optical density (OD) value at 600 nm using a spectrophotometer and to analyze all the PAEs using GC/MS.

Analysis of PAEs and DEHP metabolites

The residual PAE compounds (including DEHP, DMP, DEP, DBP, BBP, DnOP, and DINP) were analyzed using GC/MS (Shimadzu QP2010 Plus, Japan). Culture filtrates were collected at different intervals, and the filtrate extraction, cleanup, and GC/MS procedures were conducted according to the method described in our previous reports (Zhao et al. 2016a, b). The DEHP metabolites in cell-free filtrates of strain J-1 cultures grown in MSM containing 200 mg L^{-1} of DEHP were also identified by GC/MS. The metabolites identified by mass spectrometry analysis were matched with authentic standard compounds from the mass spectra library in the MS system. The details of the quality assurance/quality control (QA/QC) measures used have been presented in our previous reports (Zhao et al. 2016a, b). The recoveries of PAE compounds ranged from 85.3 to 109.7%.

Detection of the phthalate 3,4-dioxygenase gene

The phthalate 3,4-dioxygenase gene was amplified using two degenerate primers (forward primer: 5′-RGRGAGRATCGACGTTYACGC-3′, reverse primer: 5′-RCASGARGCTGAGYTTTGRGRACY-3′; Y represents A or C, S represents G or C, R represents A or G), which were designed based on ten conserved sequences of the phthalate 3,4-dioxygenase reported by NCBI (the sequence accession numbers in GenBank are provided in supplementary M1). The PCR mixture consisted of 5.0 μL 10 × PCR buffer, 5.0 mM $MgCl_2$, 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 brought to a final volume of 50 μL with sterile 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 a 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, white colonies were randomly selected and re-amplified by PCR to identify the positive clones. Representative positive clones were selected for sequencing.

Pot experiment

A pot experiment was conducted to evaluate the effects of bacterial inoculation on vegetable biomass production and DEHP residues of vegetable and soil. In the present study, soil collected from an agricultural field of the South China Agricultural University (Guangzhou, China) was air dried and sieved to 2 mm prior to use. The main soil properties are as follows (dry weight, DW): pH, 6.7; total organic C, 7.9 g kg^{-1}; total N, 1.32 g kg^{-1}; total P, 1.57 g kg^{-1}; and a sandy loam texture including sand 35.7.0%, silt 52.3%, and clay 12.0%. DEHP was spiked at 50 mg kg^{-1} of soil and then aged for 2 weeks in a dark environment. This experiment included four soil treatments with five replicates each: DEHP-spiked soil (CK), DEHP-spiked soil with inoculated bacteria (Inoculation), DEHP-spiked soil with planted vegetable (Planting), and DEHP-spiked soil with planted vegetable and inoculated bacteria (Planting + Inoculation). Ceramic pots were filled with the soils at 4.0 kg (DW) per pot and mixed with 6.0 g of chemical fertilizers consisting of urea and KH_2PO_4 (N/P/K = 4:3:4). Twenty seeds of the Chinese flowering cabbage were sown into every prepared pot. Ten days after seed germination, seedlings were thinned to maintain five plants per pot. Then, the bacterial suspension (strain J-1) was inoculated into the soil using drip irrigation to achieve a final concentration of approximately 1×10^8 CFU g^{-1} (0.9% sterile saline as control). The pots were randomly arranged in the greenhouse with natural temperature (25–32°C), and watered daily with sterile water to maintain moderate soil moisture for 45 days. The soil samples from all the treatments were periodically collected after inoculation (1, 7, 14, 21, 28, and 35 days, respectively) and stored at −70 °C until further analysis. The rhizosphere soils from the pots with planted vegetable, defined as soil adhering to roots

after removing excess bulk soil with gentle shaking, were obtained from the vegetable roots. Plants were harvested 35 days after inoculation. The fresh plants were separated into roots and shoots, and then were weighed, respectively. The plant samples were kept frozen at −70 °C until further analysis. The procedures of sample (soil or plant) ultrasonic-assisted extraction and cleanup for DEHP analysis using silicagel column were conducted as already described (Zhao et al. 2015). The recoveries of DEHP in soil and plant samples ranged from 92.0 to 96.5% and from 87.4 to 107.2%, respectively.

DNA extraction and PCR-DGGE analysis

Extractions of the total DNA from soil samples (0.5 g wet weight) collected at 7, 21, and 35 days after inoculation were performed using the EZNA™ Soil DNA kit (Omega Bio-Tek, USA) following the manufacturer's instructions. The partial sequence fragment (V3 region) of the 16S rRNA gene was PCR-amplified with the primers 341F (5′-CCTA CGGGAGGCAGCAG-3′) with GC clamp and 534R (5′-ATTACCGCGGCTGCTGG-3′) for DGGE analysis, which is most frequently used to start the study of an unknown and complex bacterial community (Muyzer et al. 1993; Sanchart et al. 2015). The PCR program was a denaturing step at 94 °C for 5 min; followed by 10 cycles at 94 °C for 30 s, 62 °C (reducing by 1 °C each cycle) for 30 s, and 72 °C for 30 s; and subsequent 20 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1.2% agarose gels. DGGE analysis was performed with a DGGE 1102 system (Chishun, China). Twenty-five microliters of each PCR product was loaded onto 10% (*w*/*v*) polyacrylamide gels containing a 40–60% linear chemical gradient (where a 100% denaturing solution contained 40% formamide and 7 M urea) and run electrophoresis in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.5) with 80 V, 60 °C for 14 h. After electrophoresis, the gels were stained with SYBR Green I solution for 30 min and documented with a UVP gel documentation system.

Soil enzyme activity and DOC analysis

The activities of soil enzymes including peroxidase (POD) and polyphenol oxidase (PPO) in soil samples were measured according to the methods described by Yu et al. (2011) with minor modifications. Briefly, the mixture of 1 g soil sample and 10 ml 1% pyrogallic acid together with 2 ml 0.5% H_2O_2 (as a substrate for POD measurement) or 10 ml 1% pyrogallic acid only (as a substrate for PPO measurement) was incubated at 30 °C for 2 h, and 4 ml citric-phosphoric acid buffer (pH 4.5) was added to the mixture. The purpurigallin produced was extracted with ether, and then measured by a spectrophotometer (Shimadzu UV-2450, Japan) at 430 nm.

For the measurement of dissolved organic C (DOC), a 10-g soil sample were shaken with 50 ml of deionized water for 1 h, followed by centrifugation (8000×*g* for 10 min) and filtration through a 0.45-μm filter membrane. The DOC concentration in the extracted solution was determined using a TOC analyzer (Shimadzu TOC-VCSH, Japan).

Kinetics analysis

The first-order kinetic model (Eq. (2)) was used to explore the biodegradation kinetics of various PAEs:

$$\ln C = -kt + A \tag{2}$$

where C is the concentration of DEHP at time t, k is the first-order kinetic constant, and A is a constant. The algorithm as expressed in Eq. (3) was used to describe the theoretical half-life ($t_{1/2}$) values of DEHP:

$$t1/2 = \frac{\ln 2}{k'} \tag{3}$$

where ln2 is the natural logarithm of 2, and k' is the rate constant (h^{-1}).

Moreover, the substrate inhibition model (Eq. (4)) was used to fit the specific degradation rate (q) at different initial concentrations [24].

$$q = \frac{q\max S}{S + Ks + \left(S^2/Ki\right)} \tag{4}$$

where q_{max} is the maximum specific DEHP degradation rate (day^{-1}), K_i is the substrate inhibition constant (mg L^{-1}), K_s is the half-saturation constant (mg L^{-1}), and S is the substrate concentration (mg L^{-1}).

Results and discussion

Isolation and identification of strain J-1

After enrichment culture with increasing DEHP, the most efficient strain isolated from landfill soil was selected and designated as J-1. Colonies of the strain J-1 grown on LB agar for 7 days were circular, opaque, convex, and yellowish white. The bacteria were Gram-positive, rod-shaped cells, 0.5–2.0 μm in length (Fig. S1). The physio-biochemical characteristics of strain J-1 were presented in Table S2. The 16S rRNA gene sequence of strain J-1 was deposited in the GenBank database (accession no. KP670414), and it shared the closest phylogenetic relationship to *Microbacterium* species with 99% of sequence similarity (Fig. 1). Taken together,

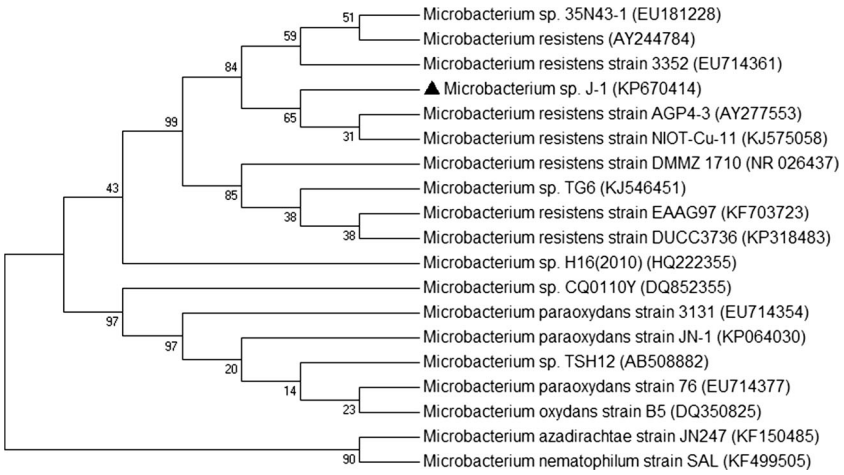

Fig. 1 Phylogenetic tree based on 16S rRNA sequences of strain J-1 and the representative *Microbacterium* species, constructed using the MEGA 5.05 software (*numbers in parentheses* represent the sequence accession numbers in GenBank; *numbers at the nodes* indicate bootstrap values from the neighborhood-joining analysis of 1000 resampled data sets; similarly hereinafter)

strain J-1 was identified as *Microbacterium* sp. This genus is widely distributed in many environments including activated sludge, arable soils, marine sediments, wastewater, and others (Tappe et al. 2013). Accordingly, *Microbacterium* species are involved in diverse biodegradation reactions and in oxidation and reduction reactions (Evtushenko and Takeuchi 2006). The degradation of xenobiotic compounds like polycyclic aromatic hydrocarbons (PAHs) (Sheng et al. 2009), sulfadiazine (Tappe et al. 2013), and chlorobenzene (Patel and Vyas 2015) by *Microbacterium* strains has been reported. However, very few studies have reported the DEHP biodegradation by this genus, which indicated that *Microbacterium* strains could effectively degrade DEHP in wastewater and polluted soil (Chen et al. 2007; Yuan et al. 2011). Here, an effective degradation bacterium of DEHP, *Microbacterium* sp. J-1, was isolated. It was different from the previously reported *Microbacterium* sp. CQ0110Y (Chen et al. 2007) based on the comparison of morphology, physio-biochemical characteristics, and 16S rRNA sequence analysis.

Optimization of the DEHP-degrading conditions by strain J-1

Based on the Box-Behnken design, RSM was employed to investigate the interactive effects of significant variables including temperature (X_1), pH (X_2), and inoculum (X_3) on DEHP degradation. The experimental design matrix and the response of the dependent variable for DEHP degradation (Y) are given in Table S3. Then, the data from Table S3 were assessed by the response surface regression procedure of SAS, and the results of the quadratic polynomial model fitting ANOVA were shown in Table S4. The statistical significance of this model was also evaluated by performing F test (Table S4) and t test (Table S5). The statistical analysis indicated that the model linear term coefficient of X_1, X_2, and X_3 and the quadric term coefficient of X_1, X_2, and X_3 showed significant effects ($p < 0.05$) on DEHP degradation. Therefore, the following fitted regression model was used to investigate the effects of temperature (X_1), pH (X_2), and inoculum (X_3) on the degradation rate of DEHP (Y):

$$Y = 8811.1 + 248.65X_1 + 334.4788X_2 + 1283.826X_3 - 1903.369{X_1}^2 - 2879.356{X_2}^2 - 1103.506{X_3}^2 \quad (5)$$

where X_1, X_2, and X_3 are the coded values for the temperature, pH, and inoculum size, respectively.

The three-dimensional response surfaces in Fig. S2 (a, b, and c) showed the respective effects of X_1 and X_2, X_1 and X_3, and X_2 and X_3 on the degradation rate of DEHP while keeping the third variable at 0 (coded value). The model predicted a maximum DEHP degradation of approximately 96% within 5 days under the optimum conditions (i.e., pH 8.3, 32 °C, and inoculum size 0.8 (OD_{600})). Compared to the traditional method of single-factor analysis, RSM was found to be a competent method for ensuring single and interactive effects of independent variables (Priji et al. 2015). Previous studies have shown that the application of response surface methodology in biodegradation processes can result in improved yields of degradation and allow the rapid and economical determination of the optimum culture conditions with fewer experiments and minimal resources (Chen et al. 2012 and 2013; Pradeep et al. 2015; Priji et al. 2015). Moreover, a mathematical model (Eq. (5)) was developed herein, which effectively optimized DEHP degradation conditions by strain J-1 within the limits of chosen factors. The correlation coefficient ($R^2 = 0.9936$) demonstrated that the predicted values of this model were well correlated with the experimental values, thereby proving the precision of the model.

Degradation kinetics of DEHP with different initial concentrations

In order to investigate the effect of the initial DEHP concentration on degradation activity, the degradation kinetics of DEHP in mineral salt medium was studied. Figure 2a showed

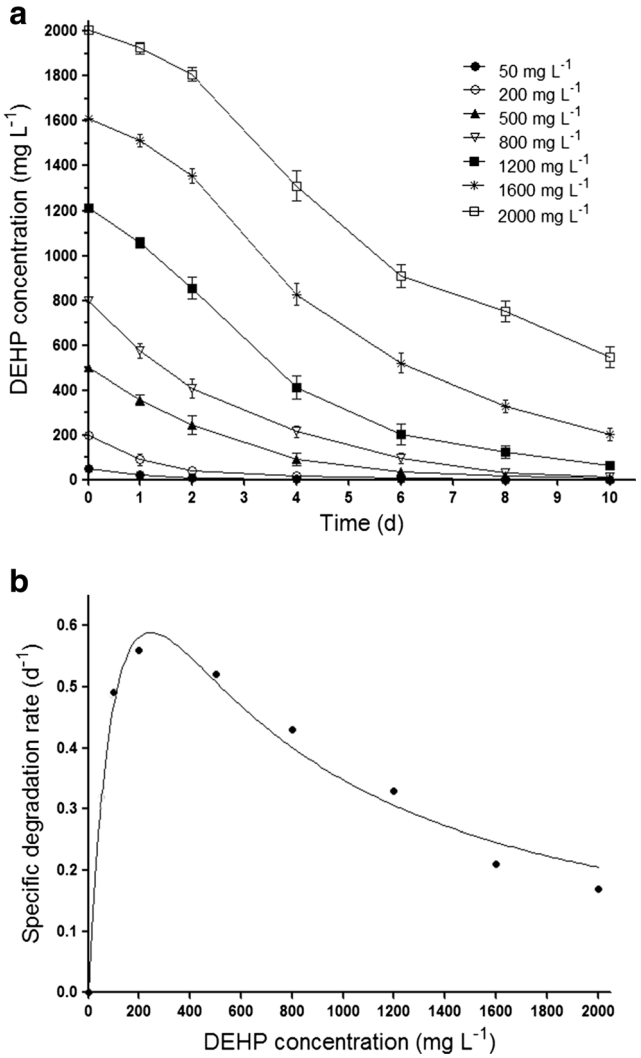

Fig. 2 Biodegradation of DEHP in mineral salt medium with different initial concentrations by strain J-1 (**a**) and specific degradation kinetics curve (**b**)

the kinetic curves of DEHP degradation at different initial concentrations (50–2000 mg L^{-1}). Strain J-1 could rapidly degrade high concentrations of DEHP up to 2000 mg L^{-1}. A lag phase was observed when DEHP concentrations ≥1200 mg L^{-1}. At low DEHP concentrations (50–800 mg L^{-1}), DEHP was almost completely degraded within 10 days. However, the degradation rate was slowed down by the increasing DEHP concentration and DEHP removal rates were 91.8, 87.2, and 72.8% at the concentration of 1200, 1600, and 2000 mg L^{-1}, respectively. This might be due to the fact that bacterial growth started slowly (Fig. S3) and required an acclimation period before accelerated degradation occurred at high xenobiotic concentrations (Chen et al. 2014).

The first-order model (Eq. (2)) was used to represent the biodegradation kinetics of DEHP with different initial concentrations (Table S6). The degradation rate constant (k) of DEHP calculated ranged from 0.1078 to 0.5330 day^{-1} with half-lives ($t_{1/2}$) of 1.30–6.43 days, respectively. The correlation coefficient R^2 ranged from 0.9238 to 0.9859, indicating that the degradation data were well fitted with the model. Previous studies reported that the half-lives of DEHP biodegradation were 3.01 days (5 mg kg^{-1} DEHP) by *Sphigomonas* sp. DK4 (Chang et al. 2004), 13.9 days (5 mg kg^{-1} DEHP) by *Corynebacterium* sp. O18 (Chang et al. 2004), and 16.99 days (200 mg kg^{-1} DEHP) by *Pseudomonas fluorescens* FS1 (Zeng et al. 2002). By contrast, *Microbacterium* sp. J-1 was much more efficient in biodegrading DEHP in this study. However, its removal efficiency was slightly lower than *Microbacterium* sp. CQ0110Y (Chen et al. 2007), which might be the result of different experimental conditions such as the initial DEHP concentration, pH, or the inoculum size.

The relationship between specific degradation rate (q) and initial DEHP concentrations was showed in Fig. 2b. The kinetic parameters of strain J-1 determined from nonlinear regression using the Graph Pad Prism software (version 5.0) were q_{max} = 1.46 day^{-1} and K_s = 180.2 mg L^{-1}. The inhibitory effect of DEHP was considered to occur in a linear manner at K_i = 332.8 mg L^{-1}. The value of R^2 was 0.9788, demonstrating that the experimental data were well correlated with the model (Eq. (4)). The critical inhibitor concentration (S_m) was established to be 244.9 mg L^{-1} by calculating the square root of $K_i \times K_s$, which revealed that the DEHP degradation activity of strain J-1 would be partially inhibited above this concentration. However, the inhibition may not occur under natural environments because the DEHP concentrations in the environments are much lower than the S_m value. In the water environment, the DEHP concentration ranged from 0.33 to 97.8 μg L^{-1} (surface water), 1.74 to 182 μg L^{-1} (sewage effluents), 27.9 to 154 mg kg^{-1} (sewage sludge), and 0.21 to 8.44 mg kg^{-1} (sediment) in samples collected from various rivers, lakes, and channels in Germany (Fromme et al. 2002). In the soil environment, the DEHP concentration ranged from 0 to 16.0 mg kg^{-1} in the agricultural fields throughout Mainland China (Cai et al. 2008; Niu et al. 2014). Therefore, the DEHP-degrading bacterium *Microbacterium* sp. J-1 can work well without DEHP inhibition in the above contaminated environments.

Kinetics analysis of other PAEs degradation by strain J-1

The strain could efficiently utilize most of the tested PAEs including DMP, DEP, DBP, BBP, and DnOP as the growth

substrates with the degradation rates of 99.8, 99.8, 99.6, 93.5, and 98.3% within 5 days, respectively (Fig. 3). But DINP was poorly degraded with a degradation rate of 56.2%. Kinetic data showed that the degradation processes were well fitted with the first-order model (R^2 ranging from 0.9272 to 0.9570) and were characterized by k ranging from 0.1300 to 1.0232 day^{-1} and half-lives ($t_{1/2}$) varying from 0.68 to 5.33 days (Table S7). Abiotic degradation was negligible in the non-inoculated controls throughout the present study. It is well known that PAEs with longer alkyl chains (such as BBP, DnOP, DEHP, and DINP) are more difficult to be biodegraded than those with shorter ones (such as DMP, DEP, and DBP), because the steric effects of PAE side chains avoid the binding of hydrolytic enzymes to PAEs, thereby inhibiting their hydrolysis (He et al. 2013; Liang et al. 2008). Nevertheless, stain J-1 could effectively degrade the PAEs with longer alkyl chains (including DEHP and DnOP, except for DINP), as well as those with shorter alkyl chains, highlighting that this strain has great potential and advantages in the bioremediation of PAE-contaminated environments.

Biochemical degradation pathway of DEHP

To confirm the metabolic profile, the intermediates were determined by GC/MS analysis and five distinct peaks were observed after 1 day culture (Fig. 4a). Based on the retention time (*RT*) and mass spectrometry analysis (*m*/*z*), MEHP (*RT*, 9.28 min; *m*/*z*, 149, 167, 57, 279), PA (*RT*, 6.55 min; *m*/*z*, 104, 76, 50, 146), PCA (*RT*, 5.99 min; *m*/*z*, 154, 107, 70, 280), and 2-ethyl hexanol (*RT*, 3.62 min; *m*/*z*, 57, 70, 83, 98) were identified apart from unutilized DEHP (*RT*, 9.80 min; *m*/*z*, 149, 167, 57, 279) (Fig. 4b–f). All the five peaks decreased gradually over time and disappeared finally at the end of 6 days. For the degradation pathway of PAEs, it is generally supposed that hydrolysis of the ester side-chain of the dialkyl phthalate through mono-alkyl phthalate occurs, leaving PA and alkyl alcohols available for further degradation (Chen et al. 2007). Based on the above intermediates, it was proposed that DEHP was hydrolyzed to MEHP and 2-ethyl hexanol by the action of esterase, and then yielded PA via further hydrolysis of MEHP. Similar pathways of DEHP degradation were also found in *Achromobacter denitrificans* (Pradeep et al. 2015), *Mycobacterium* sp. (Nakamiya et al. 2005), and *P. fluorescens* (Zeng et al. 2002). It is widely acknowledged that PA is degraded by two dioxygenase-catalyzed pathways, forming the common intermediate PCA (Liang et al. 2008). A dioxygenase catalyzes the formation of *cis*-3,4-dihydro-3,4-dihydroxyphthalate to produce 3,4-dihydroxyphthalate in Gram-positive bacteria, while the other one catalyzes the formation of *cis*-4,5-dihydro-4,5-dihydroxyphthalate to produce 4,5-dihydroxyphthalate in Gram-negative bacteria. To verify the former occurring in strain J-1 (Gram-positive), we performed amplification and analysis of the potential gene encoding phthalate 3,4-dioxygenase gene. A fragment of 736 bps gene sequence was cloned from strain J-1 using a set of degenerate primers, which was deposited in GenBank with accession no. KT359341. The sequence analysis showed that the obtained fragment was highly homologous with the nucleotide sequences of phthalate 3,4-dioxygenase genes, and the deduced amino acid sequence identity of the amplified fragment ranged from 74 to 88%. A phylogenetic tree (Fig. 5) was constructed based on the deduced amino acid sequence, indicating a close relationship of strain J-1 with the phthalate 3,4-dioxygenase of *Rhodococcus* sp. TFB (accession no.: AAY57926). To our knowledge, it is the first to report the cloning of the phthalate 3,4-dioxygenase gene from bacteria belonging to the *Microbacterium* genus. The result indicated that strain J-1 harbored the phthalate 3,4-dioxygenase gene and might hydroxylate the PA ring at positions 3 and 4 to form 3,4-dihydroxyphthalate. Collectively, DEHP was initially hydrolyzed to MEHP and 2-ethyl hexanol, and subsequently MEHP to PA, then PA is converted to PCA through hydroxylating the PA ring at positions 3 and 4 (i.e., 3,4-dihydroxyphthalate). Finally, the intermediates PCA and 2-ethyl hexanol entered the tricarboxylic

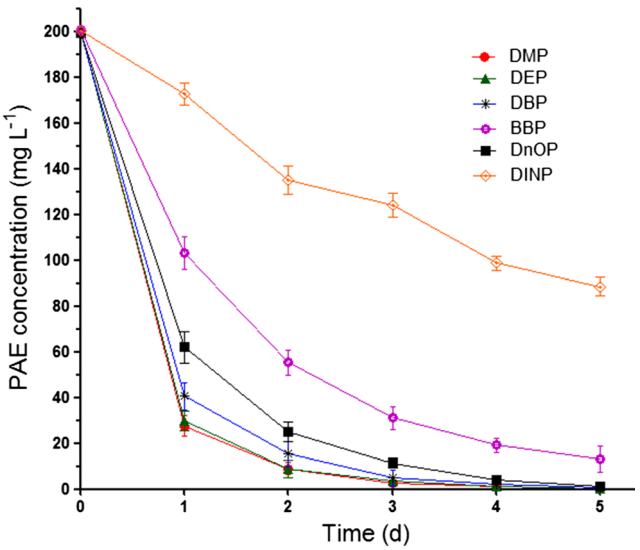

Fig. 3 Biodegradation dynamics of other PAEs by strain J-1 with the initial concentration of 200 mg L^{-1}. *Bars* indicate the standard error of three replicates

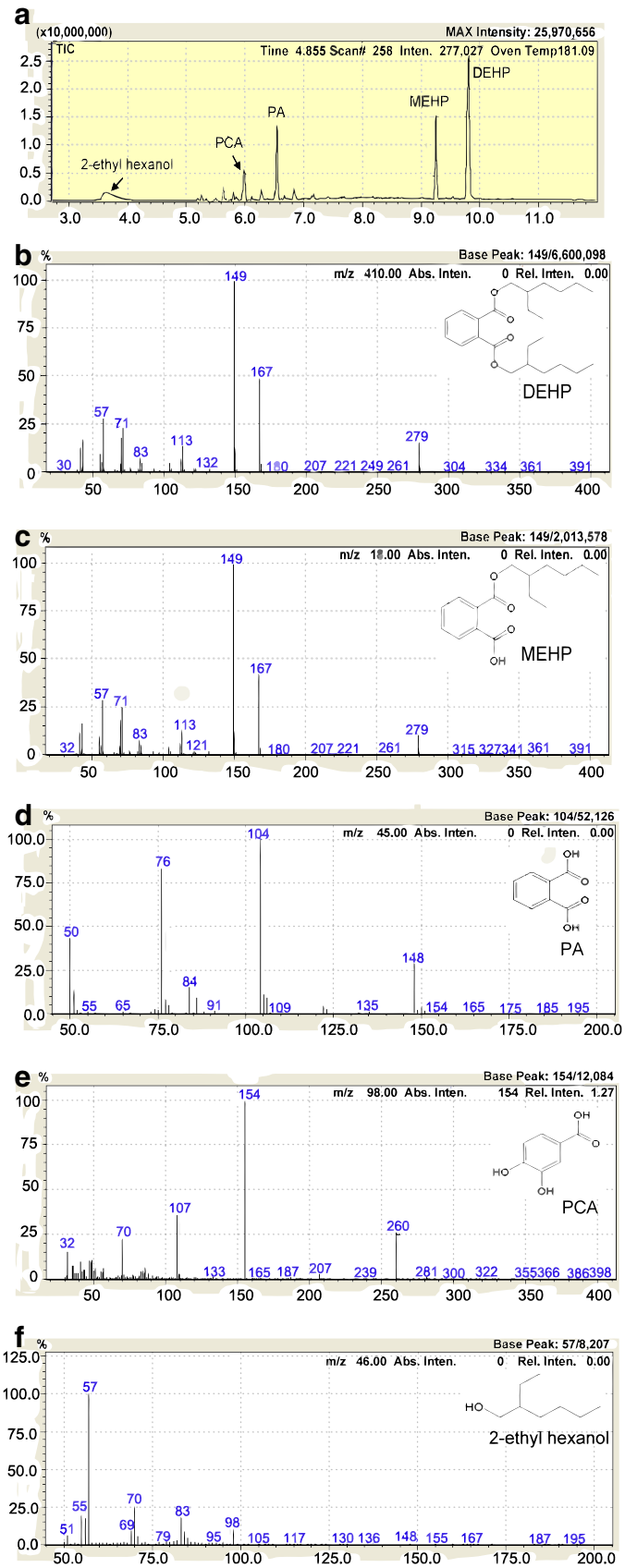

Fig. 4 GC/MS analysis of the degradation products of DEHP from 1-day cultures

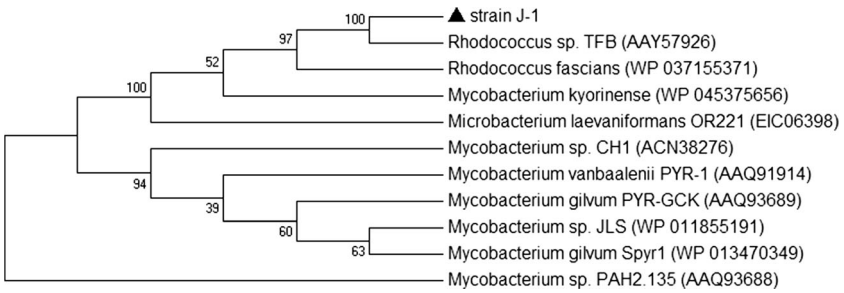

Fig. 5 Phylogenetic tree based on 3,4-phthalate dioxygenase amino acid sequences of strain J-1 and relating species, constructed using the MEGA 5.05 software

acid (TCA) cycle and were used as the substrates for bacterial growth. The tentative pathway proposed for degradation of DEHP by strain J-1 was shown in Fig. 6.

DEHP dissipation from soils and DEHP accumulation by vegetable

The DEHP residues in soils were determined to analyze the effects of vegetable cultivation and bacteria inoculation on DEHP dissipation from contaminated soils (Fig. 7a). After 35 days, only 12% of DEHP was removed from the DEHP-spiked control soil (CK), while 88, 62, and 97% of DEHP were removed from soils in treatments of bacteria inoculation, vegetable cultivation, and their combined cultures, respectively. The result showed that the presence of strain J-1 or cv. *Huaguan* alone, as well as their combined cultures, obviously accelerated DEHP dissipation from soils when compared with CK. Many studies have shown that a direct bioaugmentation of degradation bacteria into contaminated soil can greatly enhance contaminant removal (Chen et al. 2014; Zhao et al. 2016a, b), and a similar result was also observed in this study, indication of a great potential for strain J-1 to efficiently remediate DEHP-contaminated soils. Moreover, PPO activities in this study were significantly ($p < 0.05$) enhanced by inoculated strain J-1 (Fig. 8a), which is an important oxidoreductase in soil and can catalyze oxidation of aromatic compounds (Duran and Esposito 2000). Similarly, elevation of PPO activities was also observed in contaminated soil with inoculation of PAH-degrading bacteria *Acinetobacter* sp. (Yu et al. 2011). In addition, the presence of planted vegetable significantly increased ($p < 0.05$) POD activities in soil when compared with unplanted soils (Fig. 8b). It was reported that many plants could release POD into the rhizosphere soil, which had a direct effect on aromatic degradation (Husain and Ulber 2011; Kraus et al. 1999; Muratova et al. 2009). Thus, the elevated POD activity may partly explain the enhanced dissipation of DEHP from soil in the presence of vegetable. The highest dissipation rate was noted in the combined cultures of vegetable cultivation and bacteria inoculation, indicating a significant synergistic effect between cv. *Huaguan* and strain J-1 on promoting DEHP dissipation from soils. Accordingly, enhanced soil enzymatic activities (including both POD and PPO, Fig. 8a, b) by inoculated bacteria and planted vegetable may be one of the important mechanisms responsible for the high-efficient removal of DEHP from soil. As reported by Chi and Gao (2015), the microbial degradation of PAEs (including DBP and DEHP) could be enhanced by the submerged macrophyte *Potamogeton crispus*. Other studies also showed that the dissipation rates of PAHs, DDT, or petroleum oil from soil in the presence of microorganisms and plants were higher than those of only plants or microorganisms (Balseiro et al. 2016; McGuinness and Dowling 2009; Thion et al. 2013; Yousaf et al. 2010; Yu et al. 2011).

On the other hand, our previous investigation showed that the vegetable cv. *Huaguan* was prone to accumulate a high concentration of DEHP in the shoot (edible part) when grown on contaminated soil, which might pose risks to human health through the food chain (Zhao et al. 2015). In this study, inoculation of strain J-1 significantly ($p < 0.05$) decreased DEHP accumulation in both the shoot and root of cv. *Huaguan* by 74 and 83%, respectively, but no significant ($p > 0.05$) effect on biomass (Fig. 7b). Meanwhile, as the result observed at day 7 after the initial inoculation, the DEHP dissipation rates (59–69%) of inoculated soils (including only inoculated soil and planted + inoculated soil) were far greater than that of only planted soil (4%) (Fig. 7a). As a typical hydrophobic contaminant, DEHP ($\log K_{OW}$ ~7.5) often adsorbs onto the plant surface first before being incorporated in tissues (Chi and Gao 2015). The surface accumulation rates of many hydrophobic contaminants are much faster than other biological processes (Koelmans 2014). Therefore, DEHP in the rhizosphere soil or absorbed on the root surface was rapidly degraded by inoculated strain J-1 prior to being taken up into roots. Overall, the results indicated that low

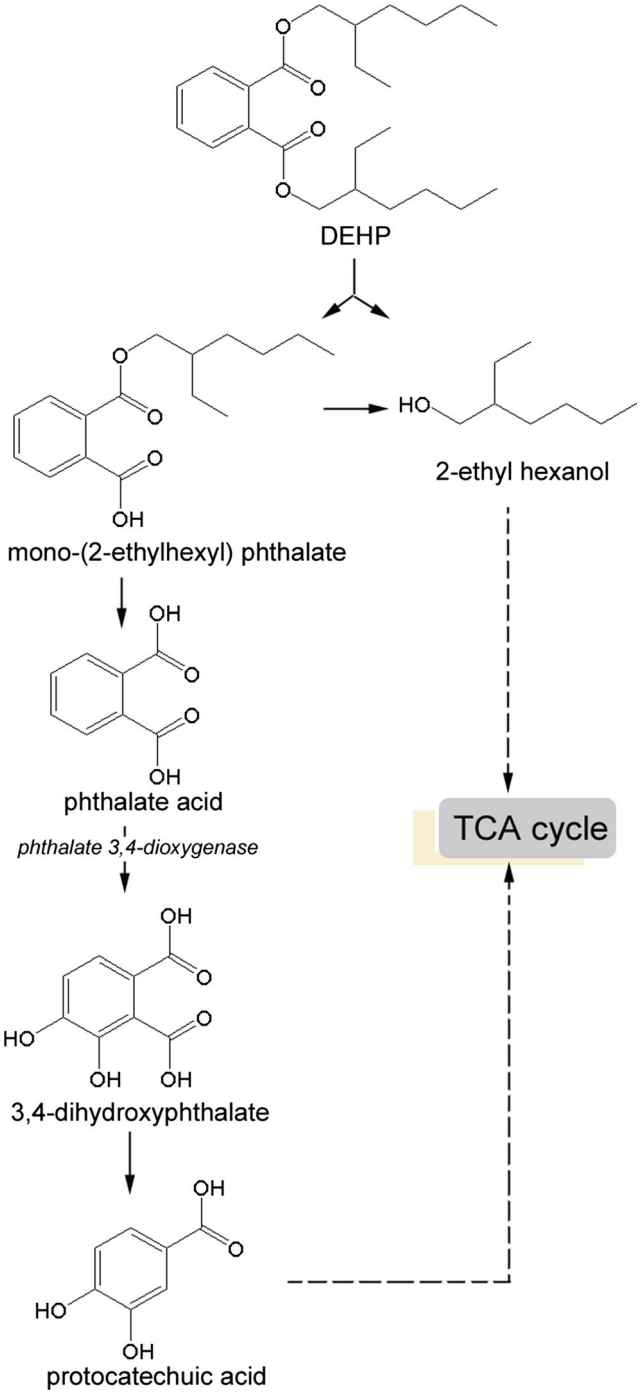

Fig. 6 The proposed pathway for DEHP degradation by strain J-1

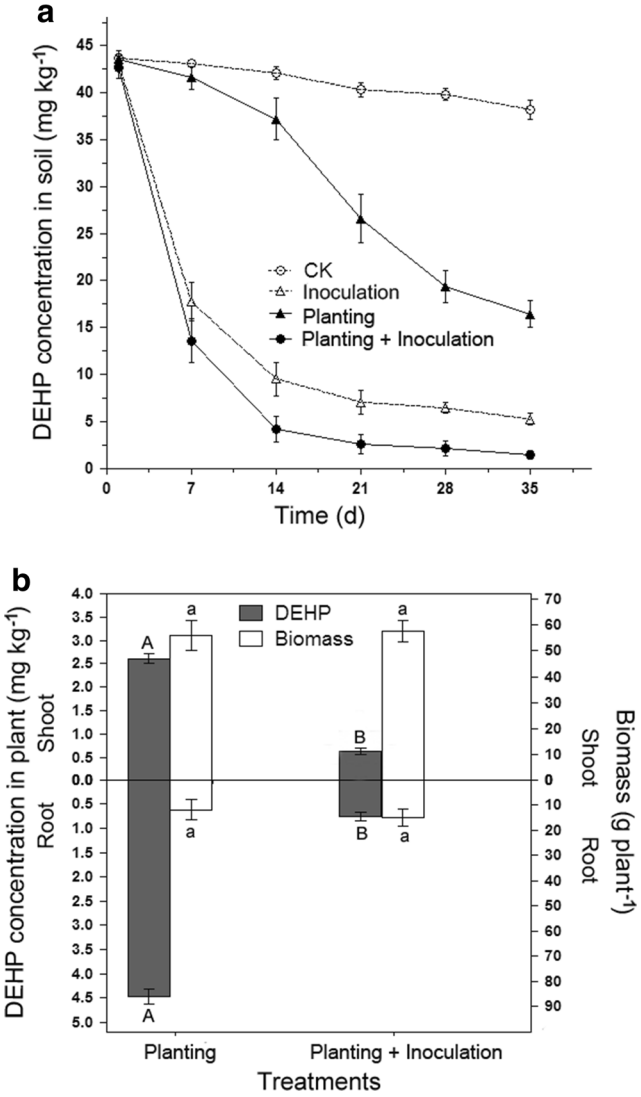

Fig. 7 DEHP residues in soils (**a**), and the biomass and DEHP accumulation of vegetable (**b**) under different soil treatments. The *different lowercase* (or *uppercase*) *letters* indicate significant differences in biomass between the same plant parts at $p < 0.05$ (*CK* DEHP-spiked soils, *Inoculation* contaminated soils with inoculation, *Planting* contaminated soils with vegetable, *Planting* + *Inoculation* contaminated soils with vegetable and inoculation; similarly hereinafter)

bioaccumulation of DEHP in the inoculated vegetable was mainly attributed to the high degradation efficiency of DEHP in soils and the root surface by strain J-1. Thus, strain J-1 has great potential and advantages in simultaneously remediating DEHP-contaminated soils and lowering DEHP accumulation in vegetable for food safety.

PCR-DGGE analysis

The survival conditions of inoculated bacteria (strain J-1) were monitored by PCR-DGGE analysis in all soil treatments at 7, 21, and 35 days after inoculation. As shown in Fig. 9, lane 13 was the DNA band of the isolated strain J-1. In the treatment with only inoculation of strain J-1 (i.e., inoculated soils, lanes 4 to 6), the band was clear and bright at the 7th day after inoculation, and then blurred at the 21th day and eventually disappeared at the 35th day. The result indicated that

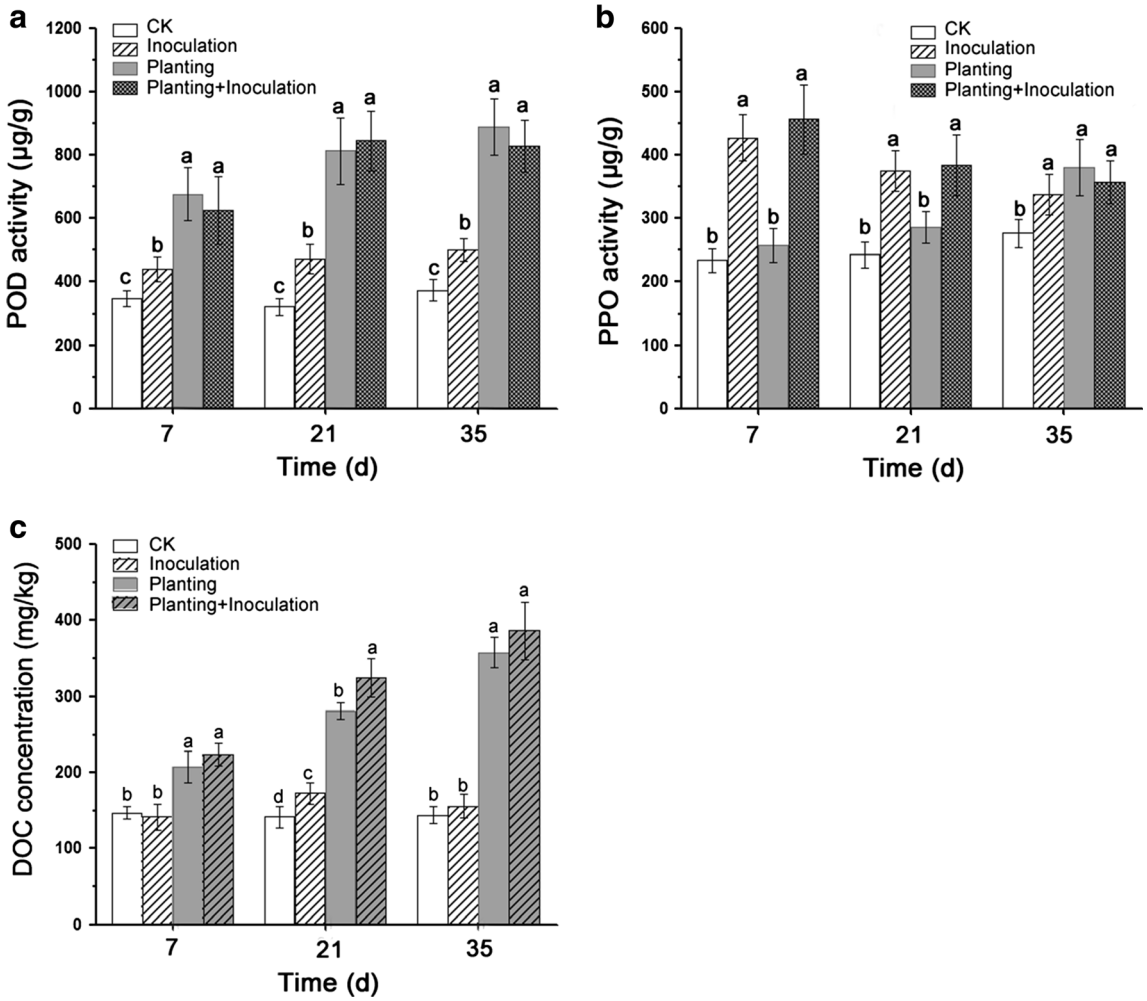

Fig. 8 The effects of bacteria inoculation and vegetable cultivation on soil enzyme activities and DOC concentrations in different soil treatments. *Bars with different lowercase letters* in the same time point are significantly different ($p < 0.05$)

strain J-1 could not survive well after 21 days of inoculation in contaminated soil, which might be due to the depletion of available nutrients or ecological competition with indigenous microorganisms (Liao et al. 2010). However, when cv. *Huaguan* was planted (i.e., planted + inoculated soils, lanes 10 to 12), the band of strain J-1 was maintained in soils until harvest (for 35 days), indicating that the vegetable planting was favorable for the survival of strain J-1 in soils, which might be attributed to root exudates and fine root turnover in the special rhizosphere environment of cv. *Huaguan* that provided readily available organic matters (Chaudhry et al. 2005; Joner et al. 2002). As expected, no target bands were detected in the soil treatments without bacteria inoculation (including CK and only planted soils). Besides, more other dominant bands were found in planted soils than those in non-plant soils, which were consistent with the result of DOC contents in these soils (Fig. 8c). The DOC concentrations in four soil treatments exhibited the following trend: planted + inoculated soil >planted soil >inoculated soil >CK (without plant and inoculation). This simple organic C pool could be easily degraded and used by bacteria as nutrients, leading to an increase in indigenous microorganisms (Gao et al. 2006). These increased microbial populations could partly contribute to increasing soil enzymatic activities, as shown in Fig. 8a, b, which probably enhanced DEHP removal from contaminated soils. Furthermore, it was reported that DOC content was closely associated with bioavailability of organic compounds (Tao et al. 2004); thus, the vegetable planting that increased DOC content of soils might influence the chemical speciation and bioavailability of DEHP in soil, which stimulated the number of DEHP-degrading microbial populations.

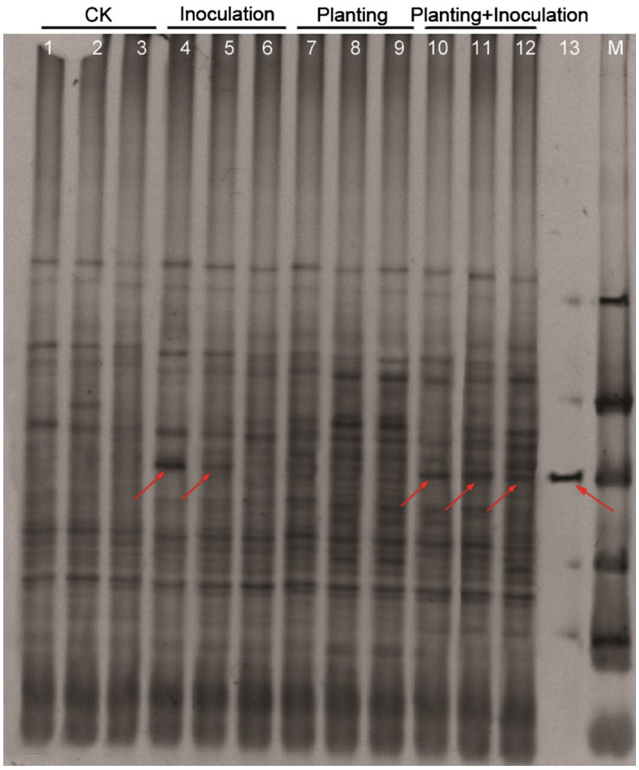

Fig. 9 Changes in DGGE profiles of soil bacterial community at 7, 21, and 35 days after inoculation under different soil treatments (*lanes 1 to 3* represent CK; *lanes 4 to 6* represent Inoculation; *lanes 7 to 9* represent Planting; *lanes 10 to 12* represent Planting + Inoculation; *lane 13* is the DNA band of strain J-1; and *lane M* is standard. *Arrows* indicate the DNA bands of strain J-1)

Conclusions

The strain *Microbacterium* sp. J-1 isolated from landfill soil was highly efficient in degrading various PAEs including DEHP. The biodegradation pathway of DEHP by strain J-1 was proposed. The inoculated strain could fit into the rhizosphere soil of vegetable (Chinese flowering cabbage) very well, without rivalry with indigenous microorganisms, and greatly enhanced DEHP dissipation from soil. The rhizosphere environment of vegetable improved the survival of this strain in contaminated soil, and in turn it effectively reduced DEHP accumulation in vegetable. Overall, inoculation of strain J-1 into the rhizosphere soil of vegetable not only enhanced DEHP removal from contaminated soil, but also lowered DEHP accumulation in vegetable, ensuring the simultaneous bioremediation of contaminated soil and safe food production.

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