# Complete degradation of the endocrine disruptor di-(2-ethylhexyl) phthalate by a novel *Agromyces* sp. MT-O strain and its application to bioremediation of contaminated soil

Hai-Ming Zhao [1], Huan Du [1], Jing Lin, Xue-Bin Chen, Yan-Wen Li, Hui Li, Quan-Ying Cai, Ce-Hui Mo [*], Hua-Ming Qin, Ming-Hung Wong

*Guangdong Provincial Research Center for Environment Pollution Control and Remediation Materials, School of Environment, Jinan University, Guangzhou 510632, China*

## HIGHLIGHTS

- This report is the first on DEHP-degradation bacteria from a novel genus *Agromyces*.
- It used various PAEs as growth substrates and degraded DEHP at high concentrations.
- The bacterium harbors the metabolic pathway for complete degradation of DEHP.
- This strain can greatly enhanced the remove rate of DEHP in contaminated soils.

## GRAPHICAL ABSTRACT
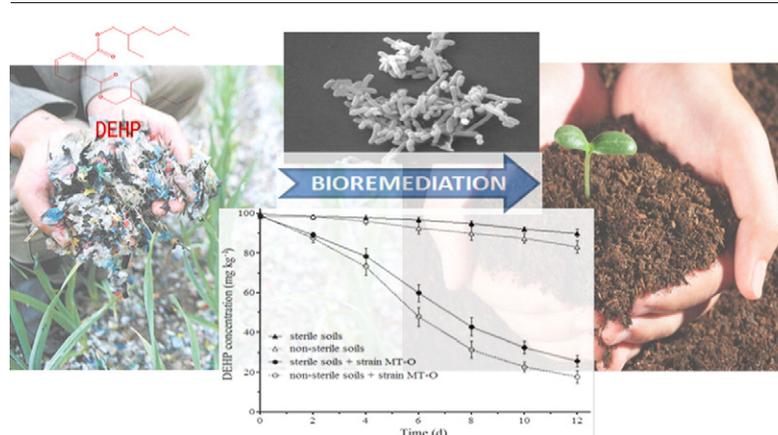
## ABSTRACT

A newly isolated strain *Agromyces* sp. MT-O could utilize various phthalates and efficiently degraded di-(2-ethylhexyl) phthalate (DEHP). Response surface methodology was successfully employed for the optimization of culture conditions including pH (7.2), temperature (29.6), and inoculum size ($OD_{600}$ of 0.2), resulting in almost complete degradation of DEHP (200 mg $L^{-1}$) within 7 days. At different initial concentrations (50–1000 mg $L^{-1}$), DEHP degradation curves were fitted well with the first-order kinetic model, and the half-life of DEHP degradation ranged from 0.83 to 2.92 days. Meanwhile, the substrate inhibition model was used to describe the special degradation rate with $q_{max}$, $K_s$, and $K_i$ of 0.6298 $day^{-1}$, 86.78 mg $L^{-1}$, and 714.3 mg $L^{-1}$, respectively. The GC–MS analysis indicated that DEHP was degraded into mono-ethylhexyl phthalate and phthalate acid before its complete mineralization. Bioaugmentation of DEHP-contaminated soils with strain MT-O has greatly enhanced DEHP disappearance rate in soils, providing great potential for efficiently remediating DEHP-contaminated environment.

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* Corresponding author.
  *E-mail address:* tchmo@jnu.edu.cn (C.-H. Mo).
[1] These authors contribute equally to this paper.

## 1. Introduction

Phthalic acid esters (PAEs) are a class of refractory organic compounds predominantly used as plasticizers to improve mechanical properties in plastic products (Gao and Wen, 2016). Due to the absence of chemical binding between the polymer mesh and the plasticizer, PAEs can be easily released from plastic products, and thus pollute various environments such as soil, air, water, and sediments (Jin et al., 2012; Lu et al., 2009; Pradeep et al., 2015; Wang et al., 2015a). Recent studies have shown that they have toxicity to humans and animals due to xeno-estrogenic and endocrine-disrupting effects (Matsumoto et al., 2008; Niu et al., 2014). Di-(2-ethylhexyl) phthalate (DEHP), a representative PAE compound, is one of the most widely used and frequently identified PAEs in various environmental samples. Its concentrations in soils of some regions in China exceed the control standard set by New York State Department of Environmental Conservation (Niu et al., 2014; Wang et al., 2012). Even at very low concentrations, DEHP can impact the primary reproduction and behavior of humans and wildlife (Vandenberg et al., 2012). Hence, DEHP has been listed as a priority pollutant by the United States Environmental Protection Agency and by China National Environmental Monitoring (Niu et al., 2014). In view of the extensive distribution and the adverse impacts of DEHP in the environments, it is of great urgency to eliminate DEHP from the environment.

Due to its chemical structure and hydrophobic property, DEHP cannot be easily removed by hydrolysis and photolysis (Li et al., 2012). Fortunately, DEHP and other PAE compounds can be biodegraded by some living microorganisms, which is generally considered to be a cost-effective and safe way to remove contaminants from environment. To date, many PAE-degrading bacterial strains have been isolated and characterized from various habitats, and have been widely applied to degrade various PAEs such as diethyl phthalate (DEP) (Lu et al., 2009; Prasad and Suresh, 2012), dimethyl phthalate (DMP) (Gu et al., 2009; Lu et al., 2009; Prasad and Suresh, 2012), di-n-butyl phthalate (DBP) (Jin et al., 2012; Prasad and Suresh, 2012; Sarkar et al., 2013; Zhao et al., 2016), benzylbutyl phthalate (BBP) (Chang et al., 2004; Xu et al., 2007; Hwang and Ka, 2012), and di-n-octylo phthalate (DnOP) (Wu et al., 2010; Zeng et al., 2004). PAE biodegradation shares a common characteristic in multiple microbes that the biodegradation rates of PAE decrease and their inhibition effects increase greatly with increasing alkyl chain length and alkyl branch chains (Prasad and Suresh, 2012; Wang et al., 2015b). Therefore, DEHP with a long side chain is more difficult to be biodegraded than other PAEs with shorter ester chains and the relevant literatures on complete biodegradation of DEHP are still fewer (Zeng et al., 2004). Besides, there has been no report about biodegradation of DEHP by Agromyces sp., which distributes in soil extensively. Although this genus currently harbors 33 characterized species (http://www.bacterio.cict.fr/collections.html) and the description of new Agromyces species continues apace, little is known about their capabilities to degrade organic pollutants.

The microbial degradation of contaminants is influenced by many factors, such as temperature, pH, microbial growth and oxygen condition (Chen et al., 2013; Li et al., 2012; Liang et al., 2008). The traditional method of "one factor at a time" approach is frequently used to optimize the various factors affecting the biodegradation of PAEs (Chen et al., 2007; Jin et al., 2012; Meng et al., 2015; Pradeep et al., 2015; Wang et al., 2012; Xu et al., 2007), but it ignores the interactive effects of these factors. Using the statistical tools is able not only to optimize the important factors but also to explore their interactions that could significantly affect the substrate degradation by isolates (Chen et al., 2013). Response surface methodology (RSM), based on statistical experimental design in complex processes, allows the rapid and economical determination of the optimum conditions with reduced experiments and minimal resources (Chen et al., 2012a; Chen et al., 2013; Pradeep et al., 2015). So far, few studies have applied RSM to optimize the microbial degradation of PAEs (Pradeep et al., 2015).

In this study, a novel bacterial strain that could efficiently degrade DEHP was isolated and characterized. The optimum conditions of DEHP degradation were determined by RSM using the Box-Behnken design, and its degradation kinetics was investigated in liquid medium and soil, respectively. Moreover, the metabolic pathway of DEHP was deduced based on the metabolites identified by gas chromatography–mass spectrometry (GC–MS).

## 2. Materials and methods

### 2.1. Chemicals and media

The chemicals were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China), including DMP, DEP, DBP, DnOP, DEHP, mono-ethylhexyl phthalate (MEHP), phthalate acid (PA), protocatechuic acid (PCA), and benzoic acid (BA) (all with purity >98%). All other chemical reagents are of analytical grade, and all solvents are HPLC grade.

Luria-Bertani medium (LB) used in this study contains (g L$^{-1}$) tryptone (10), yeast extract (5), and NaCl (10), and a mineral salt medium (MSM) contains (g L$^{-1}$) K$_2$HPO$_4$ (5.8), KH$_2$PO$_4$ (4.5), (NH$_4$)$_2$SO$_4$ (2.0), MgCl$_2$ (0.16), CaCl$_2$ (0.02), Na$_2$MoO$_4$·2H$_2$O (0.0024), FeCl$_3$ (0.0018), and MnCl$_2$·2H$_2$O (0.0015). The final pH was adjusted to 7.0 using HCl.

### 2.2. Culture enrichment and isolation

The microorganisms using DEHP as the sole carbon and energy source were isolated using an enrichment-culture technique. The initial enrichment culture was established in a 250-mL Erlenmeyer flask containing 100 mL sterilized MSM supplemented with DEHP (50 mg L$^{-1}$). The Erlenmeyer flasks were incubated in an incubator with shaking at 150 rpm and 30 °C for about 5 days. Then culture medium (5.0 mL) was transferred at 5-day intervals to new Erlenmeyer flasks containing 100 mL of fresh MSM with 100, 200, 400, 700, and 1000 mg L$^{-1}$ DEHP, respectively. After five rounds of transferring, the enrichment medium was diluted serially, and dilution method was as follows: 100 μL of the cultures were added into 900 μL of sterile water in a 1.5 mL sterile centrifuge tube, which was vortexed to mix evenly. Then, 100 μL of the above-mentioned mixture was added into 900 μL of sterile water in another 1.5 mL sterile centrifuge tube. Analogously, the dilutions of 10$^{-8}$ were achieved in duplicate. Finally, the diluted mediums were spread on enrichment agar plates with 200 mg kg$^{-1}$ DEHP for isolating individual colonies. The isolated strain could utilize DEHP as the sole carbon and energy source for growth on MSM, which was designated as MT-O (hereafter, strain MT-O), and was selected for further DEHP degradation experiments.

### 2.3. Identification and characterization of bacteria

The bacterial morphology was observed under a scanning electron microscope (SEM, Philips XL30, Netherlands; Pigeot-Rémy et al., 2012). The physio-biochemical tests were examined with reference to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Genomic DNA was extracted according to protocol of E.Z.N.A.® Bacterial DNA Kit (OMEGA BioTek, USA) and 16S rDNA gene was amplified using the universal primers F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-ACGGHTACCTTGTTTACGACTT-3′). The PCR cycling conditions consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1.25 min, with the last cycle followed by a 10-min extension at 72 °C. The service of purifying and sequencing of amplification products was provided by Sangon Corporation (Shanghai, China). The resulting sequences of the bacteria were aligned and compared with those in the GenBank database. The sequence data of the closest relatives were retrieved from NCBI GenBank and aligned using Clustal W with all parameters set at their default values. A phylogenetic tree was then constructed using the neighbor-joining method with MEGA 5.05.

### 2.4. Inoculum preparation

To prepare the inoculum, the strain MT-O growing in LB medium was harvested by centrifugation at 4000 rpm for 5 min and washed three times with 0.9% sterile saline solution. The washed bacteria were then resuspended in the saline. The colony forming units ($CFU\ mL^{-1}$) of this suspension were quantified by the dilution plate count technique (Shah and Belozerova, 2009). For all experiments, an $OD_{600}$ of 0.2, determined by measuring the optical density at 600 nm using a UV-2450 spectrophotometer (Shimadzu, Japan), was used as inoculum, unless otherwise stated.

### 2.5. Optimization of conditions for DEHP degradation using RSM

A response surface methodology (RSM) based on the Box-Behnken design was used to determine the optimal conditions for DEHP degradation by the strain MT-O. Based on the results of preliminary single factor experiments, three key factors (i.e., pH, temperature, and inoculum size) were selected as independent variables. The symbols and levels of the three independent variables used in Box-Behnken design are shown in Table S1. Using the Statistic Analysis System (SAS) ver. 9.2 (SAS Institute, Cary, NC, USA), a Box-Behnken design matrix and the response of dependent variable for DEHP degradation were generated (Table S2). Parameter measured in this experiment as dependent variable was the degradation of $200\ mg\ L^{-1}$ DEHP by the strain MT-O at day 7. All experiments were conducted in triplicate with non-inoculated samples as control (CK). The obtained data were analyzed using the response surface regression procedure of the SAS software to fit the following quadratic polynomial equation (Eq. (1)):

$$Y_i = b_0 + \Sigma b_i X_i + \Sigma b_{ij} X_i X_j + \Sigma b_{ii} X_i^2 \tag{1}$$

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

An analysis of variance (ANOVA) was also performed to identify the interaction between the variables and the responses. Model terms were selected or rejected with a 95% confidence level. Three-dimensional response surface and contour plots were drawn to show individual and interaction effects of the independent variables on the DEHP degradation rate.

### 2.6. Substrate range tests

The isolated bacterial strain MT-O was inoculated in sterile MSM supplemented with one of the following substrates as the sole source of carbon and energy ($200\ mg\ L^{-1}$): DMP, DEP, DBP, DnOP, DEHP, MEHP, PA, PCA, and BA. The culture was performed triplicately in 250 mL-Erlenmeyer flasks and incubated at the optimum cultural conditions for 5 days. For each substrate, MSM without inoculation was served as a control. Substrate utilization was assessed by microbial growth by plate count method and measuring the increase of the biomass ($OD_{600}$).

### 2.7. Biodegradation of DEHP by the strain MT-O in MSM

Growth experiments with DEHP as the sole carbon source were conducted in 250-mL Erlenmeyer flasks containing 50 mL sterile MSM with $200\ mg\ L^{-1}$ DEHP. The seed suspension was aseptically inoculated into the MSM in triplicate and incubated for 7 days at 150 rpm under the optimum conditions. Non-inoculated medium served as a control. Samples of cell-free filtrates were collected every day to measure the $OD_{600}$ and DEHP residues. Meanwhile, the metabolites of DEHP in the samples collected at 1, 3, 5, and 7 days were identified using GC-MS (QP2010 Plus, Shimadzu, Japan).

To study the DEHP degradation kinetics by the strain MT-O, DEHP at different initial concentrations ($50–1000\ mg\ L^{-1}$) was served as the sole carbon source in MSM. The culture was also incubated at 150 rpm under the optimum conditions for 7 days. The samples were collected every day to measure DEHP residues.

### 2.8. Biodegradation of DEHP by the strain MT-O in contaminated soil

Soil samples were collected from an agricultural field of South China Agricultural University (Guangzhou, China). The characteristics of the soil were as follow (dry weight): total organic carbon, $7.9\ g\ kg^{-1}$; total N, $1.32\ g\ kg^{-1}$; total P, $1.57\ g\ kg^{-1}$; organic matter, 1.5%; and pH, 6.7. The soil has a sandy loam texture (sand 35.7.0%, silt 52.3%, clay 12.0%). To investigate the potential of DEHP degradation by the strain MT-O in soils, 200 g of the soil (sieved <2 mm) was placed in 500-mL Erlenmeyer flask and treated with DEHP ($100\ mg\ kg^{-1}$ soil) in an acetone solution. After thorough mixing and solvent evaporation, the bacterial suspension was inoculated into soil (in triplicate) using drip irrigation to achieve a final concentration of approximately $1 \times 10^7\ cells\ g^{-1}$. The inoculum was thoroughly mixed under sterile condition. In addition, to rule out the influence of indigenous microorganism in soil, the soil was sterilized by autoclaving at 121 °C for 1 h and then was inoculated with the strain MT-O. The triplicate samples of sterile and non-sterile soils without the strain MT-O were kept as controls, respectively. All treatments were adjusted by the addition of sterile water to 40% of water-holding capacity and then incubated in dark at 30 ± 1 °C. Soil samples (20 g) were collected periodically to measure residual DEHP concentration.

### 2.9. Chemical analysis and identification of intermediates

The residual DEHP and its degradation intermediates in the culture filtrates (the initial concentration of DEHP was $200\ mg\ L^{-1}$) of the strain MT-O were analyzed by GC–MS. The filtrate extraction and cleanup procedures were conducted following our previous report (Zhao et al., 2016) with slight modifications. Briefly, the liquid cultures were mixed with 20 mL of ethyl acetate by vibration, and then the aqueous and organic phases were separated by centrifugation at 6000 rpm for 10 min. The aqueous phase was extracted twice. The organic phase was evaporated to near dryness and the residue was dissolved in 10 mL methanol. All the tests were conducted in triplicate. Finally, the extracts passed through a 0.22 μm membrane filter and 1.0 μL filtrates were injected into GC–MS. Additionally, soil extraction and cleanup procedures were conducted as our previous report (Zhao et al., 2015) with slight modifications. 1.0 g soil sample (dry weight) was ultrasound extracted in 20 mL of dichloromethane for 10 min and then centrifuged at 3000 rpm for 5 min to obtain supernatants. This process was then repeated twice for a total of three extractions. The supernatants were pooled and loaded onto a glass chromatography column (35 cm length × 1 cm i.d., pre-eluted with 20 mL of dichloromethane), which was packed bottom-up with anhydrous sodium sulfate, silica gel, and alumina, and eluted three times with 10 mL of dichloromethane. The eluates were evaporated to near dryness and the residue was redissolved in 1 mL dichloromethane for GC–MS analysis. The following detection conditions of GC–MS were employed: an HP-5 column (0.25 μm × 0.25 mm × 30 m) with helium as carrier gas at a flow rate of $1.0\ mL\ min^{-1}$, an injection temperature of 250 °C, and an ion source temperature of 220 °C. The GC oven temperature was programmed as follows: 100 °C hold for 2 min, raised at $15\ °C\ min^{-1}$ to 129 °C, then at $40\ °C\ min^{-1}$ to 280 °C (hold for 5 min). Mass spectra were acquired in the electron ionization (EI) mode using an electron impact ionization of 70 eV and scanning at 50–600 amu. The scans collected for the metabolites were identified by comparing the results with both the standard solution and the mass spectra library in the MS system.

The GC–MS program and details of the quality assurance/quality control measures used have been presented in our previous reports

**Table 1**
Analysis of variance (ANOVA) for the fitted quadratic polynomial model for DEHP degradation.

| Source | DF | SS | MS | F-value | P-value* |
|--------|----|----|----|---------|----------|
| $X_1$ | 1 | 194.3406 | 194.3406 | 197.8777 | <0.0001 |
| $X_2$ | 1 | 31.64101 | 31.64101 | 32.21689 | 0.0024 |
| $X_3$ | 1 | 5.3792 | 5.3792 | 5.477103 | 0.0664 |
| $X_1 X_1$ | 1 | 490.114 | 490.114 | 499.0342 | <0.0001 |
| $X_1 X_2$ | 1 | 0.198025 | 0.198025 | 0.201629 | 0.6722 |
| $X_1 X_3$ | 1 | 1.2996 | 1.2996 | 1.323253 | 0.3020 |
| $X_2 X_2$ | 1 | 190.6789 | 190.6789 | 194.1493 | <0.0001 |
| $X_2 X_3$ | 1 | 0.0169 | 0.0169 | 0.017208 | 0.9007 |
| $X_3 X_3$ | 1 | 39.21028 | 39.21028 | 39.92392 | 0.0015 |
| Model | 9 | 884.2077 | 98.24531 | 100.0334 | <0.0001 |
| Error | 5 | 4.910625 | 0.982125 | | |
| Total | 14 | 889.1184 | | | |

$R^2 = 0.9945$ (adjusted $R^2 = 0.9845$), coefficient of variation (CV) = 1.14%. DF refers to degrees of freedom, SS refers to sum of sequences, MS refers to mean square.
 * $P < 0.05$ indicates that the model terms are significant.

(Zhao et al., 2015). Quantitative analysis was performed using the external calibration based on a five-point calibration curve (0–4.0 mg L$^{-1}$). The instruments were calibrated daily using calibration standards. The detection limit of DEHP in samples, based on three fold of the signal-to-noise ratio, was 2.5 μg L$^{-1}$. The average concentration of DEHP was 6.3 μg L$^{-1}$ in all procedural blanks ($n = 12$). This value was subtracted from sample values. The DEHP recoveries in aqueous samples and soil samples ranged from 97.67% to 104.72% and from 87.42% to 107.17%, respectively.

### 2.10. Data analysis

To describe the effect of the initial DEHP concentration on degrading efficiency, the first-order kinetic model (Eq. (2)) was used to determine the removal rate of DEHP:

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

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 (Chen et al., 2013).

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

where $q_{max}$ is the maximum specific DEHP degradation rate (d$^{-1}$), $K_i$ 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$^{-1}$).

## 3. Results and discussion

### 3.1. Isolation and identification of the strain MT-O

After enrichment and selection with increasing DEHP, the strain with superior degrading ability isolated from landfill soil was chosen and designated as MT-O. Colonies of the strain MT-O grown on LB agar for 7 days were circular, opaque, convex, and dark brown, lack of aerial mycelia. The bacterium was Gram-positive, rod-shaped cells with 0.3–0.5 × 0.8–4.0 μm in size (Fig. S1). The physio-biochemical characteristics of the strain MT-O are presented in Table S3. The 16S rRNA gene sequence with 1423 bps was deposited in the GenBank database with the accession number KP670413. Phylogenetic analysis of the 16S rRNA gene sequences revealed that the strain MT-O was grouped among *Agromyces* species and closely clustered with *Agromyces mediolanus* strain c70 (GenBank accession number FJ950561) and *Agromyces* sp. (GenBank accession number JN867352) with high identities of 99% (Fig. S2). Based on morphology, physio-biochemical characteristics, and 16S rRNA gene analysis mentioned above, the strain MT-O was identified as *Agromyces* sp. Previous studies indicate that the bacteria from genera *Agromyces* can utilize certain hydrocarbons (Rivas et al., 2004) and organo-phosphonate compounds (Panas et al., 2006), but the biodegradation of aromatic compounds by the isolates from this genus has not been reported. The present work first described the biodegradation of DEHP by this genus bacteria and supplemented PAE-degrading bacterium pool isolated from soil.

### 3.2. Optimization of culture conditions for DEHP degradation by strain MT-O

RSM based on the Box-Behnken design was used to investigate the main and interactive effects of significant variables, including pH ($X_1$), temperature ($X_2$), and inoculum size ($X_3$), on the degradation of DEHP
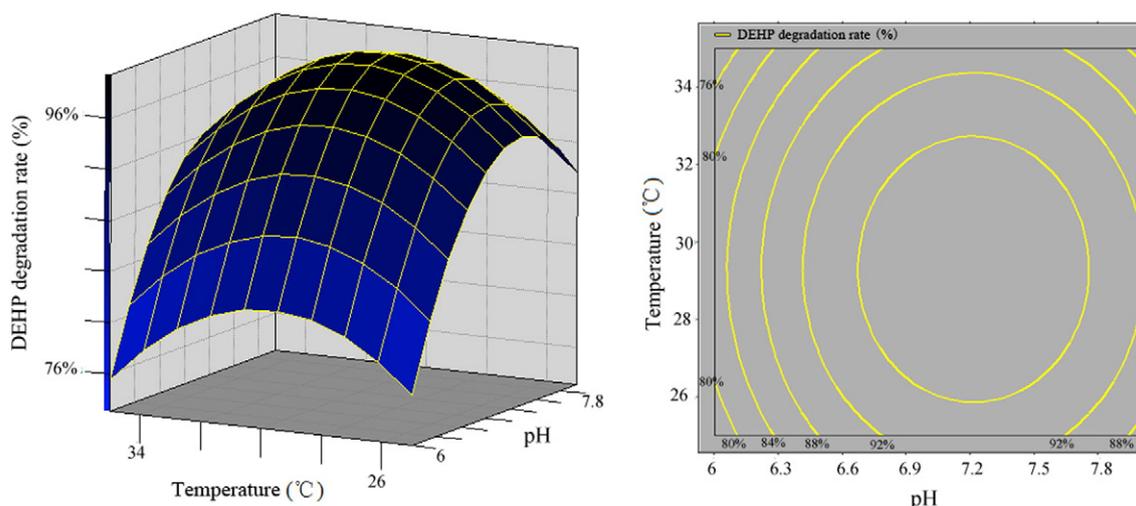


**Fig. 1.** Response surface and contour plots for the effect between pH ($X_1$) and temperature ($X_2$) on degradation rates of DEHP by the strain MT-O. Inoculum size ($X_3$) was set at 0.2 (OD$_{600}$).

by the strain MT-O. The Box-Behnken design matrix and the response of dependent variable for DEHP degradation are given in Table S2. The data obtained for the degradation percentage of DEHP ($Y_1$) represent the combined effects of the three factors at various levels. By using the response surface regression procedure of SAS software package, the data from Table S2 were assessed, and the quadratic polynomial model fitting in the term of analysis of variance (ANOVA) are shown in Table 1. Moreover, the statistical significance of the fitted quadratic polynomial model for DEHP degradation was evaluated using $F$-tests (Table 1) and $t$-tests (Table S4). Only statistically significant terms ($P < 0.05$) were included in the model. A first-order effect ($X_3$) and three interaction effects ($X_1X_2$, $X_1X_3$, and $X_2X_3$) were not significant ($P > 0.05$) and removed from the model (Table 1). Therefore, the following fitted regression model (equations in terms of coded values for the regressor) was used to investigate the effects of pH ($X_1$) and temperature ($X_2$) on the degradation rates of DEHP:

$$Y_1 = 97.140257 - 4.92875X_1 - 1.98875X_2 - 11.52125X_1^2 - 7.18625X_2^2 - 3.25875X_3^2.$$

The overall effects of the process variables on the response were further analyzed, and the large value of the regression coefficient ($R^2 = 0.9845$) indicated that most of the variation in the response can be explained by the regression model equation. The high value of the adjusted $R^2$ (0.9845) further supported the accuracy of this model (Table 1). Besides, the low coefficient of variation (CV = 1.14%) also demonstrated good precision and reliability of the experiments. Thus, the established quadratic polynomial model for DEHP degradation by the strain MT-O was adequate to represent the actual relationship between the response and the variables.

Estimation of the regression parameter revealed that the inoculum size ($X_3$) had insignificant effects ($P > 0.05$) on the DEHP degradation by the strain MT-O. Thus, with the fixed value of the inoculum size at 0.2 ($OD_{600}$), three-dimensional response surface and contour were plotted to directly illustrate the effects of pH and temperature on DEHP degradation by the strain MT-O (Fig. 1). The plot of DEHP degradation by the strain MT-O had a theoretical maximum value of 97.52% at the stationary point where the corresponding pH and temperature were 7.2 and 29.6 °C, respectively. That is, the optimal conditions for DEHP degradation by the strain MT-O were pH 7.2, temperature 29.6 °C, and an inoculum size of 0.2 ($OD_{600}$). It shows that the mathematical model is effective to predict and optimize DEHP degradation by the strain MT-O within the selected factors.

### 3.3. Utilization of DEHP for bacterium growth in MSM under the optimal condition

The DEHP degradation by the strain MT-O and its growth were investigated simultaneously in MSM under the optimal condition, to which 200 mg L$^{-1}$ DEHP was added as the sole carbon and energy source. The results showed that the strain MT-O could utilize DEHP for growth substrate, leading to a rapid DEHP degradation without a following lag phase (Fig. S3). About 90% of the DEHP was degraded by the strain MT-O within 4 days. After 7 days of incubation, DEHP was completely degraded. As a control, no significant change in DEHP concentration was observed in the flasks which contained medium without inoculation. Meanwhile, the density of the strain MT-O cells increased to a maximum level of 0.7 ($OD_{600}$) within 4 days, then this value slightly decreased (Fig. S3). Compared with previous reports (Chen et al., 2007; Pradeep et al., 2015), the time of complete biodegradation of DEHP by the strain MT-O was longer, which might be due to the differences in the inoculum size, environmental conditions, and bacteria characteristics (Wang et al., 2015b). Nevertheless, the strain MT-O could directly utilize DEHP as a sole carbon source and degrade it without a lag phase, indicating excellent environmental adaption. These
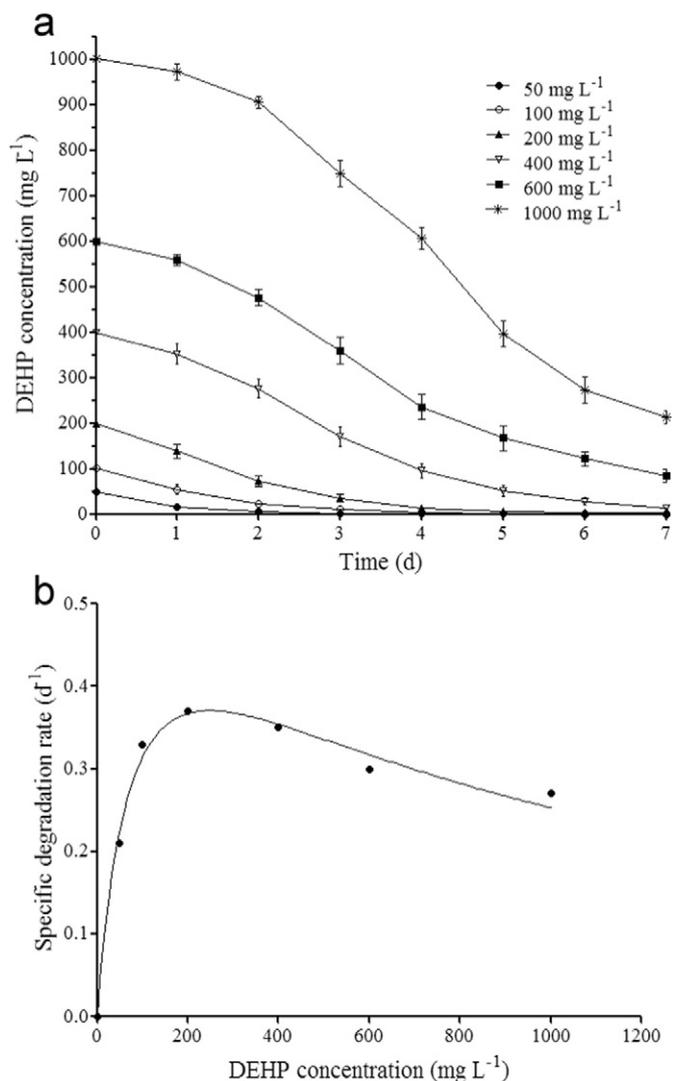


**Fig. 2.** Degradation of DEHP by the strain MT-O at different initial concentrations (a) and specific degradation kinetics curve (b). Error bars indicated the standard error of three replicates.

characteristics of the strain MT-O highlights that it can be employed for bioremediation of variable contaminated environments.

### 3.4. Biodegradation of DEHP at different initial concentrations

To investigate the DEHP degradation kinetics by the strain MT-O, DEHP at different initial concentrations (50–1000 mg L$^{-1}$) was used as the sole carbon source in MSM, and the dynamic curves of DEHP degradation are shown in Fig. 2a. The results demonstrated that the strain MT-O could rapidly degrade DEHP even at the concentration of 1000 mg L$^{-1}$. At low concentrations (50–200 mg L$^{-1}$), DEHP was completely removed within 7 days. The rapid degradation of DEHP may be related to the fact that the strain MT-O could readily gain carbon

**Table 2**
Kinetic parameters of DEHP degradation in MSM with different initial concentrations.

| Initial concentration (mg L$^{-1}$) | Kinetic equations | $t_{1/2}$ (d) | $R^2$ |
|---|---|---|---|
| 50 | $\ln C = -0.8360t + 3.4726$ | 0.83 | 0.9631 |
| 100 | $\ln C = -0.6742t + 4.4623$ | 1.03 | 0.9742 |
| 200 | $\ln C = -0.6708t + 5.6172$ | 1.03 | 0.9842 |
| 400 | $\ln C = -0.5059t + 6.3916$ | 1.37 | 0.9602 |
| 600 | $\ln C = -0.2968t + 6.6119$ | 2.34 | 0.9713 |
| 1000 | $\ln C = -0.2372t + 7.1502$ | 2.92 | 0.9277 |

source and energy by degrading the DEHP, which promoted the growth of bacteria. However, along with the increased concentrations of DEHP, the efficiency of degradation slowed down, and its removal rates reached about 75.0%, 58.5% and 35.0% at the initial concentrations of 400, 600, and 1000 mg $L^{-1}$, respectively. We noticed that at a high concentration of DEHP (400–1000 mg $L^{-1}$), enhanced degradation
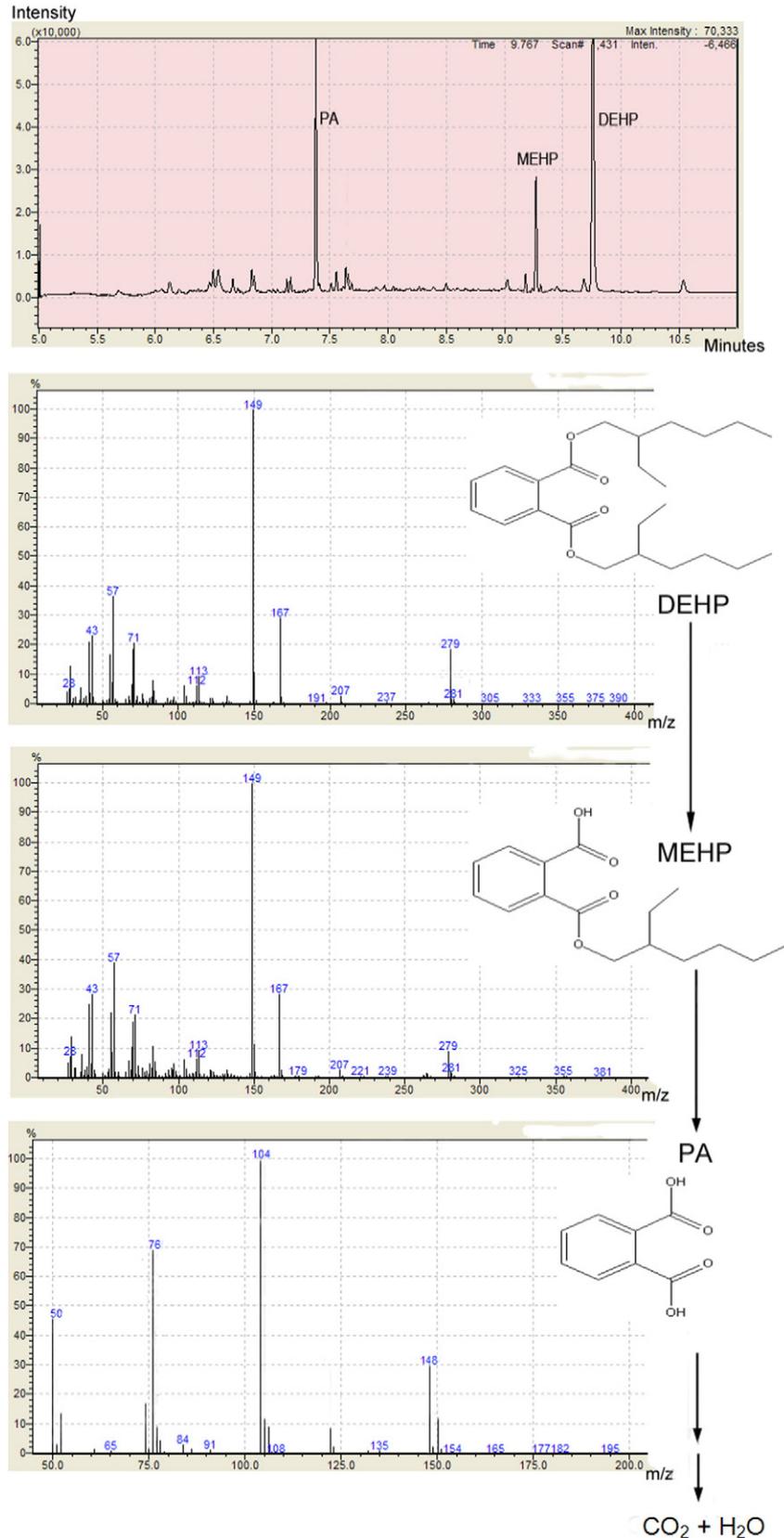


Fig. 3. GC–MS chromatograms and spectra of DEHP degradation intermediates at 3 days in MSM. DEHP: di-(2-ethylhexyl) phthalate, MEHP: mono-ethylhexyl phthalate, PA: phthalate acid.

occurred after a prolonged culture. This might be due to the fact that at high xenobiotic concentrations, bacterial growth starts slowly and requires an acclimation period before accelerated degradation (Chen et al., 2013).

The kinetic parameters for all initial concentrations of DEHP calculated from the above equations (Eqs. (2) and (3)) are presented in Table 2. The degradation process fitted well to the first-order kinetics model ($R^2$ ranging from 0.9277 to 0.9842). The biodegradation half-life ($t_{1/2}$) of DEHP by the strain MT-O varied from 0.83 to 2.92 days, which were much shorter compared with those in the literature (Chang et al., 2004; Chao and Cheng, 2007; Zeng et al., 2002, 2004). These results showed that the degradation of DEHP was enhanced significantly by the strain MT-O and its degradation rates were evidently depended on the initial concentrations. Furthermore, to better understand the effects of initial DEHP concentrations on its degradation, the substrate inhibition model (Eq. (4)) adapted from Chen et al. (2013) was used to describe the specific degradation rate ($q$) at different initial concentrations. The $q$ of the strain MT-O increased with the increase of DEHP concentration within low concentrations ($<200$ mg L$^{-1}$), and then decreased with increasing DEHP level (Fig. 2b), indicating substrate inhibition to the degradation ability of the strain MT-O. Using the non-linear regression analysis by graph-pad prism software (Version 5.0) according to Chen et al. (2014), the kinetic parameters $q_{max}$, $K_s$ and $K_i$ were determined to be 0.6298 day$^{-1}$, 86.78 mg L$^{-1}$, and 714.3 mg L$^{-1}$, respectively. The maximum special degradation rate ($S_m$) occurred at a low DEHP concentration (about 200 mg L$^{-1}$). The determination coefficient ($R^2$) was 0.9888, suggesting that the experimental data were well fitted with the model. With the further increase in DEHP concentrations, the decline in $S_m$ was clearly observed (Fig. 2b). In previous studies, the initial PAE concentrations in the medium, causing substrate inhibition to microbial metabolism, were also widely discussed (Fang et al., 2010; Wen et al., 2014). For instance, Fang et al. (2010) reported that the high initial DBP concentrations could constrain the DBP biodegradation by *Enterobacter* sp. T5. The values of $K_i$ for DMP degradation by *Sphingomonas* sp. strain PA-02 (Zeng et al., 2008) and for DBP degradation by *Arthrobacter* sp. strain C21 (Wen et al., 2014) were 154.3 and 204.6 mg L$^{-1}$, respectively. By contrast, the strain MT-O displayed a higher tolerance to PAE concentrations. In general, the concentrations of DEHP and other PAE compounds in the environment are far lower than the above values of inhibition coefficients, thus the strain MT-O can efficiently degrade DEHP without PAE inhibition in natural environment.

### 3.5. Substrate utilization tests

After 5 days of incubation in MSM containing supplemented substrate, the strain MT-O was able to utilize all of the PAE compounds (including DMP, DEP, DBP, DnOP, and DEHP) as the sole carbon source for its growth (OD$_{600}$ ≥ 0.3) (Table S5). The results indicated that this strain could utilize a series of PAEs with long or short alkyl-chains, which is similar to *Rhodococcus* sp. JDC-11 in a previous report (Jin et al., 2012). This is attributed to the fact that these PAEs share a similar structure particularly the ones with short alkyl-chains (Liang et al., 2008). In addition, the strain MT-O was also able to utilize the common intermediates of PAE degradation, such as MEHP, PA, and PCA, suggesting that this strain may degrade DEHP through MEHP, PA, and PCA as intermediates.

### 3.6. Identification of DEHP degradation intermediates

To explore the pathways of DEHP degradation by *Agromyces* sp. MT-O, intermediates of DEHP were identified by GC–MS. At the beginning of the experiment (0 day), an apparent peak occurred at retention time of 9.77 min, corresponding with the DEHP standard. Along with biodegradation (at 3 days), the peak area at 9.77 min decreased and two other apparent peaks appeared (Fig. 3). Two new compounds, MEHP and PA, with retention time of around 9.27 and 7.38 min, respectively,

were detected and identified based on the similarities of their fragments and molecular ions as well as retention time that corresponded with authentic compounds. All the three peaks decreased gradually until 5 days. At 7 days none of the above compounds were detected. Hence, no persistent accumulative metabolite was detected at the end of experiment, which was consistent with the findings that the strain MT-O could use these compounds as a sole carbon source, respectively. Similar to previous studies, the intermediate products, such as MEHP and PA, were identified in the DEHP degradation by *Gordonia* sp. HS-NH1 (Yan et al., 2014), *Microbacterium* sp. strain CQ0110Y (Chen et al., 2007), and *Bacillus subtilis* No. 66 (Quan et al., 2005). Although no PCA (involved in PA degradation) was detected in this experiment, it should be noted that the possible intermediates PCA was readily utilized by the strain MT-O in substrate tests (Table S5). This observation was likely due to these intermediates existing in MSM with short lag times or being masked by unknown compounds (Nakamiya et al., 2005). MEHP and PA could be monitored because they were primary intermediates during DEHP degradation. Therefore, DEHP was degraded by hydrolysis of the ester linkage between each alkyl chain and the aromatic ring, forming first the MEHP and subsequently PA (Fig. 3), and then the intermediates were further utilized for bacterial growth by the strain MT-O, finally resulted in complete mineralization. In addition, phthalate has the basic structure of an esterified benzene-dicarboxylic acid with two alkyl chains, and its primary biodegradation involves the sequential hydrolysis of the ester linkage between the alkyl chain and the aromatic ring (Xu et al., 2007). It further confirms that the strain MT-O can efficiently utilize all of the PAE-substrates tested as the sole carbon source and energy for growth (Table S5). Overall, *Agromyces* sp. MT-O could be used for the bioremediation of PAE-contaminated environment because its complete degradation can be achieved by this single strain of bacteria within a short time.

### 3.7. Biodegradation of DEHP in soils

Recently, many studies have investigated the occurrence of PAEs in agriculture soil in China with DEHP concentrations up to dozens of mg kg$^{-1}$ (Cai et al., 2008; Wang et al., 2013). Microbial biomass, basal respiration, and catalase activity in soil were inhibited when PAEs concentration approached 100 mg kg$^{-1}$ (Gao and Chen, 2008). Thus, we set 100 mg kg$^{-1}$ DEHP in soil to investigate the efficiency of DEHP degradation by the strain MT-O in contaminated soil. The results suggested that the strain MT-O was able to efficiently degrade DEHP in contaminated
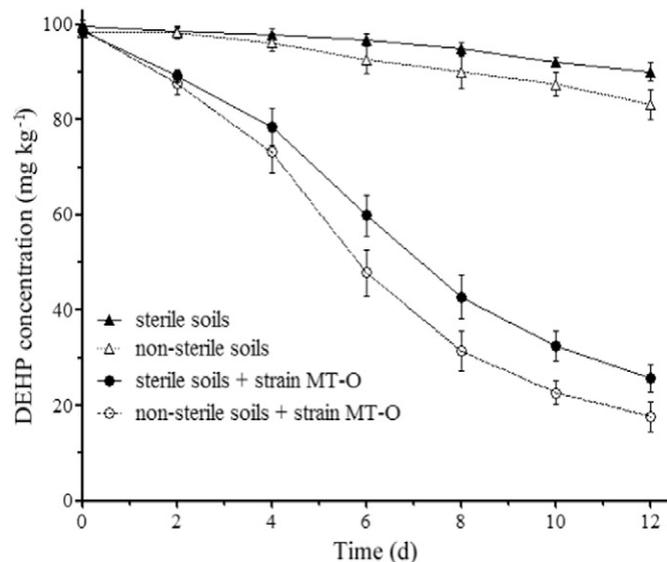


Fig. 4. Degradation of DEHP by the strain MT-O in contaminated soils. Error bars indicated the standard error of three replicates.

**Table 3**
Kinetic parameters of DEHP degradation in contaminated soils by the strain MT-O.

| Soil treatments | Kinetic equations | $t_{1/2}$ (d) | $R^2$ |
|---|---|---|---|
| Sterile soils | $\ln C = -0.0085t + 4.6118$ | 81.55 | 0.9432 |
| Non-sterile soils | $\ln C = -0.0143t + 4.6092$ | 48.47 | 0.9578 |
| Sterile soils + MT-O | $\ln C = -0.1189t + 4.7167$ | 5.83 | 0.9740 |
| Non-sterile soils + MT-O | $\ln C = -0.1553t + 4.7432$ | 4.46 | 0.9770 |

soil even at high concentration (100 mg kg$^{-1}$). In the non-sterilized soil inoculated with the strain MT-O, 82.1% of added DEHP in soil was removed after 12 days of incubation, while in control without inoculum, DEHP was removed only 15.4% (Fig. 4). In case of sterilized soil inoculated with the strain MT-O, 73.9% of added DEHP was removed after 12 days, while a non-inoculated control DEHP was removed only 9.5% (Fig. 4). Moreover, no lag phase was observed during the DEHP biodegradation by the strain MT-O in soils, which was consistent with the result of DEHP degradation by the strain MT-O in MSM. In addition, kinetic data showed that the degradation process was fitted well with the first-order model ($R^2 = 0.9432$–$0.9770$) with the kinetic constant $k$ ranging from 0.0085 to 0.1553 day$^{-1}$ (Table 3). The half-life ($t_{1/2}$) of DEHP was remarkably reduced by 75.72 in sterilized soil and 44.01 days in non-sterilized soil as compared to the controls (Table 3), respectively, implying that the strain MT-O can efficiently remove DEHP from contaminated soils. Furthermore, the residue of DEHP in the non-sterilized soil was significantly lower ($P < 0.05$) than that in the sterilized soil, indicating that the indigenous soil microorganisms could enhance the ability of the strain MT-O to eliminate DEHP in soil. The possible reason is that the introduced isolate and indigenous soil microorganisms may have a synergistic effect on removal of pollutants (Chen et al., 2012b). Overall, the strain MT-O can efficiently degrade DEHP in soil, suggesting that this strain has vast potential to the bioremediation of DEHP-contaminated soil.

## 4. Conclusions

A novel bacterium *Agromyces* sp. MT-O was isolated and characterized, which could utilize a wide range of PAEs as growth substrates, and efficiently degrade DEHP. This is the first report about biodegradation of PAE compounds by a bacterial strain from the *Agromyces* genus. Moreover, we presented evidences that this strain harbors the metabolic pathway for complete degradation of DEHP and has advantages as a bioremediation organism in removing DEHP residues from soil. These results indicate that this strain has great potential for bioremediation of PAEs. Further researches on environmental influence and genetic aspects involved PAE biodegradation are still required.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.03.171.

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