Degradation of di-2-ethylhexyl phthalate (DEHP) by indigenous isolate *Acinetobacter* sp.

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Abstract
A bacterial strain capable of degrading di(2-ethylhexyl) phthalate (DEHP) from artificially contaminated water (with over 90% removal within 5 days of incubation) was isolated from the activated sludge at a regional wastewater treatment plant in Macau SAR (Special Administrative Region), China. The isolate was identified as *Acinetobacter* sp. by the 16S rRNA gene sequence analysis. Two major experimental parameters, temperature (25, 30, 35°C) and pH (3-9), were further optimized to enhance the biodegradation efficiency for DEHP. The optimum temperature was 30°C while there were no significant differences at pHs 6 to 9 ($p=0.87$). The growth and degradation kinetics of the isolate toward DEHP followed the inhibition model, at the maximum degradation rate, the half saturation constant, and the inhibition constant of 124.8 mg/L·day, 272.3 mg/L, and 720.5 mg/L, respectively, for degradation and at the maximum specific growth rate, the half saturation constant, and the inhibition constant of 0.1192 day^{-1}, 137.6 mg/L, and 850.3 mg/L, respectively, for growth, after the inhibition model for growth and degradation was simulated using Matlab software. The maximum degradation rate was achieved at the initial DEHP concentration of 400 mg/L. The first-order rate constant (k) and the half-life of DEHP was estimated at 0.854-0.625 and 19.44-26.6 h, respectively, at the concentration levels studied (100-400 mg/L). The respective DEHP degradation pathway for the isolate is also proposed through the identification of the following intermediates: mono-(2-ethylhexyl) phthalate (MEHP), phthalic acid (PA), protocatechuate, β-carboxy-*cis*,*cis*-muconic acid, and 3-katoadipate.

Keywords
Acinetobacter sp.; biodegradation; DEHP; inhibition kinetics; degradation pathway

INTRODUCTION

Di(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used plasticizers and is usually applied in such plastic products as polyvinylchloride (PVC), toys, and medical devices. DEHP is considered one of the most resistant phthalate esters due to its long hydrocarbon chain (Chang et al., 2004). Most plastics or polymeric materials contain DEHP physically bound to the matrices, which may result in the phthalate leaching out during the lifetime of a product or after its disposal (Latorre et al., 2012b). It has been reported that the human exposure to DEHP may have dysfunction of endocrine, reproductive, and nervous systems (Heudorf et al., 2007; Lomenick et al., 2010; Lo et al., 2014; Net et al., 2015; Zong et al., 2015; Chen et al., 2016). Since 1987 DEHP has been classified as a possible human carcinogen by the United States Environmental Protection Agency (Sakiti et al., 2013). In China, DEHP has been detected at high levels in rivers, sediments, and wastewaters (He et al., 2013; Wang et al., 2014; Li et al., 2015). Wang et al. (2011) reported that the average concentration of DEHP in industrial wastewater, well water, and pond water was 42.43 μg/L, 14.20 μg/L, and 135.68 μg/L, respectively, with all three over the standard (8 μg/L) for drinking water and ambient surface water in China.

DEHP can be removed from the contaminated environment by biological or physicochemical processes (Magdouli et al., 2013). More attention have been paid to the biological methods due to their environmentally friendly nature, low cost, and contaminant mineralization. DEHP is susceptible to the microbial degradation, and studies had shown this phthalate could be degraded by microorganisms as the sole carbon and energy sources (Nakamiya et al., 2005; Chen et al., 2007;

Baek et al., 2009; Meng et al., 2015; Pradeep et al., 2015a). The biodegradation process of contaminants can be affected by many parameters such as temperature, pH, and type of microorganisms. In this study, a bacterium which has a remarkable degradation capability toward DEHP was isolated from the activated sludge at a local wastewater treatment plant. Batch experiments were performed at different temperatures and pHs to further optimize the DEHP biodegradation. The respective growth kinetics and biodegradation pathway were further elucidated and proposed.

MATERIALS AND METHODS

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (U.S.A.). The stock solution (10 g/L) was prepared in dimethylformamide (DMF) and kept at 4°C until use. All other chemicals used were of analytical grades.

Microbial enrichment and isolation

The activated sludge samples were collected from a regional wastewater treatment plant (Macau SAR, China). The microorganisms were first enriched in nutrient broth (NB; 3 g/L beef extract + 5 g/L peptone), then subcultured in a basic salt medium (BSM) with an increasing DEHP concentration supplied as sole carbon source on a weekly basis (10-500 mg/L) at pH 7±0.2 in 160-mL serum bottles. The bottles were incubated on a shaker (25ºC and 150 rpm). The BSM contained: NaCl 1.0 g/L, K_2HPO_4 1.0 g/L, NH_4Cl 0.5 g/L, and $MgSO_4$ 0.4 g/L. The pure culture able to use DEHP as growth substrate was further isolated from the nutrient agar (NA; 3 g/L beef extract + 5 g/L peptone + 15 g/L agar) plates.

Microbial identification

The 16S rRNA gene (rDNA) from the isolate was amplified by the bacteria specific universal primers: 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-GGTTACCTTGTTACGACTT-3'). The fragment generated was then purified by agarose gel electrophoresis and band extraction before sequenced. The nucleotide sequences were determined by the Guangzhou Magigen Biotechnology Co, Ltd (Guangzhou, China) and the sequence similarities were determined using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) from NT database (NBCI).

Analytical methods

The DEHP concentration was analyzed by Dionex UltiMate 3000 HPLC (Thermo Scientific, U.S.A.) equipped with AcclaimTM C18 reversed-phase column (5 μm, 4.6 x 150 mm) and Dionex UltiMate 3000 diode array detector. The injection volume of liquid sample was 20 μL and the ratio of mobile phase (acetonitrile:deionized water) was 9:1. The flow rate of eluent was 0.5 mL/min and the oven temperature was 45°C. The intermediate metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS) (Thermo Scientific, U.S.A.) with electro spray ionization (ESI) source, using a full scan for the mass range of 50-400. The polarity of the scan was positive and the probe temperature was 300°C. The magnitude of optical density of the culture was measured at 600 nm (OD_{600}) using a UV mini-1240 spectrophotometer (Shimadzu, Japan).

Experimental setup

For the effects of different temperatures (25, 30, 35°C) and pHs (3-9) on the DEHP biodegradation, 5 mL of the culture (10%, v/v) were inoculated into the serum bottles containing 45 mL BSM solution and 100 mg/L DEHP. The bottles were covered with stoppers and sealed with aluminium crimp. The control was prepared under the same experimental conditions but without

microorganisms. The concentration of DEHP was measured in every 24 h for 5 days. All the apparatus and media were autoclaved (Hirayama Mfg. Corp., Saitama, Japan; at 121°C, 103.5 kPa) for 20 min before use. For the kinetics study, the experimental procedure used was the same except with different initial DEHP concentrations (10-500 mg/L) at pH 7±0.2 and 30°C. The concentrations of DEHP and the optical density (OD_{600}) were determined in every 24 h for 5 days. All the treatments were incubated in the dark at 150 rpm and 30ºC, in duplicates, and each bottle was measured twice for the DEHP concentration. The one-way analysis of variance (ANOVA) was used to determine for any significant differences in the effects of temperature and pH on the biodegradation of DEHP.

RESULTS AND DISCUSSION

Microbial identification

The phylogenetic tree for the isolate is shown in Figure 1. Through the 16S rRNA sequencing analysis, the isolate is considered belonging to the genus *Acinetobacter* of Moraxellaceae family. It is a Gram-negative coccus shape bacterium and most related (more than 98% identical) to *Acinetobacter genomosp*. This isolate belongs to the same genus as the one isolated by Latorre et al. (2012a), the first *Acinetobacter* strain reported capable of degrading DEHP.

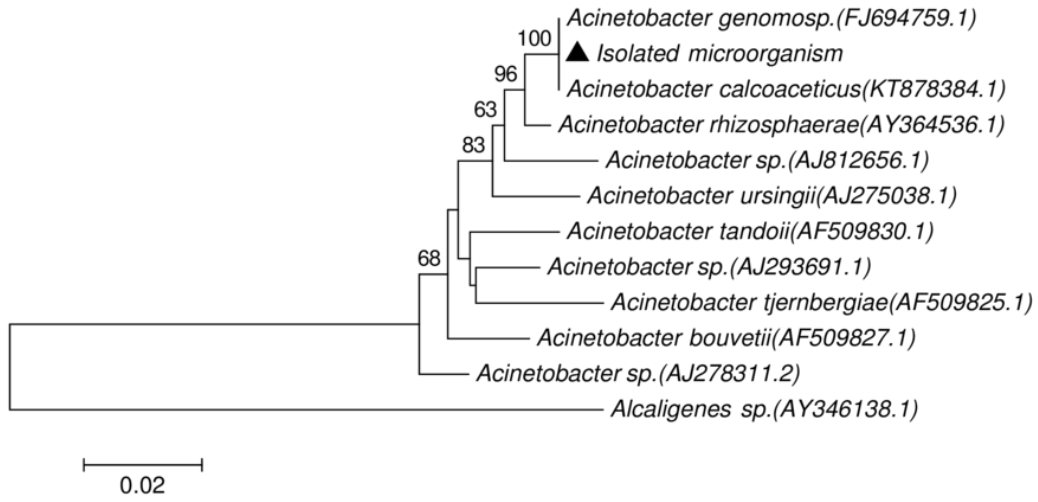

Figure 1. Phylogenetic tree for the isolate *Acinetobacter* sp.

Effects of temperature and pH on DEHP biodegradation

The highest biodegradation efficiency for DEHP was obtained at 35°C, even though there was no significant difference in DEHP biodegradation at 35°C and 30°C (p=0.22). The biodegradation efficiency for DEHP at 35°C and 30°C was 10.32% and 6.88%, respectively, higher than at 25°C. On the other hand, the biodegradation efficiencies under the acidic condition (pH 3-5) were much lower compared to the neutral and alkaline conditions (pH 6-9). This lower DEHP biodegradation efficiency in the acidic medium may be due to the fact that the enzyme activities for the PAEs degradation are known particularly sensitive to the low pH (Fang et al., 2010). The low microbial growth also suggested *Acinetobacter* sp. can hardly survive under the acidic condition, resulting in low degradation efficiency for DEHP. In general, the biodegradation efficiency for DEHP at pHs 6-9 was more than 90% and there were no significant differences between the pH values (p=0.87).

DEHP biodegradation kinetics

Figures 2 and 3 show DEHP biodegradation and cell growth at different initial concentrations, respectively. For the cell growth, no lag phase was observed, except when the initial concentration of DEHP was higher than 500 mg/L, further suggesting the *Acinetobacter* sp. growth may be inhibited by the toxicity of DEHP at this concentration level. The isolate showed the lag phase in the first two days and after that, it started the exponential growth with OD_{600} reaching 0.3 (similar when the isolate grown at 400 mg/L). Amir et al. (2005) also reported that the inhibition of PAEs may affect the membrane of microbial cells in the activated sludge. The obtained results clearly indicate the cell growth inhibited at high DEHP concentration levels, a general trend among PAE-degrading microorganisms (Wen et al., 2014).

Based on the inhibitory effect of DEHP at >400 mg/L on the growth of *Acinetobacter* sp., Andrew's substrate inhibition equation (Eq. 1) was applied to estimate the biodegradation kinetics parameters for the isolate (Shim and Yang, 1999) as follows:

$$D = D_{max} \frac{S}{K_s + S + \frac{S^2}{K_i}} \qquad \text{Eq. (1)}$$

where D (mg/L•day) and D_{max} (mg/L•day) are degradation rate and maximum degradation rate, respectively, S (mg/L) is the substrate (DEHP) concentration, K_s (mg/L) is the half-saturation constant, and K_i (mg/L) is the inhibition constant. A higher K_i value means the microbial culture is less sensitive to the substrate inhibition (Shim and Yang, 1999). Similarly, the following inhibition kinetics (Eq. 2) was applied to estimate the kinetics parameters associated with the growth of the isolate on DEHP.

$$\mu = \mu_{max} \frac{S}{K_s + S + \frac{S^2}{K_i}} \qquad \text{Eq. (2)}$$

where μ (day^{-1}) and μ_{max} (day^{-1}) are specific growth rate and maximum specific growth rate, respectively. The inhibition model for degradation rate and specific growth rate was further simulated using the Matlab software. The respective values of D_m, μ_m, K_s, and K_i are shown in Table 1. The highest DEHP degradation rate was achieved when the initial concentration of DEHP was 400 mg/L.

When the initial concentration of DEHP was lower than 500 mg/L, the biodegradation followed the first-order kinetics. The linear regression of the natural logarithm of the DEHP concentration (100-400 mg/L) was plotted against the incubation time, and the slope of such regression was considered with the first-order constant of DEHP (k, day^{-1}) (Kim et al., 2007). The half-live ($t_{1/2}$) of DEHP was calculated as follows (Eq. 3):

$$t_{1/2} = \frac{0.693}{k} \qquad \text{Eq. (3)}$$

The first-order constant (k) and the half-life of DEHP was 0.854-0.625 and 19.44-26.6 h, respectively, at the concentration range studied (100-400 mg/L). Benjamin et al. (2015) reported that the half-life of DEHP during biodegradation in nature was 29, 45, and 14 days in composting activated sludge, in lagoon sludge, and in sediment, respectively. The half-life of DEHP during biodegradation using *Pseudomonas fluorescens* was 10.07 days when the initial concentration of

DEHP was lower than 50 mg/L (Feng et al., 2002). In current study, the isolate *Acinetobacter* sp. was found active in the DEHP degradation, and to further enhance the microbial activity, the addition of microelements would be one possibility since some micronutrients played important roles in the metabolic activity of microorganisms in the activated sludge (Lu and Biao, 2015).

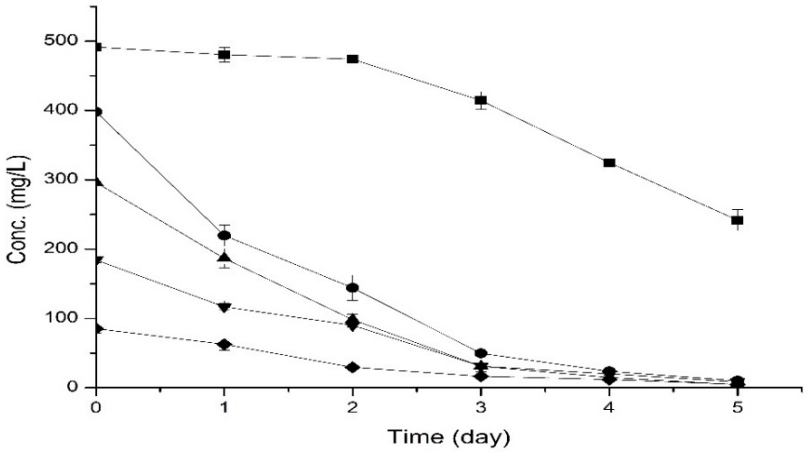

Figure 2. DEHP biodegradation at different initial concentrations: (-■-) 500; (-●-) 400; (-▲-) 300; (-▼-) 200; and (-◆-) 100 mg/L.

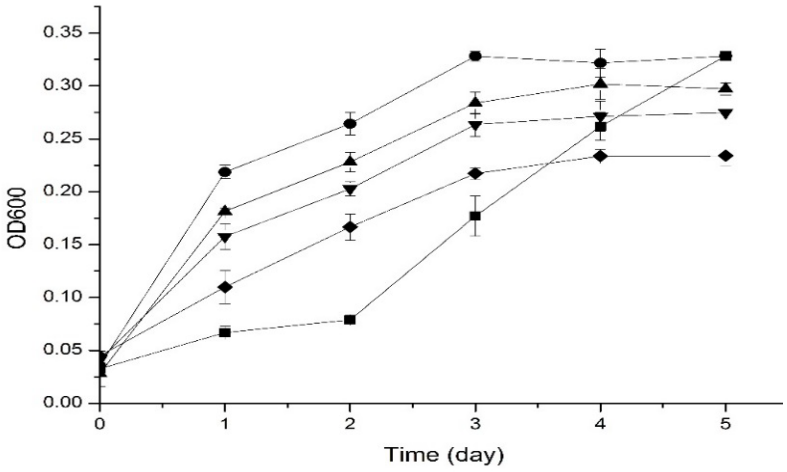

Figure 3. Cell growth of *Acinetobacter* sp. at different initial DEHP concentrations: (-■-) 500; (-●-) 400; (-▲-) 300; (-▼-) 200; and (-◆-) 100 mg/L.

Table 1. Biodegradation kinetics parameters for the isolate *Acinetobacter* sp. grown on DEHP (values were estimated from simulation).

Parameter	Degradation rate	Specific growth rate
D_{max} (mg/L•day)	124.8	-
μ_{max} (day^{-1})	-	0.1192

K_s (mg/L)	272.3	137.6
K_i (mg/L)	720.5	850.3

Proposed DEHP biodegradation pathway

The aerobic biodegradation pathway for phthalates follows two stages. First, the biodegradation from phthalic di-esters (PDEs) to phthalic mono-esters (PMEs) occurs and then the PMEs are degraded to phthalic acid (PA). Secondly, the PA is mineralized all the way to CO_2 and H_2O (Liang et al., 2008; Benjamin et al., 2015). During the DEHP biodegradation, mono-(2-ehtylhexyl) phthalate (MEHP) as the first metabolite is generated through hydrolysis, followed by the generation of PA after further hydrolysis of MEHP (Li et al., 2016). In current study, the metabolic products of the DEHP degradation by the isolate *Acinetobacter* sp. were analysed by LC-MS and the intermediate products were determined based on the mass-to-charge ratio (m/z). The m/z value for the first peak was 277, which matched with the molecular weight of MEHP (278). This result indicates that the first step of the DEHP biodegradation was probably hydrolysis, resulting in the removal of one hydrocarbon chain from DEHP. For this process, esterase is the main enzyme produced by microorganism to catalyse hydrolysis, resulting in the formation of MEHP and 2-ethylhexanol (Magdouli et al., 2013; Pradeep et al., 2015b). Other intermediates were also measured in current study, at the m/z values of 79, 105, 115,145, and 186 while the m/z values of 105 and 115 were excluded as common background ions by ESI. The metabolic products of the DEHP biodegradation were approximated based on the results from the MS as well as from the previous studies (Liang et al., 2008; Magdouli et al., 2013; Benjamin et al., 2015; Karandikar et al., 2015). The compound with the m/z value of 145 might be di-ethyl hexanoic acid, the oxide of di-ethyl hexanol dechained from DEHP during hydrolysis. Based on the metabolic pathway for the phthalates degradation reported by others, the metabolic products of the DEHP biodegradation identified in this study include MEHP, PA, protocatechuate, and other metabolites like β-carboxy-*cis*,*cis*-muconic acid (m/z=186) and 3-katoadipate (m/z=79). The proposed biodegradation pathway is shown in Figure 4.

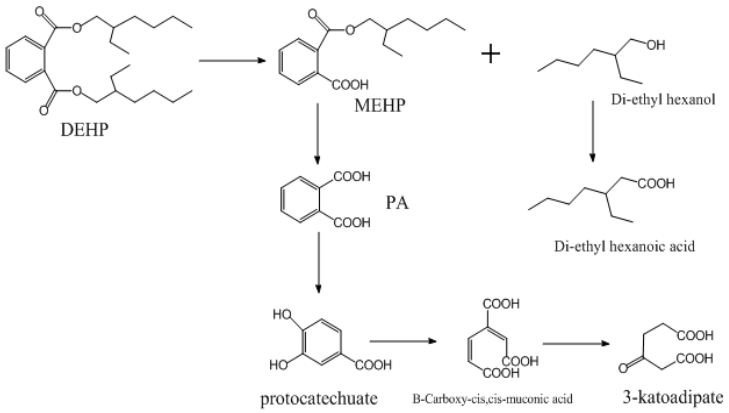

Figure 4. The potential degradation pathway for the DEHP biodegradation by the isolate.

CONCLUSION

An indigenous bacterial isolate, identified as *Acinetobacter* sp., was evaluated for the DEHP degradation. The optimal temperature for the biodegradation is considered 30ºC and the neutral and alkaline conditions are shown favourable to this isolate. The kinetics results showed that high concentrations of DEHP (500 mg/L) were inhibitory to both biodegradation and cell growth. Some metabolic products were identified and the pathway for the DEHP degradation by the isolate is

proposed. The biological process developed in this study could be further scaled up by using an appropriate bioreactor configuration and applied to treat different types of wastewater, especially the ones containing high concentration levels of DEHP and other PAEs, mainly generated from the plastics industries.

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