International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (2), pp. 001-007, February, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Biodegradation of di-n-butyl phthalate by a newly isolated *Diaphorobacter* sp. strain QH-6

Decai Jin[1,2], Ping Wang[2], Zhihui Bai[1]*, Bo Jin[3], Zuopeng Lv[4], Dongbin Wei[1] and Guoqiang Zhuang[1]

[1]Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China.
[2]Graduate University of Chinese Academy of Sciences, Beijing, 100083, China.
[3]School of Earth and Environment Sciences, School of Chemical Engineering, The University of Adelaide, Adelaide, SA 5005, Australia.
[4]School Chemical Engineering and Technology, China University of Mining and Technology, Xuzhou, 221116, China.

Accepted 10 January, 2019

Di-n-butyl phthalate (DBP), one of the most popular phthalic acid esters (PAEs), is commonly found in wastewater treatment plant. In this study, a bacterial strain capable of using DBP as sole carbon and energy source was isolated from activated sludge. This strain was identified as *Diaphorobacter* sp., designated as QH-6, based on the 16S rRNA and *gyrB* gene sequence analysis. For the first time, we studied the biodegradation ability of PAEs by genus *Diaphorobacter*. The high performance liquid chromatography (HPLC) analysis revealed that the optimum conditions for DBP degradation were pH 7.0 to 8.0, temperature 30 to 35°C, and agitation rate 150 to 225 r/min. Under these conditions, 500 mg/L of DBP could be completely degraded with a half-life of 5.20 h. We investigated the effects of heavy metals (Cr^{6+} and Cu^{2+}) on the DBP degradation. The results demonstrated that the heavy metals at a wide concentration range of 5 to 30 mg/L can restrain the DBP degradation. Furthermore, substrate utilization tests showed QH-6 can also utilize other PAEs and the degradation intermediates.

Key words: *Diaphorobacter* sp., dibutyl phthalate, biodegradation, heavy metal.

INTRODUCTION

With the development of urbanization, water source contamination by wastes from households and industry has become a considerable issue worldwide. Many municipal wastewater treatment plants (WWTPs) were built to deal with this problem. But people should not neglect the increasing number of organic compounds were detected in effluents of municipal WWTPs (Nakada et al., 2004). These organic compounds include many endocrine disrupting chemicals (EDCs), which can interfere with the normal functioning of the endocrine systems of many aquatic and terrestrial organisms.

PAEs as one type of additive to improve mechanical properties of the plastic resin are widely utilized in plastics industry (Cartwright et al., 2000). Some of PAEs are suspected to be mutagens and carcinogens, and considered as EDCs (Lu et al., 2009). Unfortunately, because of bounding covalently to the plastic resin, PAEs are prone to migrate into the environment during use or after disposal (Wang et al., 2004). Up to date, phthalates have become ubiquitous pollutants in variety of environments (Chatterjee and Karlovsky, 2010). Previous studies confirmed that metabolic breakdown of PAEs by microorganisms is the major routes for degradation of these widespread pollutants (Chang et al., 2009). Many bacterial strains with individual PAEs degrading ability have been isolated from a variety of habitats under either aerobic or anaerobic conditions in recent years (Lertsirisopon et al., 2006; Trably et al., 2008). However, there is still lack of detailed information about which types of bacteria capable of degrading these contaminants in activated sludge treatment plants. Among these contaminants, DBP and DEHP (the prevailing PAEs) were the main organic compounds which

*Corresponding author. E-mail: zhbai@rcees.ac.cn or gqzhuang@rcees.ac.cn Tel: +86 10 62 849 613. Fax: +86 10 62 923 563.

are difficult to be degraded in WWTPs (Wang et al., 2004; Huang et al., 1994). Therefore, in order to get an in-depth understanding of PAEs biodegradation and to improve treatment efficiency and water quality in WWTPs, there is a need to screen more novel bacterial strains which have specific abilities to degrade PAEs in WWTPs.

The aims of present study are to isolate and characterize a novel highly effective DBP-degrading bacterium from the activated sludge, which will enrich our knowledge of DBP-degrading microbial community. Moreover, the effects of heavy metals on DBP biodegradation by the isolated bacterium are also studied. It is expected that the bacterial strain isolated in this study would be used for bioaugmentation of the biological treatment process for high strength phthalate-containing industrial and domestic wastewaters.

MATERIALS AND METHODS

Chemicals

The dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate, di-n-octyl phthalate (DOP), and diisooctyl phthalate (DIOP), were purchased from Alfa Aesar (Ward Hill, MA), and have a purity above 98%. The methanol was of high-performance liquid chromatography grade (Sigma, USA). All other chemicals and solvents were of analytical reagent grade.

Enrichment culture and isolation of bacteria

The activated sludge sample was collected from a wastewater treatment plant with A2/O process in the suburbs of Beijing, China. The enrichment and isolation process was the same as described by Jin et al. (2010).

Identification of pure culture

The genomic DNA was extracted using an EZ-10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc., Canada) according to the manufacturer's introductions. The 16S rRNA gene was amplified using the bacterial primers F27 and R1492 (Jin et al., 2010). The 1.2-kbp nucleotide sequences of the *gyrB* gene of the bacterial strain was amplified using polymerase chain reaction (PCR) with universal primer sets UP1 and UP2r as described by Yamamoto and Harayama (1995). The 16S rRNA and *gyrB* sequence obtained from strain were then subjected to a BLAST homology search (http://www.ncbi.nlm.nih.gov/BLAST).

Substrate utilization tests

For substrate utilization tests, the isolated strain was inoculated in the liquid MSM containing 200 mg/L following substrates (DMP, DEP, DBP, DOP, DIOP, phthalic acid, protocatechuic acid, phenanthrene, and toluene respectively). For each substrate, medium without inoculation was performed as negative control. The substrate utilization was based on the microbial growth, which was determined by measuring the increase of the biomass (OD600).

Analysis of DBP residue

For all experiments, the initial concentration of DBP was 500 mg/L. In order to analyze the residual DBP concentration in the culture media, the liquid cultures were mixed with 20 ml of ethyl acetate by vibrating, and then the aqueous and organic phases were separated by centrifugation at 12, 000 r/min for 3 min. The aqueous samples were then extracted twice, the ethyl acetate was evaporated to dryness and the residue was redissolved in 10 ml of methanol. Approximately 1 ml of the DBP-containing methanol was passed through a 0.22 μm membrane filter. Finally, aliquots of 20 μl filtrates were injected into Agilent 1100 Series HPLC systems (USA). A Kromail C18 column (4.6 × 200 mm × 5 μm) was used for the separation, and the UV wavelength was 228 nm. The mobile phase consisted of methanol: water solution (90:10, v/v) and the flow rate was 0.5 ml/min.

RESULTS

Isolation and identification of novel DBP-degrading bacteria

Isolation of PAEs degrading microorganisms were successfully achieved by establishment of highly enriched aerobic cultures. One strain which can grow well on agar plate with 500 mg/L DBP was selected for further study and designed as QH-6. The strain QH-6 was found to be Gram-negative and asporogenous, and has rod-shaped cells with rounded ends. A scanning electron micrograph of QH-6 is shown in Figure 1. Furthermore, the partial 16S rRNA gene of strain QH-6 was sequenced and deposited in the GenBank database under accession number HQ588349. This isolated microorganism revealed a 99.8% similarity with 16S rRNA gene sequences of *Diaphorobacter* sp., the corresponding phylogenetic tree is given in Figure 2. In order to further confirm the classification of strain QH-6, *gyrB* gene was also amplified and sequenced. The result showed the *gyrB* of QH-6 (HQ588350) were 99% identical to that of *Acidovorax ebreus* TPSY (Synonym: Diaphorobacter sp. TPSY), which agreed with the 16S rRNA analysis. Based on 16S rRNA and *gyrB* gene sequence analysis, QH-6 was identified as belonging to the genus *Diaphorobacter*.

Effects of different environmental conditions on microbial growth and DBP degradation

Initially, influence of different pH on DBP degradation and microbial growth were investigated and the results are shown in Figure 3a. We found that the concentration of DBP in the culture medium increased as the pH varied from 7.0 to 10.0, and completed DBP degradation occurred at pH 7.0 where the maximum biomass was achieved. Although the DBP degradation rate of the QH-6 dropped obviously when pH was below 6 and above 10, DBP was still degraded approximately 70%. These indicated that QH-6 has a capability to grow in a wide range of pH, while preferring to degrade DBP in a slightly

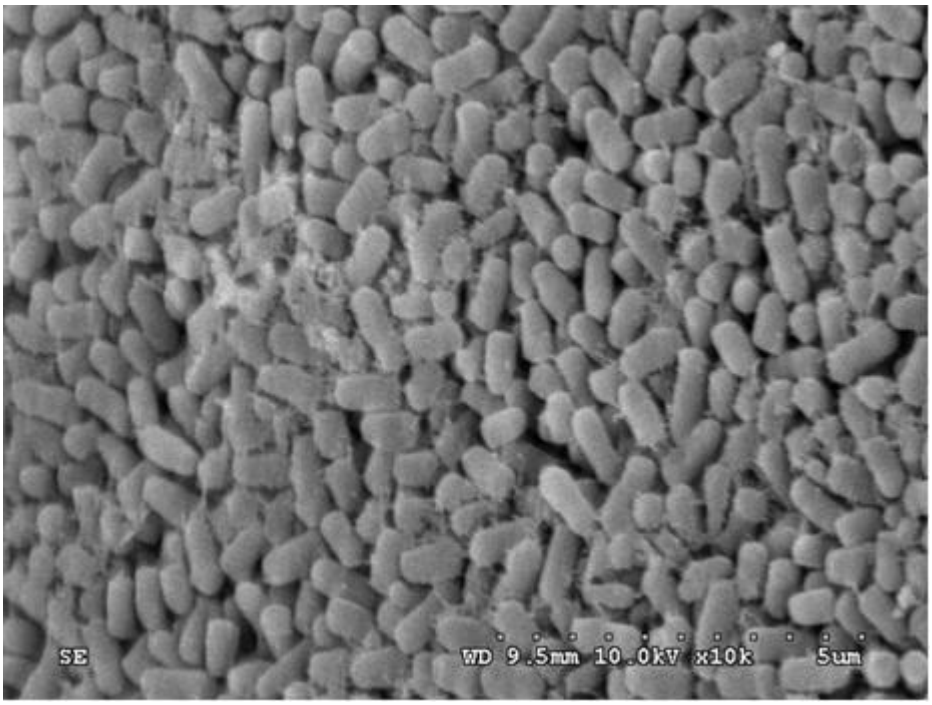

Figure 1. Scanning electron micrograph of strain QH-6 (×10,000).

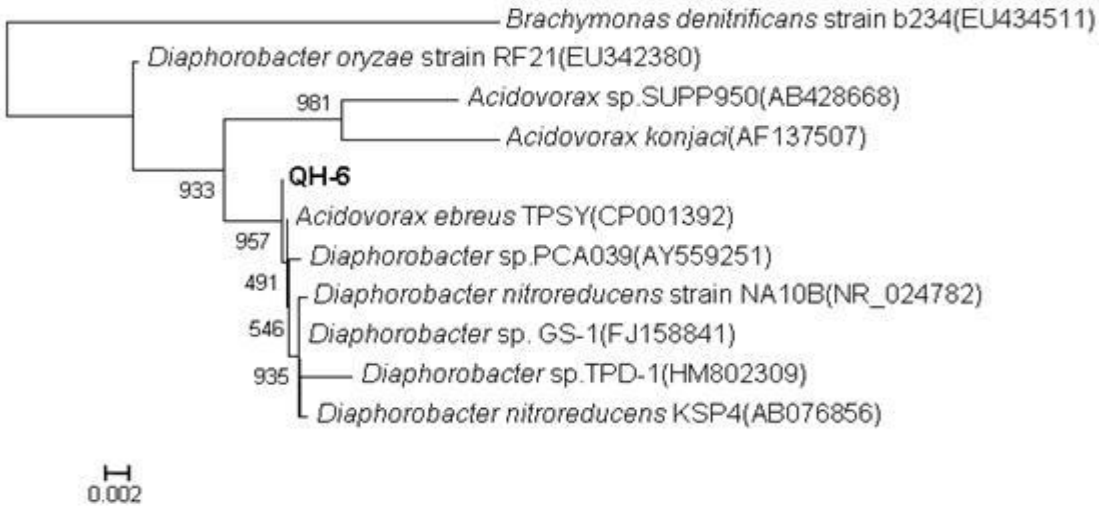

Figure 2. Phylogenetic tree derived from 16S rRNA gene sequence of QH-6 and sequences of related species. Distances were calculated using neighbor-joining method. Numbers at branch points are bootstrap values (based on 1,000 samplings). GenBank accession numbers are included in brackets. *Brachymonas denitrificans* strain b234 (EU434511) was used as outgroup. Scale bars represent 0.002 substitutions per site.

alkaline environment. In our present work, the most optimum pH for DBP degradation and the microbial growth of QH-6 was 7.0. Temperature is also a vital factor may influence DBP degradation. Here, we investigated the DBP degradation rates by QH-6 under different temperature at pH 7.0. After incubating 12 h, the degradation rates at the tested temperatures were measured and the results are presented in Figure 3b. The degradation rate of QH-6 dropped apparently when the temperature reached up to 40°C or down to 20°C. It indicated that the QH-6 demonstrate an excellent DBP degradation ability at a moderate temperature. At a

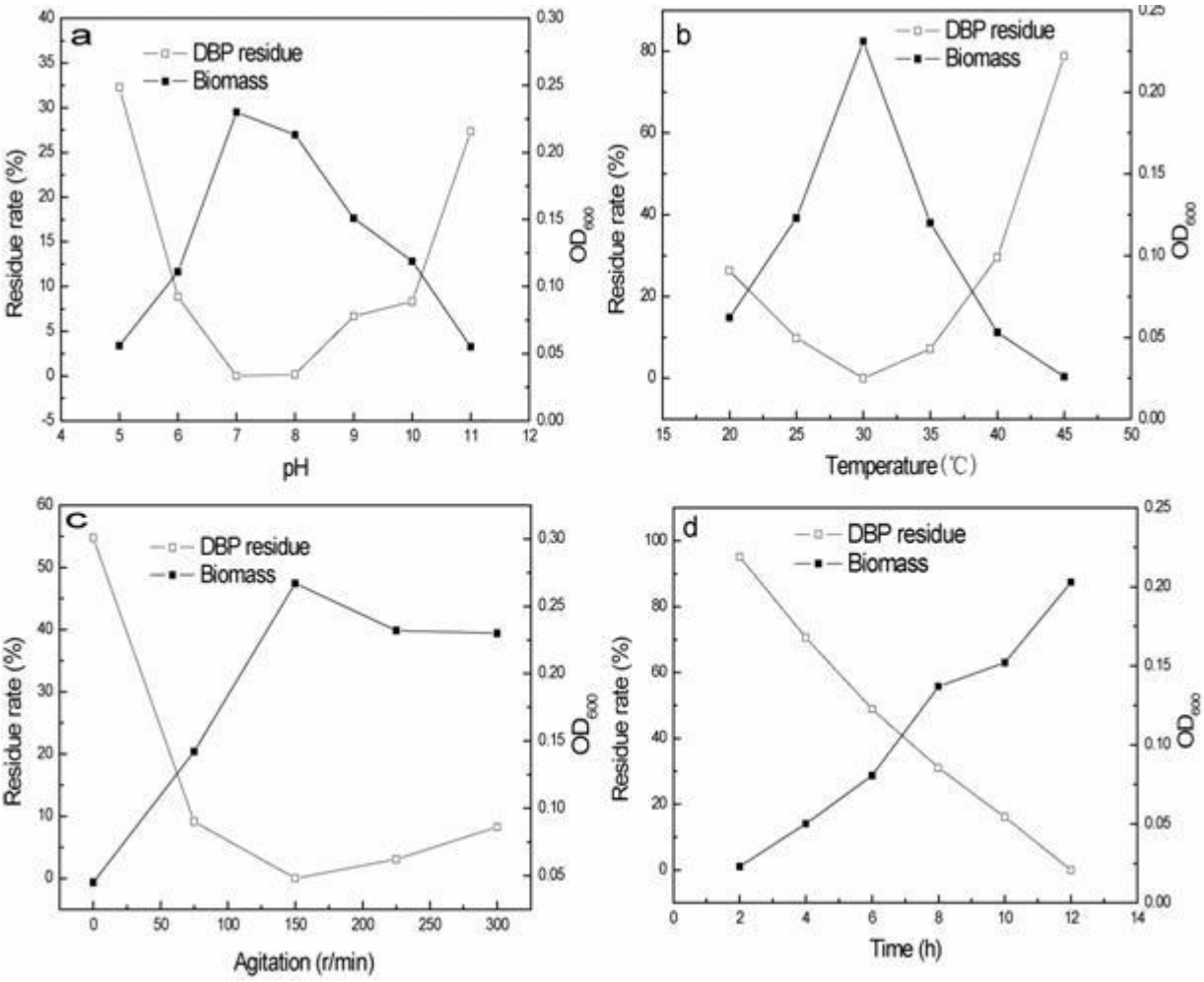

Figure 3. Effect of pH (a), temperature (b), and agitation rate (c) on biomass and DBP biodegradation. (d) Eff ect of incubation time on DBP biodegradation under optimal conditions. Data are averages of triplicate experiments.

temperature range of 25 to 35°C, the DBP degradation rate showed not obvious fluctuations and no residues of DBP were detected at 30°C, indicating that the optimal temperature for the DBP degradation and QH-6 growth was at 30°C. In addition, the influence of aeration conditions on the degradation rate was also investigated at 30°C and pH 7.0. The results were shown in Figure 3c. No DBP was detected when the agitation rate was increased to 150 r/min. However, no distinctive change was shown in the degradation rate when the shaking rate of incubator was between 75 and 300 r/min. Therefore, the agitation rate of 150 r/min was used for all the subsequent experiments. Under optimal conditions, strain QH-6 can completely degraded 500 mg/L of DBP within 12 h (Figure 3d). A first-order kinetics model, $\ln C = -kt + A$, was used to calculate the half life, where C is the initial concentration (mg/L), k is the biodegradation rate constant, t is the time period, A is the constant. From the result, it can be found that the half-life of degradation was about 5.20 h when the concentration of DBP was 500 mg/L.

Effects of heavy metals on DBP degradation by QH-6

Heavy metal pollution is widespread existence in water environment. Heavy metals can be toxic for many species and have a significant dose-dependent impact on microbial growth of microorganisms and their biochemical activities. Herein, we investigated the effect of heavy metal ions (Cr^{6+} and Cu^{2+}) at a range from 0 to 30 mg/L on the DBP degradation by QH-6. As shown in Figure 4, the supplement of the heavy metals had a significant effect on the DBP degradation by strain QH-6. The QH-6 can completely utilize DBP in absence of Cr^{6+} and Cu^{2+} in the batch experiments within 12 h. However, the QH-6 is extremely sensitive to heavy metals Cr^{6+} and Cu^{2+}, and the depressing effect on DBP degradation is obvious. Despite all that, it is interesting to note that DBP degradation rates still reached 13.68 and 29.07% within

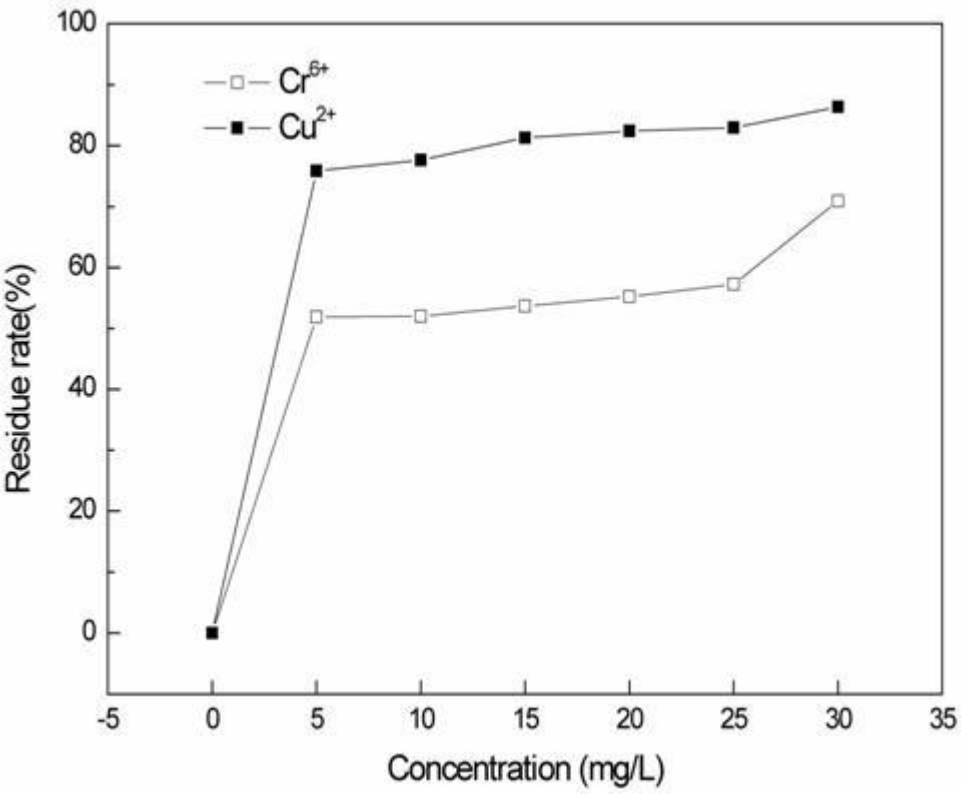

Figure 4. Effect of heavy metals (Cr^{6+} and Cu^{2+}) on DBP biodegradation.

Table 1. Substrate utilization profile for strain QH-6.

Substrate	Utilization	Substrate	Utilization	Substrate	Utilization
DMP	++	DOP	+	Protocatechuic acid	+
DEP	++	DIOP	+	Phenanthrene	–
DBP	++	Phthalic acid	+	Toluene	–

++: Higher utilization, +: Lower utilization, –: No utilization.

12 h, respectively, when the concentrations of Cu^{2+} and Cr^{6+} were 30 mg/L.

Substrate utilization tests

The substrate utilization tests indicated that the isolate had different abilities in degrading different phthalate esters (Table 1). QH-6 could growth better in a medium containing DMP, DEP and DBP than in DOP and DIOP. This finding indicated that the shorter alkyl chains could be rapidly and preferentially degraded by the isolated strain. These results were in agreement with previous reports (Jin et al., 2010; Chang et al., 2004). Furthermore, strain QH-6 was found to be able to utilize the intermediate products of phthalic acid and protocatechuic acid, suggesting that QH-6 may degrade DBP through PA and PCA as intermediates.

DISCUSSION AND CONCLUSIONS

Activated sludge in wastewater treatment plant contains a large number of microorganisms, which play a major role in the biodegradation of organic matter, removal of inorganic compounds, inactivation of human or animal pathogens and detoxification of pollutants (Schuppler et al., 1995). DBP, as one of the most commonly used PAEs, is widely existed in wastewater (Huang et al., 1994). In the present study, a novel bacterial strain (QH-6) capable of utilizing DBP as the sole source of carbon and energy was isolated from activated sludge sample, and then as identified as belonging to the genus *Diaphorobacter* with a 99% similarity based on a 16S

rRNA and *gyrB* gene sequence analysis. Although the genus *Diaphorobacter* was capable of degrading pyrene (Klankeo et al., 2009), limited reported referred to its ability of degradation other organic compounds. The DBP degradation by QH-6 under different conditions was investigated and the results showed that the optimum pH, temperature, and agitation rate are quite similar to many reported strains (Jin et al., 2010; Li et al., 2006). Compared to some reported PAEs-degrading strains such as genera *Delftia*, *Enterobacter*, *Ochrobactrum*, *Deinococcus* and *Pseudomonas* (Lertsirisopon et al., 2006; Patil et al., 2006; Fang et al., 2010; Wu et al., 2010), our isolated bacterium shows a high degradation ability and promising biochemical activities at a broad range of processing conditions.

It is well known that the presence of heavy metals in wastewater can significantly affect the performance of biological waste treatment processes (Chua et al., 1999). In recent years, effects of heavy metals on microbial populations and microbial processes have been well documented (Sokhn et al., 2001). However, little is known about the effect of heavy metals on the degradation of PAEs by degraders from activated sludge. Because Cr^{6+} and Cu^{2+} exists widely in the industrial wastewater and the contaminated environment, both of them were selected as the heavy metal for evaluating potentially toxic effects on DBP biodegradation. The results showed that both heavy metals can adversely affect the rate of DBP degradation by strain QH-6. It was speculated that the Cu^{2+} and Cr^{6+} might chemically combined to the enzymes involved in DBP degradation. Moreover, Cu^{2+} showed more toxic than Cr^{6+} in inhibiting QH-growth for the DBP degradation, which was in agreement with previous report (Onga et al., 2010). Nevertheless, the DBP degradation rate by QH-6 still reached 13.68 to 40.24% when the heavy metals present in batch culture within 12 h. Meanwhile, the diversity of degradable substrates test showed that QH-6 can not only utilize PA and PCA, which are the two major metabolites of DBP degradation, but also grew with DMP, DEP, DOP and DIOP as the sole source of carbon and energy, indicating the strain QH-6 may possesses a complete pathway for DBP degradation and has a great potential for bioremediation in regions contaminated with a variety of PAEs.

In summary, we describe a novel DBP-degrading bacterium isolated from an activated sludge in a municipal wastewater treatment plant using a culture enrichment technique. This strain can completely degraded 500 mg/L of DBP within 12 h and also utilize other PAEs as well. Furthermore, this study also showed that the presence of heavy metals could influence the degradation rate of DBP. This is the first detailed report on DBP degradation by strain from the genus *Diaphorobacter*. In particular, characteristics of using a well variety of PAEs and high degradation ability make strain QH-6 be a promising candidate for bioremediation of PAEs-contaminated sewage and soil.

ACKNOWLEDGEMENTS

This work was supported by the “Knowledge Innovation” Program of Chinese Academy of Sciences (No. KZCX2-YW-JC407-3) and the National Natural Science Foundation of China (No. 20777089).

REFERENCES

Cartwright CD, Owen SA, Thompson IP, Burns RG (2000). Biodegradation of diethyl phthalate in soil by a novel pathway. FEMS Microbiol. Lett., 186(1): 27-34.

Chang BV, Lu YS, Yuan SY, Tsao T, Wang MK (2009). Biodegradation of phthalate esters in compost-amended soil. Chemosphere, 74(6): 873-877.

Chang BV, Yang CM, Cheng CH, Yuan SY (2004). Biodegradation of phthalate esters by two bacteria strains. Chemosphere, 55(4): 533-538.

Chatterjee S, Karlovsky P (2010). Removal of the endocrine disrupter butyl benzyl phthalate from the environment. Appl. Microbiol. Biotechnol., 87(1): 61-73.

Chua HP, Yu HF, Sin SN, Cheung MWL (1999). Sub-lethal effects of heavy metals on activated sludge microorganisms. Chemosphere, 39(15): 2681-2692.

Fang CR, Yao J, Zheng YG, Jiang CJ, Hu LF, Wu YY, Shen DS (2010). Dibutyl phthalate degradation by *Enterobacter* sp. T5 isolated from municipal solid waste in landfill bioreactor. Int. Biodeter. Biodegr., 64(6): 442-446.

Huang X, Zhang XJ, Fu Q (1994). Assessment for biodegradability of organics in municipal wastewater and controlling measure for its refractory organics. Chin. J. Environ. Sci., 15: 15-19.

Jin DC, Liang RX, Dai QY, Zhang RY, Wu XL, Chao WL (2010). Biodegradation of di-n-butyl phthalate by *Rhodococcus* sp. JDC-11 and molecular detection of 3,4-phthalate dioxygenase gene. J. Microbiol. Biotechnol., 20(10): 1440-1445.

Klankeo P, Nopcharoenkul W, Pinyakong O (2009). Two novel pyrene-degrading *Diaphorobacter* sp. and *Pseudoxanthomonas* sp. isolated from soil. J. Biosci. Bioeng., 108(6): 488-495.

Lertsirisopon R, Soda S, Sei K, Ike M, Fujita M (2006). Biodegradability of four phthalic acid esters under anaerobic condition assessed using natural sediment. J. Environ. Sci., 18(4): 793-796.

Li J, Chen J, Zhao Q, Li X, Shu W (2006). Bioremediation of environmental endocrine disruptor di-n-butyl phthalate ester by *Rhodococcus rubber*. Chemosphere, 65(9): 1627-1633.

Lu Y, Tang F, Wang Y, Zhao J, Zeng X, Luo Q, Wang L (2009). Biodegradation of dimethyl phthalate, diethyl phthalate and di-n-butyl phthalate by *Rhodococcus* sp. L4 isolated from activated sludge. J. Hazard. Mater., 168(2-3): 938–943.

Nakada N, Nyunoy H, Nakamura M, Hara A, Iguchi T, Takada H (2004). Identification of estrogenic compounds in wastewater effluent. Environ. Toxicol. Chem., 23(12): 2807-2815.

Onga SA, Toorisakaa E, Hirataa M, Hano T (2010). Adsorption and toxicity of heavy metals on activated sludge. Science Asia, 36: 204-209.

Patil NK, Kundapur R, Shouche YS, Karegoudar TB (2006). Degradation of a plasticizer, di-n-butyl phthalate by *Delfia* sp. TBKNP-05. Curr. Microbiol., 52(5): 369-374.

Schuppler M, Mertens F, Schön G, Göbel UB (1995). Molecular characterization of nocardiform actinomycetes in activated sludge by 16S rRNA analysis. Microbiology, 141: 513-521.

Sokhn J, De Leij FA, Hart TD, Lynch JM (2001). Effect of copper on the degradation of phenanthrene by soil microorganisms. Lett. Appl. Microbiol., 33(2): 164-168.

Trably E, Batstone DJ, Christensen N, Patureau D, Schmidt JE (2008). Microbial dynamics in anaerobic enrichment cultures degrading di-n-butyl phthalic acid ester. FEMS Microbiol. Ecol., 66(2): 472-483.

Wang JL, Zhao X, Wu WZ (2004). Biodegradation of phthalic acid esters (PAEs) in soil bioaugmented with acclimated activated sludge. Process Biochem., 39(12): 1837-1841.

Wu XL, Wang YY, Liang RX, Dai QY, Chao WL (2010). Degradation of

di-n-butyl phthalate by newly isolated *Ochrobactrum* sp. Bull. Environ. Contam. Toxicol., 85(3): 235-237.
Yamamoto S, Harayama S (1995). PCR amplification and direct sequencing of *GyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl. Microbiol. Biotechnol., 61(3): 1104-1109.