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Biodegradation of di-n-butyl phthalate ester by newly isolated *Raoultella* sp. ZJY

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A bacterial strain ZJY that could degrade di-n-butyl phthalate was isolated from sludge of river of Hangzhou city, and identified as *Raoultella* sp. by morphological, physiological characteristics and the analysis of 16S rRNA gene sequence. The results of DBP-degrading characteristics showed that the optimal temperature and pH for dibutyl phthalate biodegradation was 30 °C and 7.0, respectively. The degradation was best fitted by first-order kinetic equation and the half-life was 40.81 h at 418 mg/L dibutyl phthalate of initial concentration. GC-MS analysis of the metabolites revealed that initial step of dibutyl phthalate was degradation was transesterification. To our knowledge, this is the first report on the biodegradation characteristics of DBP by a member of the *Raoultella* genus.

Key words: *Raoultella* sp; Di-n-butyl phthalate; Biodegradation; Phthalic acid esters

INTRODUCTION

Phthalic acid esters (PAEs) are major industrial products used as plasticizers in the industrial production of plastics. In recent years, phthalic acid diesters have appeared as important pollutants in environmental samples as endocrine disrupting chemicals which influence the genitals. Among rats and adult men, phthalate exposure resulted in decreased testicular weight, seminiferous tubular atrophy and increased DNA damage in sperm [1, 2]. Some of the PAEs including dimethyl phthalate (DMP), di-n-butyl phthalate (DBP), and di-n-octyl phthalate (DOP) have been listed as priority pollutants by the China National Environmental Monitoring Center [3] and the US Environmental Protection Agency [4].

Since the rates of photolysis and chemical hydrolysis of phthalate esters are very slow, metabolic breakdown by microorganisms is considered to be one of the major routes for the environmental degradation of these widely spread pollutants in aquatic and terrestrial systems. In recent years, various microorganisms are mainly responsible for the degradation of phthalic acid esters [5], these include *Gordonia* sp. [6], *Sphingomonsa* sp. [7], *Arthrobacter* sp. [8], *Rhodococcus* sp. [9], *Acinetobacter* sp. [10]. Numerous studies have demonstrated the microbial bio-degradation pathways of phthalate esters. The metabolism of phthalate esters is initiated in bacteria by their hydrolysis to mono-phthalate esters, which are further degraded by ester-hydrolysis to phthalate. Phthalate is further metabolized in aerobic bacteria by two different dioxygenase-initiated pathways through the common intermediate, protocatechuate (3,4-dihydroxybenzoate). Protocatechuate is further degraded into organic acids through either ortho- or meta-cleavage pathway by ring cleavage enzymes, which eventually converted them to CO_2 and H_2O through Krebs cycle [11-15]. In this paper, the aerobic degradation of selected phthalate esters by a pure culture of *Raoultella* sp. ZJY isolated from river sludge, was studied. To our knowledge, there is no report that *Raoultella* strains have the capacity to mineralize PAEs. In addition, we investigated the effects of temperature, pH, DBP concentration and the biochemical degradation pathway on the biodegradation performance of the strain ZJY. These results allow for a better understanding of the mechanism of PAEs degradation of the genus *Raoultella*.

EXPERIMENTAL

Chemicals

DMP, Diethyl phthalate (DEP), DBP, di(2-ethylhexyl)phthalate (DEHP), mono-butyl phthalate (MBP) and mono-methyl phthalate (MMP) were purchased from Sinopharm Chemical Reagent Co.,Ltd, all were >98% pure. HPLC-grade methanol was purchased from Tianjin Siyou Fine Chemicals Co., Ltd. (Tianjin, China). Other chemicals were of analytical-reagent grade, and were purchased locally.

Enrichment culture, isolation and identification of bacteria

The sludge samples were collected from sludge of the river of Hangzhou city. 1 g sample was mixed with 100 ml of a basic inorganic salt medium (BSM) ($K_2HPO_4 \cdot 3H_2O$ 1.0 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.4 g, $CaCl_2$ 0.0755 g, $FeCl_3 \cdot 6H_2O$ 0.0143 g in 1 L of distilled water, the pH of the medium was adjusted to 7.0), and supplemented with DBP (DBP dissolved in methanol) to 125 mg l^{-1} of final concentration as the sole carbon and energy source. After

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acclimation for 7 days, the enriched medium was used to inoculate nutrient agar plates under aseptic conditions. The plates were incubated at 30 °C, and colonies were observed after 72 h. The colonies were then transferred to fresh plates, and the incubation process was repeated until pure cultures were obtained.

The identification of bacteria was based on their morphology, biochemical characteristics and analysis of the 16S rRNA gene. Cell morphology was observed by a transmission electron microscope (JEOL JEM-1230, Japan). The 16S rRNA gene was amplified from genomic DNA using universal bacterial primers F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and R1492 (5'-GGT TAC CTT GTT ACG ACT T-3') [16]. The PCR products were cloned into the pGEM-T Easy vector (Sangon, Shanghai, China) and sequenced. The sequence data of the closest relatives NCBI GenBank (http://www.ncbi.nlm.nih gov./BLAST) were aligned by ClustalX. A phylogenetic tree was then constructed using the neighbor-joining method with MEGA 7.0 software.

Substrate utilization tests

The isolated strain was inoculated into MSM containing 125 mg l^{-1} of one of the following substrates: DMP, DEP, DBP, DEHP, MBP, MMP, phthalic acid (PA), catechol and phenol as the carbon source to examine the ability of the strain to utilize these compounds. Substrate utilization was based on microbial growth as indicated by an increase in biomass ascertained by OD_{600} measurements.

Environmental factors effect on DBP biodegradation

The effect of different initial pH on DBP degradation was studied at initial pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. Meanwhile, the degradation of DBP at different temperatures was studied at different temperatures: 25, 30, 37, and 42 °C. The degradation efficiency was determined by HPLC.

Based on the optimal pH and temperature established above, the DBP degradation kinetics of strain was studied. The effect of initial DBP concentrations on the degradation was investigated between 62.7 and 627 mg l^{-1} and the degradation efficiency was determined by HPLC.

Degradation of mixed PAEs

To study the degradation ability of mixed PAEs, the isolated strains and PAEs (a mixture of DMP, DEP, DBP and DEHP (each at 125 mg l^{-1}) were incubated aerobically at 30 °C and 180 rpm on a rotary shaker for 48 h. As a control, medium inoculated with heat-killed cells was maintained at the same conditions. The degradation efficiency was determined by HPLC.

Analysis of DBP and its metabolites

The concentration of DBP was analyzed by HPLC (Agilent 1100 series, USA) equipped with a Diamonsil-C18 column (4.6 mm×250 mm×5 μm; Dikma Technologies Inc, China). A mixture of methanol and H_2O (90:10 by volume) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. The UV detector wavelength was 235 nm and the sampling quantity was 20 μl.

DBP degradation metabolites were identified using a Hewlett Packard 6890N gas chromatograph (Hewlett Packard, USA) equipped with an Agilent 5975C mass selective detector (Agilent, USA). The column used was a HP-5MS（30m×250 m×0.25 m） capillary column. The temperature program consisted of 1 min hold at 60 °C, an increase to 220 °C at 30 °C min^{-1}, and 2 min hold, an increase to 250 °C at 5 °C min^{-1}, and 2 min hold, an increase to 280 °C at 5 °C min^{-1}, and 3 min hold. The injection volume was 1 μl and the carrier gas was helium (1.0 ml min^{-1}). The mass spectrometer was operated at an electron ionization energy of 70 eV. Instrumental library searches, comparison with available authentic compounds, and mass fragmentation pattern were used to identify the metabolites.

Statistical analysis methodology

Each treatment in the experiment was performed in triplicate. Software Origin 8.0 was used to draw the figures with error bars.

RESULTS AND DISCUSSION

Identification and characteristics of strain ZJY capable of DBP degradation

A bacterial strain ZJY that could degrade DBP was isolated from river sludge of Hangzhou city. The strain had a short-rod shape, a size of (0.8-1.2) μm × (0.4-0.7) μm. It was a Gram-negative, non-sporulating, and non-pigmented bacterium without flagella. The morphological features of strain ZJY are shown in Fig. 1.

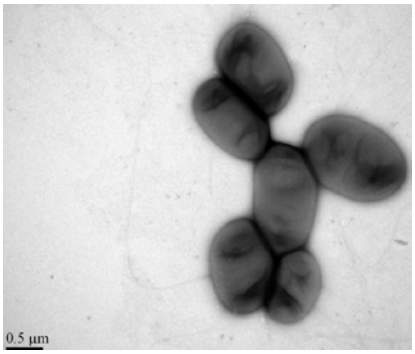

Fig. 1. Transmission electron micrograph of strain ZJY

Based on 16S rRNA gene sequence similarity, it was identified as a member of the genus *Raoultella* (GenBank accession no. KY317922.1). The phylogenetic relationship of strain ZJY with its close relatives is shown in Fig. 2.

Substrate utilization tests

The substrate utilization tests indicated that the strain ZJY was able to use PA, DMP, DEP, DBP, MBP and MMP as the sole carbon sources while no growth was observed in the presence of DEHP, catechol, phenol (Table 1). Members of the genus *Raoultella* are widespread in the nature and can be obtained from various sources such as plants, soil, and water. Although studies have shown that *Raoultella* species can degrade a variety of pollutants such as dimethoate, trinitrotoluene and petroleum [17,18], none prior to the present study have shown that *Raoultella* sp. degrades PAEs

Effects of pH and temperature on DBP biodegradation

The effect of pH on DBP degradation by *Raoultella* sp.ZJY is shown in Fig. 3A. The DBP degradation rate was the highest (about 95.7%) when pH was 7.0 after incubation for 48 h. When the pH was 5.0 and 9.0, the degradation rate was 9.8% and 54%, respectively, after incubation for 48 h. The highest DBP degradation rate was achieved at 30 °C (about 98%) after incubation for 48 h (Fig. 3B). When the temperature was 37 °C, 25 °C and 42 °C, the degradation rate was 81.8%, 49.8% and 6.7%, respectively, after incubation for 72 h.

Therefore, pH 7.0 and 30 °C were chosen for all subsequent experiments as the optimal conditions for DBP degradation by the strain ZJY.

Effect of the initial DBP concentration on DBP biodegradation

The degradation of DBP by *Raoultella* sp. ZJY was investigated at different initial concentrations (from 62.7 to 627 mg/l) (Fig. 4). DBP biodegradation by *Raoultella* sp. ZJY was assumed to fit the Monod first-order kinetic equation as follows: lnC=-Kt+A (where C is DBP concentration, t expresses time, K is the first-order rate constant and A is a constant). Table 2 shows the DBP degradation kinetic equations at different initial DBP concentrations. The results showed that when the initial DBP concentration was <627 mg l^{-1}, DBP biodegradation reaction fitted well with first-order kinetics. When the initial DBP concentration was 418 mg l^{-1}, the half-life of DBP was 40.81 h.

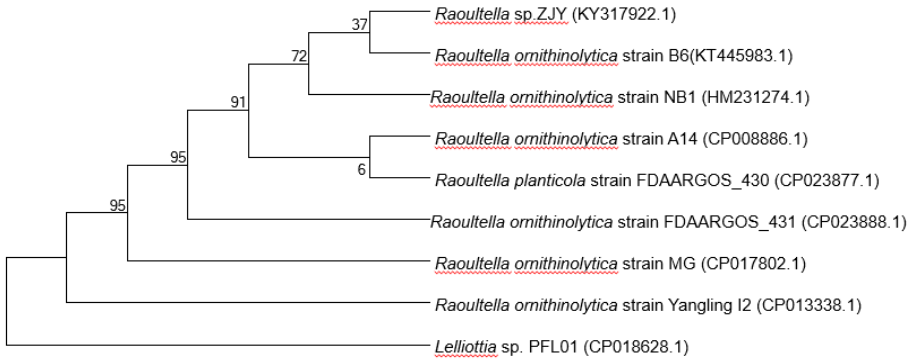

Fig. 2. Phylogenetic tree of *Raoultella* sp. ZJY.

Note: Distances were calculated using neighbor-joining method. The numbers at the branch nodes are bootstrap values (%). Accession numbers of the bacterial isolates are shown in brackets.

Table 1. Substrate utilization tests by *Raoultella* sp. ZJY

Substrates	DMP	DEP	DBP	DEHP	PA	MBP	MMP	Catechol	Phenol
Growth	+	++	++	-	+	+	+	-	-

++：means vigorous growth; +：means growth; −：means no growth

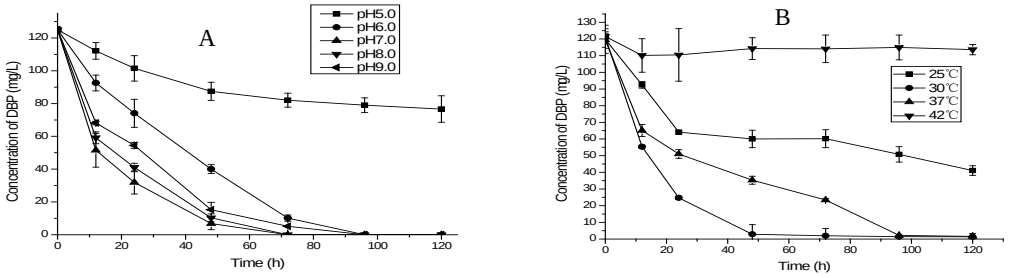

Fig.3. Effect of pH (A) and temperature (B) on degradation of DBP by *Raoultella* sp. ZJY.

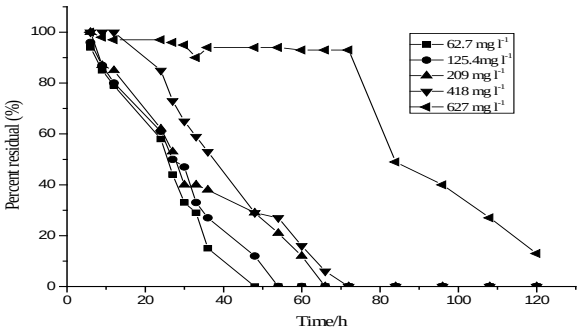

Fig. 4. Effect of different initial DBP concentrations on degradation by *Raoultella* sp. ZJY.

Table 2. Kinetics of aerobic DBP degradation by *Raoultella* sp. ZJY

Initial concentration /mg l^{-1}	Kinetic equations	$t_{1/2}$ /h
62.7	lnC =4.9264 -0.1413 t	22.13
125.4	lnC=5.0156-0.1203 t	24.25
209	lnC=5.0782-0.0862 t	27.02
418	lnC=5.2115-0.0634 t	40.81

Degradation of mixed PAEs

The degradation of a PAEs mixture consisting of DMP, DEP, DBP and DEHP (125 mg/l respectively) was performed (Fig. 5). The results showed that DBP was degraded very quickly, with more than 98.5% removed in 72 h. The degradation rate of DEP and DMP was 96% and 89%, respectively, after incubation for 72 h. This results indicated that PAEs with longer alkyl chains are easily degraded, while PAEs with shorter alkyl chains are harder to degrade. Previous studies have reported similar findings [19].

In the substrate utilization tests, no growth of strain ZJY was observed in the presence of DEHP as the sole carbon source. However in PAEs mixture, the degradation rate of DEHP was 10% after incubation for 72 h. This result might be due to the adsorption of microbial cells.

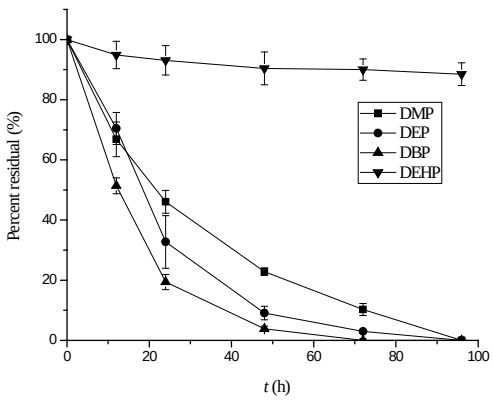

Fig.5. Degradation of mixed PAEs by *Raoultella* sp. ZJY

Biochemical degradation pathway

To explore the metabolic pathways for DBP degradation by *Raoultella* sp. ZJY, metabolites of DBP were identified by GC-MS. Four major metabolites besides the parent compound DBP (Fig. 6A), butyl-methyl phthalate (Fig. 6B), DMP (Fig. 6C) and PA (Fig. 6D) were identified. GC-MS analysis of the metabolites revealed that the initial step of dibutyl phthalate degradation was transesterification in the presence of CH_3OH. In the first step, butyl-methyl phthalate was formed by the first transesterification, and DMP was formed by the second transesterification, then followed hydrolysis of DMP to PA. The above results are similar to those previously reported [20]. Based on the above results, a conjectural metabolic pathway for the degradation of DBP by *Raoultella* sp. ZJY may be proposed (Fig. 7).

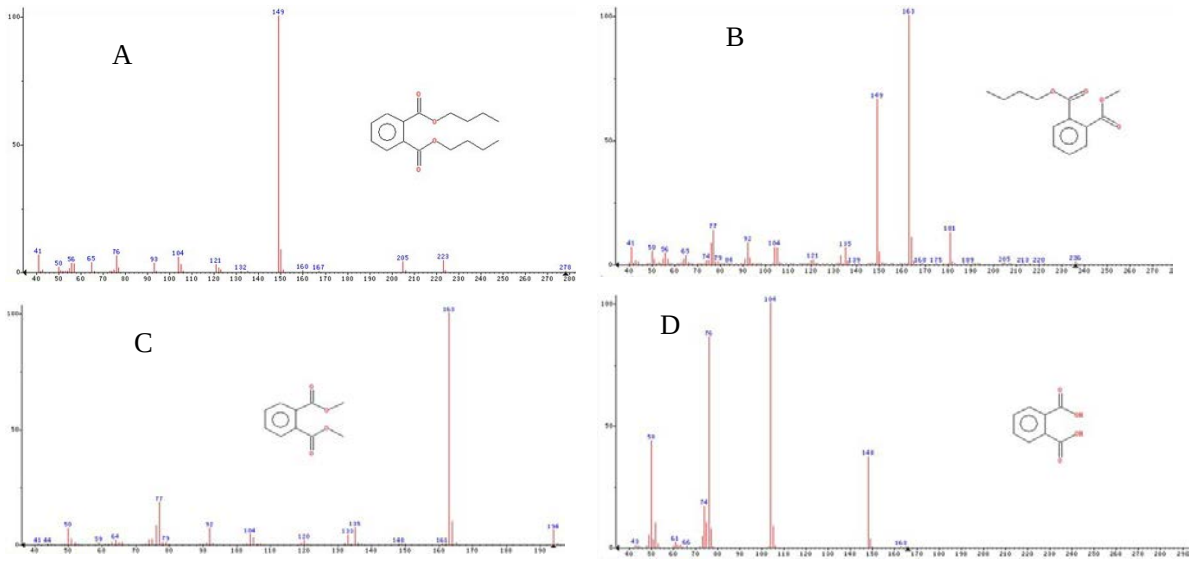

Fig. 6. GC-MS analysis during the degradation of DBP by *Raoultella* sp. ZJY

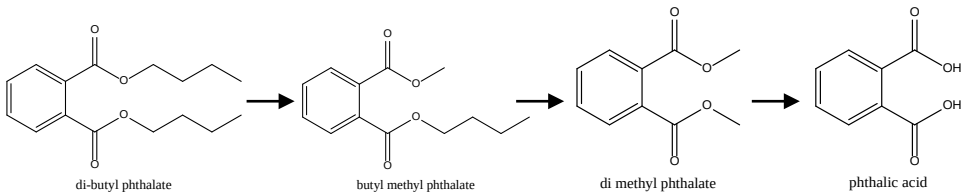

Fig. 7. The degradation pathway of DBP by *Raoultella* sp. ZJY

CONCLUSIONS

A bacterial strain ZJY that could degrade di-n-butyl phthalate was isolated from sludge of the river of Hangzhou city. Strain ZJY was identified as *Raoultella* sp. based on the analyses of morphological, physiological characteristics and 16S rRNA gene sequence. To our knowledge, this is the first report on the biodegradation characteristics of DBP by a member of the *Raoultella* genus. The optimal temperature and pH for dibutyl phthalate biodegradation were 30 °C and 7.0, respectively. The strain ZJY was able to use PA, DMP, DEP, DBP, MBP and MMP as the sole carbon sources. GC-MS analysis of the metabolites revealed that the initial step of dibutyl phthalate degradation was transesterification.

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REFERENCES

1. O. S. Fatoki, A. O. Ogunfowokan, *Environ. Int.*, **19**, 619 (1993).
2. Y. Y. Wang, Y. Z. Fan, J. D. Gu, *Int. Biodeter. Biodegr.*, **53**, 93 (2004).
3. J. L. Wang, P. Liu, Y. Qian, *Chemosphere*, **31**, 4051 (1995).
4. US EPA, Code of Federal Regulation, 40 CFR Part 136 (1992).
5. S. M. Duty, N. P. Singh, M. J. Silva, D. B. Barr, J. W. Brock, L. Ryan, R. F. Herrick, D. C. Christiani, R. Hauser, *Environ. Health Perspect.*, **111**,1164 (2003).
6. D. Jin, Z. Bai, D. Chang, D. Hoefel, B. Jin, P. Wang, D. Wei, G. Zhuang, *Journal of Hazardous Materials*, **221-222**, 80 (2012).
7. W. Wirongrong, S. Pareenart, N. Monnapa, T. Sirinthra, M. Skorn, L. Suvit, *International Biodeterioration & Biodegradation*, **99**, 45 (2015).
8. Y. Wang, B. Miao, D. Hou, X. Wu, B. Peng, *Process Biochemistry*, **47**, 936 (2012).
9. D. Jin, R. Liang, Q. Dai, R. Zhang, X. Wu, W. Chao, *J. Microbiol. Biotechnol*., **20**,1440 (2010).
10. J. Wu, X. Liao, F. Yu, Z. Wei, L. Yang, *Appl. Microbiol. Biotechnol.*, **97**, 2483 (2013)
11. S.W. Mary, M.E. Stephanie, S.B. Gertrud, X. Ye, J. S. Manori, C. Zhu, W. James, M. C. Antonia, *Environ. Health Perspect.*, **116**,1092 (2008).
12. R.W. Eaton, *J. Bacteriol.*, **183**, 3689 (2001).
13. R. L. Stingley, B. Brezna, A. A.Khan, C. E. Cerniglia, *Microbiology*, **150**, 2749 (2004).
14. P. Keyser, B. G. Pujar, R. W. Eaton, *Environ. Health Persp.*, **18**, 159 (1976).
15. S. E. Williams, E. M. Woolridge, S. C. Ransom, J. A. Landro, P. C. Babbitt, J. W. Kozarich, *Biochemistry*, **31**, 9768 (1992).
16. C. R. Fang, J. Yao, Y. G. Zheng, C. J. Jiang, L. F. Hu, *International Biodeterioration & Biodegradation*, **64**,442(2010)
17. Y. Liang, FH. Zeng, GZ. Qiu, XY. Lu, XD. Liu, *Biodegradation*, **20**, 363 (2009).
18. H. Claus, T. Bausinger, I. Lehmler, N. Perret, G. Fels, U. Dehner, J. Preuss, H. König, *Biodegradation*, **18**, 27 (2007).
19. B. V. Chang, C. M. Yang, C. H. Cheng, S. Y. Yuan, *Chemosphere*, **55**, 533 (2004).
20. Y. Okamoto, C. Toda, K. Ueda, K. Hashizume, N. Kojima, *Journal of Health Science*, **57**, 293 (2011).