Degradation of dibutyl phthalate and diethyl phthalate by indigenous isolate *Bacillus* sp. MY156

Yimin Xie, Xiaoyuan Guo, and *Hojae Shim**

Department of Civil and Environmental Engineering, Faculty of Science and Technology, University of Macau, Macau SAR 999078, China

Abstract. Dibutyl phthalate (DBP) and diethyl phthalate (DEP) are among the most extensively utilized plasticizers and widely exist in the environment causing adverse effect on human health. Previous studies on these phthalic acid esters (PAEs) biodegradation mainly involved individual substrates. However, these pollutants are more likely to appear as a mixture. This work explored the biodegradation of DBP and DEP and their interactions using the indigenous isolate *Bacillus* sp. MY156. The results demonstrate DBP could be completely degraded by strain MY156 with high efficiency, while DEP showed relatively lower removal efficiency (72%) in 120 h. The binary substrate removal results revealed the coexistence of DBP and DEP had no significant influence on the degradation of each under the optimal pH and temperature. Nevertheless, slight retardation of the removal efficiency for DEP observed in mixture condition suggested their removal was probably impacted by the substrate competition. The dehydrogenase activity assay illustrated the particular activities of dehydrogenases induced by PAEs with longer alkyl chains were higher than those of dehydrogenases induced by PAEs with shorter alkyl chains. The isolate *Bacillus* sp. MY156 showed a positive application potential for the bioremediation of PAEs-contaminated environment.

Corresponding author: Dr. H. Shim (hjshim@um.edu.mo)

1. Introduction

Phthalate acid esters (PAEs) are well known as plasticizers, increasingly used in the commercial products due to their properties of changing physical characteristics of polyvinyl chloride (PVC) for improving flexibility and durability of the polymer [1]. PAEs show ubiquity in various environments such as wastewater, seawater, soil, sediment, and vegetables [2-4]. DBP and DEP are among the most widely occurred and abundant PAEs existing in the environment [5]. Due to their negative effects as mutagens and carcinogens, these contaminants result in increasing concerns to human health [6]. They have also been identified as top priority contaminants by China National Environmental Monitoring Center, Environmental Protection Agency of the United States, and European Union.

DBP and DEP widely exist together in the environment. They can be degraded naturally by hydrolysis, photodegradation, and biodegradation. However, as a result of the inefficiency of long reaction period needed and risk for causing secondary pollution by intermediates of natural degradation, microbial degradation is considered as a highly efficient treatment for dealing with PAEs contaminated issues due to its economical and environmentally friendly characters during the remediation progress [7, 8]. Bacteria can achieve decomposition of organic contaminants and utilize them as carbon and energy sources. Most bacterial strains present extreme tolerance to pollutants and own a short growth period [9].

Generally, owing to deeply complex and recalcitrant characters, PAEs with long alkyl chains are harder to be treated compared with phthalates with short alkyl chains [10, 11]. In comparison, however, several researches have also proved some bacteria behaved priory removal efficiency when treating phthalates with long-side chains rather than the shorter ones [12, 13], even though there has been no exact explanation and clear understanding for this phenomenon. Enzyme activity could be identified as a solution for these unknown questions as it is related to the spatial steric resistance generated by the side chain rather than to the length of side chains [14].

In current study, we aimed to obtain an indigenous strain isolated from the activated sludge with degrading ability in treating phthalates contaminants. The optimal reaction conditions and metabolic pathways for degradation of DBP and DEP were also investigated. For a clear exploration of the interaction mechanism for degradation, the dehydrogenase activity was examined.

2. Materials and methods

2.1 Chemicals

DBP and DEP, methanol, and acetonitrile were purchased from Aladin. These chemicals were of highest purity and analytical grade. Other chemicals such as *n*-hexane and Tween 80 were of analytical grades. Serum bottles and solutions used were sterilized at 121°C for 20 min before use.

2.2 Bacterium and medium

The strain was enriched and isolated from activated sludge collected at the aeration tank of a regional wastewater treatment plant. The sludge suspension (5 mL) were first added in

Luria-Bertani (LB) medium (10 g/L peptone, 10 g/L NaCl, 5 g/L yeast extract). After LB medium, 5 mL were serially transferred into basal salt medium (BSM) containing the increasing concentration of DEP and DBP (10-600 mg/L). BSM consisted of 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), with pH adjusted at $7\pm$ 0.2 by HCl or NaCl [15]. Serum bottles (150 mL) were then incubated on shaker at 150 rpm and 30°C. Tween 80 was utilized to enhance the solubility of DEP or DBP in the reaction system.

2.3 Isolate identification

The isolated strain was analysed via 16S rRNA gene sequence analysis. The universal primer for 16S rRNA gene amplification was 27F (5'-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The purification was conducted via sepharose gel before sequence analysis. The PCR recycled production was sent to Genewiz Inc. for automatic two-directional sequencing analysis. The sequence similarities were recognized via BLAST and have been submitted in NCBI GenBank with the accession number of SUB10877930.

2.4 Analytical methods

For the analysis of residual PAEs, 1 mL of the solution was collected at 12-h interval for DBP and 24-h interval for DEP, respectively. Samples were first filtered through 0.22 µm membrane filters (Millipore®), followed by the *n*-hexane extraction thrice. Then, it was dried using nitrogen gas and dissolved in methanol to a final volume of 0.5 mL for the HPLC analysis. The concentration of DBP and DEP were detected using Dionex UltiMate 3000 HPLC (Thermo Scientific, U.S.A.) equipped with diode array detector and AcclaimTM C18 reversed-phase column (5 mm, 4.6x150 mm). Acetonitrile and deionized water (pH adjusted to 3 using formic acid) were utilized as mobile phase (7:3 and 5:5 for DBP and DEP, respectively) and the injection volume was 20 µL. The ratio of them were. The flow rate and column oven temperature were set at 0.5 mL/min and 35°C.

The inhibitory effect of substrate was estimated for the biodegradation kinetics parameters for the isolate as follows [16].

$$D = \frac{D_m S}{K_s + S + S^2/K_i} \tag{1}$$

$$\mu = \frac{\mu_m S}{K_s + S + S^2/K_i} \tag{2}$$

Where D (mg/L/d) and D_m (mg/L/d) represent degradation rate and maximum degradation rate, respectively, and μ (day^{-1}) and μ_m (day^{-1}), specific growth rate and maximum specific growth rate, respectively, S (mg/L), the concentration of substrate, and K_s (mg/L) and K_i (mg/L), half-saturation constant and inhibition constant, respectively.

Analysis of dehydrogenase activity was in terms of measurement of reaction product from 2,3,5-triphenyl-2H-tetrazolium chloride [17]. Micro-morphology of the strain was observed via scanning electron microscope (Hitachi SU8020, EMAXevolutin X-Max80).

2.5 Experimental setup

For the effects of different pHs (4-10), 2% (v/v) inocula were transferred to the bottles containing 100 mg/L substrate. All the bottles were sealed using stoppers (90% Teflon/10% silicone; Ohio Valley Specialty, U.S.A.) and aluminous crimp. Due to the fast degradation of DBP by the isolate, the concentration of DBP was examined after 3 days of the inoculation, while DEP was analysed after 5 days reaction. The control was prepared without inoculum under same conditions.

For the degradation kinetics, different concentrations (50-500 mg/L) of DBP and DEP were employed, while for the effect of temperature, three different temperatures (25, 30, and 40°C) were tested at 300 mg/L DBP and 100 mg/L DEP. For the effect of mixture, the same condition (300 mg/L DBP and 100 mg/L DEP) was set. The concentrations of PAE were determined in 12 h for DBP and 24 h for DEP interval for 120 h, and the bacterial growth was monitored as optical density at 600 nm wavelength (OD_{600}).

3. Results and discussion

3.1 Isolation and characterization of PAEs-degrading strain

After enrichment for 6 weeks, several PAEs-degrading strains were isolated from the activated sludge, and the strain that showed significant ability in degrading DBP and DEP was selected for further experiments. The isolate designated as MY156 was aerobic, Gram positive, and appeared white and rod shaped with approximately 3 mm diameter. The 16S rRNA sequencing analysis revealed the strain isolated belongs to *Bacillus* sp. with the highest (greater than 99.86%) genetic relationship with *Bacillus subtilis*. Most microorganisms with positive responses to PAEs have been reported belonging to the genera of *Sphingomonas, Rhodococcus, Arthrobacter, Bacillus*, and *Flavobacterium*.

3.2 Biodegradation of single PAE

The concentrations of DBP and DEP without inoculum did not show any significant changes ($p > 0.05$), indicating the abiotic loss was negligible. After a short lag phase, the isolate MY156 showed efficient degradation ability (75-100% removed) of DBP and DEP when the concentration below 200 mg/L within 5 days. The degradation kinetics for DBP and DEP at different initial concentrations were fitted by the inhibition model (Table 1). DBP at 400 mg/L, there was an increasing growth trend but with a decrease for the growth at higher than 400 mg/L (Fig. 1), and similar to DEP, the inhibition effect appeared at 300 mg/L.
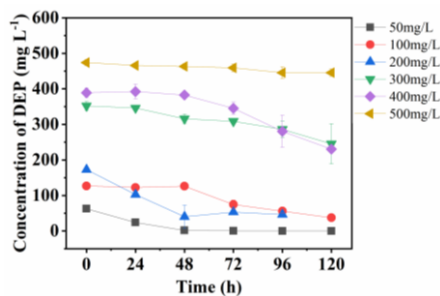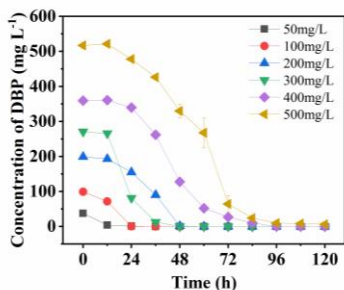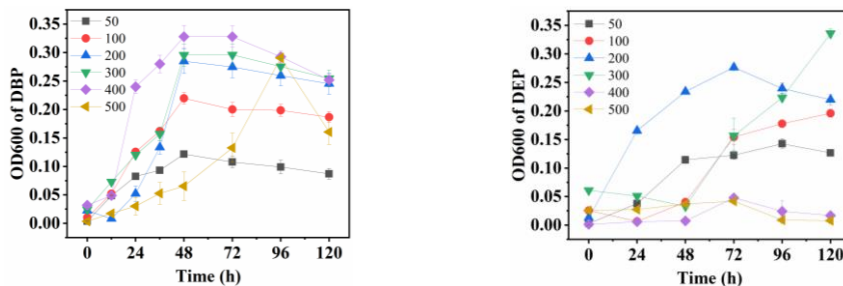
Fig. 1. Biodegradation of DBP and DEP and cell growth.

Table 1

Biodegradation kinetics parameters estimated for the isolate grown on DBP or DEP.

|	D_m (mg/L/d)	μ_m (d^{-1})	K_s (mg/L)		K_i (mg/L)	
			Degradation	Growth	Degradation	Growth
DBP	135.5	0.13	112.5	56.2	800	1,601.42
DEP	38.3	0.10	40.2	35.5	249	281.69

3.3 Biodegradation of binary PAEs

3.3.1 Biodegradation of DBP/DEP mixture

Most previous studies have been focusing on degradation of PAE as single substrate. In comparison, however, most PAEs co-exist in the environment, affecting microbial degradation capacity. MY156 showed preference of DBP as substrate, and the addition of other PAE may lead to inhibitory effect compared to when only single contaminant present [18]. Figure 2 shows biodegradation of DBP and DEP when present singly and in mixture. Due to the influencing factors such as energy and growth status, enzyme activity, the residual nutrient and substrate as well as oxygen in the reaction system, the degradation rate decreased with time [19].

3.3.2 Dehydrogenase activity

Enzyme activity can be utilized as a general measure of bacterial growth and reflect the influence of PAEs on microbial activities [20]. Dehydrogenase is generally used to describe the microbial activity and its ability to utilize organic substance [17]. The dehydrogenase activities for individual DBP and DEP were lower than the mixture during the log phase of MY156 growth (Fig. 2; $p < 0.05$). The dehydrogenase activity is generally analyzed for the general bacterial activities and the utilization of organic substance. The extra carbon source might increase the dehydrogenase activity [21]. This result shows the dehydrogenase

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activity was promoted with the increase of PAE concentration and the substrate type involved.
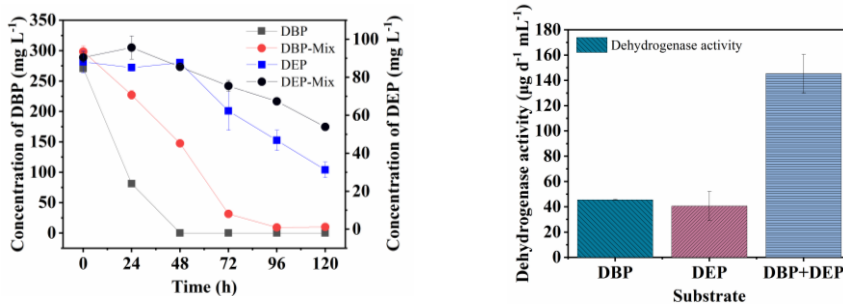
Fig. 2. Bioremoval of DBP and DEP (singly and in mixture) and dehydrogenase activity after 48 h.

3.3.3 Micro-morphology

SEM shows MY156 was rod shape and longer in DBP than in DEP, and several cells became wrinkled on the surface and shrunk of the volume in DEP (Fig. 3). It might be due to the change of cell morphology to protect important cellular elements [22]. Meanwhile, the cell structure will change accordingly to increase the contaminant contact area, leading to higher biodegradation efficiency of *Bacillus* in PAEs added treatment [23].

(a) Scanning electron micrograph of strain MY156 in DBP.

(b) Scanning electron micrograph of strain MY156 in DEP.

(c) Scanning electron micrograph of strain MY156 in mixture of DBP and DEP.

Fig. 3. Morphological characteristics of *Bacillus sp*. MY156 under scanning electron microscope (×10,000; Scale bar, 200 nm)

3.4 Effects of pH and temperature

At the initial concentration of 300 mg/L DBP and 100 mg/L DEP, the results showed the optimal pH for biodegradation of individual PAE was 7, while the efficient degradation occurred under the alkaline condition (Fig. 4). The initial pH has been reported significant for PAEs degradation, considering pH can affect microbial activity, contaminant binding, and hydrolase activity [24]. The biodegradation activity has been reported closely related to pH due to its influence on sorption ability of PAEs and catalytic enzyme activity [25].

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On the other hand, the biodegradation efficiency for DBP at 30°C and 35°C was 24.4% and 21.7% higher than at 25°C, respectively, while the degradation efficiency for DEP at 30°C and 25°C was 25.0% and 14.5% higher than at 40°C, respectively (Fig. 4). Since high temperature would affect the growth and degradable ability of the strain, mainly affecting the denaturation or deactivation of microbial enzymes, the degradation of PAEs is reduced as the temperature enhanced to 40°C [25]. Therefore, the optimal temperature for biodegradation of DBP and DEP was considered 30°C.
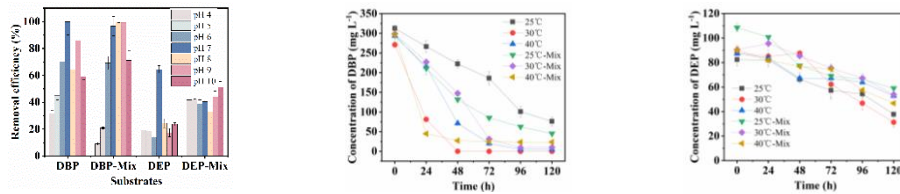

Fig. 4. Effects of pH and temperature on PAEs biodegradation.

4. Conclusion

The indigenous isolate *Bacillus* strain MY156 was capable of degrading DBP and DEP with significant removal efficiencies. MY156 showed a substrate preference on DBP compared to DEP. The biodegradation rate for DEP singly was higher than in mixture with DBP, implying DBP with inhibitory effect on DEP degradation. The enzymatic activities results further showed the dehydrogenase was stimulated by PAEs. The micro-morphology of strain MY156 revealed the damage on cell structure caused by DEP can be reduced by adding DBP.

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