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Biodegradation of di-n-butyl phthalate by an isolated *Gordonia* sp. strain QH-11: Genetic identification and degradation kinetics

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ABSTRACT

Di-n-butyl phthalate (DBP) is one of the most widely used phthalic acid esters (PAEs), which have shown increasing environmental concerns worldwide. A bacterial strain designated as QH-11, was isolated from activated sludge and found to be capable of utilizing DBP as carbon and energy sources for growth. 16S rRNA and *gyrb* gene sequence analysis revealed that strain QH-11 was most closely related to *Gordonia* sp. Kinetics studies of DBP degradation by the strain QH-11 revealed that DBP depletion curves fit with the modified Gompertz model ($R^2 > 0.98$). Meanwhile, substrate utilization tests showed that strain QH-11 could utilize other common PAEs and also the main intermediate product phthalic acid (PA). A gene encoding the large subunit of the phthalate dioxygenase, which is responsible for PA degradation, was successfully detected in strain QH-11. Furthermore, the results of reverse transcription quantitative PCR demonstrate that mRNA expression level of phthalate dioxygenase increased significantly after strain QH-11 was induced by DBP and PA.

1. Introduction

Phthalic acid esters (PAEs) are a group of chemicals widely used in the manufacture of plastics and many other industrial and consumer products [1]. Due to their widespread application and the increasing amounts of phthalates discharged into various ecosystems (including river, ground and drinking waters, open oceans and lakes), significant environmental and human health concerns now exist [2]. In fact, as a result of the mutagenicity, teratogenicity, and carcinogenicity of PAEs [3,4], di-n-butyl phthalate (DBP) and other phthalates have now been listed as priority pollutants by both China National Environmental Monitoring Centre [5] and the US Environmental Protection Agency [6]. Human exposure is widespread where phthalates are commonly found in the blood, tissue, breast milk, and urine of many people [7]. Among the various PAEs, DBP is one of the most widely used phthalates, which have shown a rapid growing application and consumption worldwide [8].

As the hydrolysis, photolysis and volatilization of PAEs occur very slowly, microbial degradation is believed to be a promising removal process for PAEs in aquatic and terrestrial environments. Previously, a number of PAEs-degrading bacterial strains have been isolated and characterized from various habitants [1], and the rate of PAEs degradation in the absence of oxygen is much slower than under aerobic conditions [9,10]. Recently, the actinomycete *Gordonia* has been found to play an important role in degrading environmental pollutants [11] and researchers have identified *Gordonia* strains which can metabolize these PAEs as sole sources of carbon and energy, including those which were isolated from river sludge [12], creosote-contaminated soil [13], and sediment [14]. To our knowledge, there is no report that *Gordonia* strains with the capacity to mineralize PA, which is the major intermediate product during PAEs degradation. In addition, there appears to be no investigations reporting the quantification of phthalate dioxygenase expression in *Gordonia* sp.

This study reports the aerobic degradation of selected phthalate esters by a pure culture of *Gordonia* strain QH-11 which was isolated from activated sludge. A kinetic model of DBP degradation and transformation was proposed, and a comparison of phthalate dioxygenase expression levels within strain QH-11 was performed using PA, DBP and glucose as sole carbon and energy source. These results allow for a better understanding of the mechanism of PAEs degradation and metabolic diversity of the genus *Gordonia* in response to PAEs degradation.

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2. Materials and methods

2.1. Chemicals

Dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate, di-n-octyl phthalate (DOP), di(2-ethylheyl) phthalate (DEHP), and diisooctyl phthalate (DIOP) were purchased from Alfa Aesar (Ward Hill, MA), with a purity greater than 98%. Methanol, ethyl acetate and hexane were of high-performance liquid chromatography grade (Dikma Technologies Inc., Beijing). All other chemicals and solvents were of analytical reagent grade.

2.2. Isolation and identification of bacteria

Activated sludge obtained from a wastewater treatment plant with anoxic–anaerobic–oxic process in Beijing was used as a source of PAEs degrader. The enrichment and isolation process was conducted as described by Jin et al. [15]. Pure cultures were phylogenetically characterized using 16S rRNA and *gyrb* gene sequencing. Combinations of universal primers 27f/1492r [16] and UP-1/UP-2r [17] were used for PCR amplification of the 16S rRNA and *gyrb* genes, respectively. The PCR thermal cycling consisted of an initial denaturation at 95 °C for 10 min, followed by 35 cycles at 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 90 s, plus a final step at 72 °C for 10 min. Both PCR products were cloned and sequenced. The obtained sequences were subjected to BLAST homology search (http://www.ncbi.nlm.nih.gov/BLAST).

2.3. Substrate utilization experiments

The isolated bacterial strain was inoculated in liquid mineral salts medium (MSM) containing 200 mg/l of following substrates: DMP, DEP, DBP, DOP, DIOP, DEHP, mono-n-butyl phthalate (MBP), PA, and protocatechuic acid (PCA), respectively. Substrate utilization was assessed by microbial growth by measuring the increase of the biomass (OD_{600}) combined with visible turbidity after 36 h of incubation. For each substrate, liquid medium without inoculation was used as a negative control.

2.4. Degradation experiments

The isolated strain was grown on an enriched medium (1.0% peptone, 0.5% beef extract, and 0.5% NaCl) for 24 h at 30 °C on a rotary shaker (150 rpm). Then cells were harvested and washed three times with a 0.02 mol/l phosphate buffer (pH7.0). The washed cells were re-suspended in the same buffer, resulting in a cell suspension with an OD_{600} of 0.2. In addition, a stock solution of DBP (10,000 mg/l) was prepared by dissolving the DBP in methanol. After transferring appropriate volume of this stock solution to a 50 ml sterile flask, 20 ml of sterilized MSM was added until the methanol was completely volatilized. 1 ml of the prepared cell suspension was then inoculated into the medium for biodegradation assessment. All tests were conducted in triplicate.

2.5. Molecular detection of phthalate 3,4-dioxygenase gene

The phthalate 3,4-dioxygenase gene was amplified using the degenerate primers as described by Jin et al. [15], the reference strains for primers designed including *Arthrobacter keyseri*, *Terrabacter* sp. DBF63, *Rhodococcus* sp. TFB, *Rhodococcus* sp. DK17, *Rhodococcus* sp. RHA1, and *Mycobacterium vanbaalenii* PYR-1. The PCR mixture consists of 5 μl of the 10× PCR buffer (Mg^{2+} plus), 1 μl dNTP (10 mmol), 1 μl of each primer, 0.5 μl *Taq* polymerase, and 100 ng of the DNA extraction product. Double-distilled water was added to a final volume of 50 μl. The PCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, plus a final step at 72 °C for 10 min. The PCR product was purified and cloned into pGEM-T vector (Promega, Switzerland). The recombinant plasmids were then transformed into *E. coli* DH5α. The representative positive clones were selected for sequencing (Tsingke, Beijing).

2.6. Real time quantification of phthalate 3,4-dioxygenase gene expression in strain QH-11

Bacterial strain QH-11 was transferred to fresh liquid medium (20 ml) that contained 500 mg/l of DBP and PA. MSM containing 1000 mg/l of glucose was used as control. Total RNA was extracted at early logarithmic phase of bacterial growth (at 30 h, 25 h, and 30 h, respectively) using RNAprep Cell/Bacteria Kit according to the manufacturer's protocol (Tiangen, Beijing, China). RNA purity was quantified using a NanoDrop-1000 (NanoDrop Technologies, Wilmington, USA) as well as native agarose gel electrophoresis. Only samples with A260/A280 ratio greater than 1.8 were used. Reverse transcription of 1 μg of RNA was performed using Promega Reverse Transcription System. The qPCRs were performed using the Promega GoTaq qPCR Master Mix using the Mx4000 (Stratagene) real-time thermocycler, according to the company's instructions. Primers used for PCR amplification of phthalate 3,4-dioxygenase gene were 5′-CGGTCGCATCATCGGTCTT-3′ as the forward primer and 5′-CCCTTGCTCTGCTTGGTGTA-3′ as the reverse primer, amplifying a 209 bp fragment. For 16S rRNA gene, the forward primer 5′-TACCTGGAGAAGAAGCACCG-3′ and the reverse primer 5′-CTCAAGTCTGCCCGTATCG-3′ were used, amplifying a 170 bp fragment. The following conditions were used for PCR: 94 °C for 10 min, followed by 45 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. 16S rRNA gene of strain QH-11 was used as the control gene. Relative phthalate 3,4-dioxygenase gene expression was determined using the ΔΔCt method [18].

2.7. Analysis of DBP and its metabolites

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. The aqueous and organic phases were separated by centrifugation at 12,000 rpm 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 mm × 200 mm × 5 μm) was used for the separation, and the UV wavelength was 228 nm. For DBP, the mobile phase consisted of a methanol:water solution (90:10, v/v), for PA, the mobile phase was methanol:1% acetic acid water (40:60, v/v), and both the flow rate were 0.5 ml/min. All the tests were conducted in triplicate.

The metabolites were analyzed using GC/MS under the following conditions: DB-5MS (60 m × 0.25 mm × 0.25 μm) column, helium carrier gas at flow rate of 1.0 ml/min, injection temperature of 260 °C, and transfer line temperature of 280 °C. The oven temperature was programmed from 80 to 280 °C. The effluent from the GC column was directly connected to the MS, while the spectra were measured in the electron impact (EI) ionization mode at 70 eV and scanned at 50–550 amu for 2 ms. The scans collected for the metabolites were identified by comparing the results with the mass spectra library in the MS system.

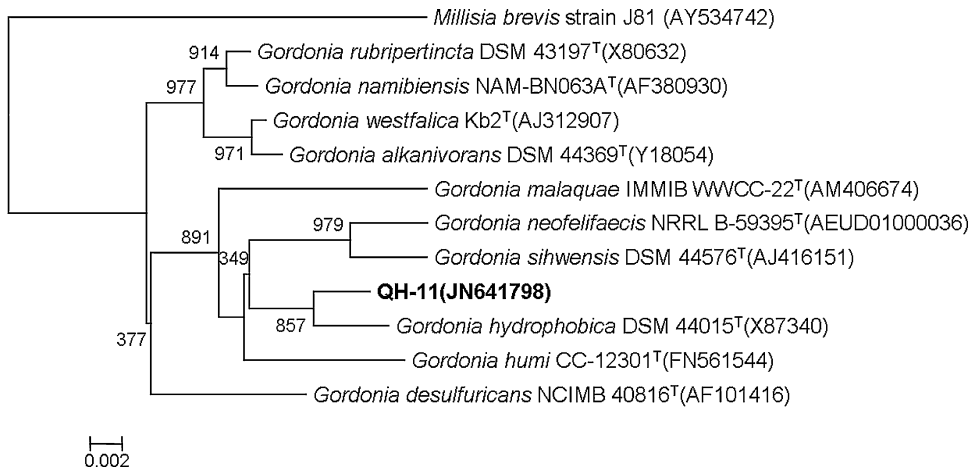

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence of QH-11 and sequences of related species. Distances were calculated using neighbor-joining method. Numbers at branch points are bootstrap values (based on 1000 samplings). *Millisia brevis* strain J81 (AY534742) was used as out-group. Scale bars represent 0.002 substitutions per site.

3. Results

3.1. Isolation and characterization of strain QH-11

Following 10 weeks enrichment, several DBP-degrading strains were isolated from the activated sludge. One strain shown to utilize DBP and PA was designated as QH-11 and selected for further investigation. QH-11 was a Gram-positive bacterium that formed white colonies when grown on LB agar. Phylogenetic of the 16S rRNA gene (Fig. 1) revealed strain QH-11 clustered with members of the genus *Gordonia*, and had a 99% sequence similarity with *Gordonia hydrophobica*. In addition, the results from the amplification of *gyrb* sequences also indicated that strain QH-11 belongs to genus *Gordonia*. However, a lower level of similarity (less than 90%) was revealed between the *gyrb* gene of QH-11 (JN641799) and the *gyrb* gene sequences deposited in the NCBI (date not shown). The combination of 16S rRNA and *gyrb* gene sequence data suggest that strain QH-11 belonged to genus *Gordonia* sp., but may represent a new species.

3.2. Utilization of other PAEs and common metabolites

After 36 h incubation in MSM containing supplemented substrates, strain QH-11 showed a wide range utilization capacity of other PAEs with three common degradation metabolites (MBP, PA and PCA) (Table 1). Phthalate monoester and phthalate were found to be the most common intermediates from PAEs degradation, and both have important toxicological implications. This broad-spectrum utilization suggests that the *Gordonia* sp. QH-11 had evolved a complete degradation pathway and could be used as a potential candidate for remediation of PAEs contaminated-sites.

3.3. Effects of pH, temperature and initial concentrations on DBP degradation by QH-11

The effects of pH and temperature on the aerobic degradation of DBP in the culture medium were tested after incubation 24 h. The results showed that the optimal conditions for DBP degradation was pH 7.0 and 30 °C. No significant differences were observed when strain QH-11 was incubated at pH 7.0 and 8.0, 30 °C and 35 °C (data not shown). Therefore, pH 7.0 and 30 °C were chosen for all subsequent experiments. At the same time, control experiments were carried out under the same conditions without bacteria. No obvious degradation was detected in the control experiments. The recovery rates of DBP after sample pretreatment were measured between 96% and 105%.

In order to determine the effect of initial DBP concentrations on degrading efficiency, biodegradation of DBP by *Gordonia* sp. QH-11 was conducted under DBP concentrations ranging 100, 200, 300, 500 and 750 mg/l. The changes in concentrations of DBP with time are shown in Fig. 2 with the modified Gompertz model [19,20]. The modified model can be expressed as

$$S = S_0 \left\{1 - \exp\left[-\exp\left(\frac{eR_m}{S_0}(t_0 - t) + 1\right)\right]\right\}$$

where S is the substrate concentration (mg/l); S_0 is the initial substrate concentration (mg/l); R_m is the maximum substrate transformation rate (mg/l/h); t_0 is the lag phase time (d), and t is the incubation time (h).

Fig. 2 reveals that the substrate depletion curves were described well by the modified Gompertz model. An apparent lag phase was observed during DBP degradation, however, DBP can be rapidly degraded by *Gordonia* sp. QH-11 after lag phase. No residues were detected within 45 h at all of the concentrations tested.

Table 2 shows the DBP degradation kinetics parameters associated with the degradation trails. Lag phase prolonged from 6.44 h

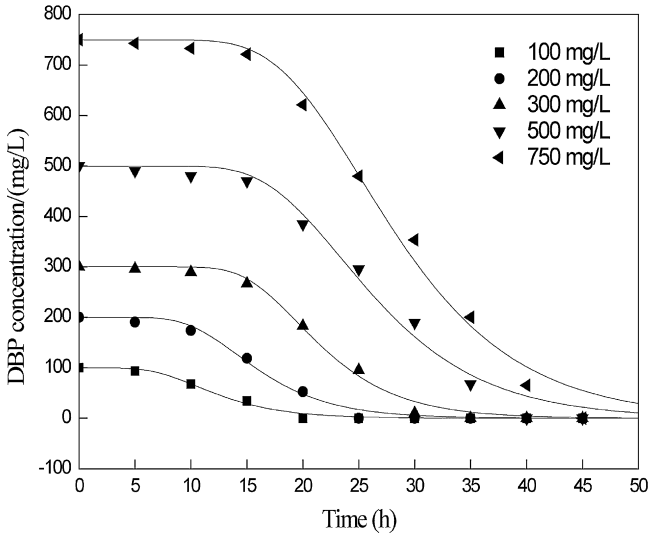

Fig. 2. Best-fitted curves for DBP degradation using modified Gompertz mode at different initial concentrations by *Gordonia* sp. QH-11.

Table 1
Substrate utilization profile for strain QH-11.

Substrate	Utilization	Substrate	Utilization	Substrate	Utilization
DMP	+	DOP	++	Phthalic acid	++
DEP	++	DIOP	+	Protocatechuic acid	+
DBP	++	DEHP	+	Benzoic acid	++

(++) Vigorous growth; (+) growth; the concentration of each substrate was 200 mg/l.

Table 2
Comparison of calculated maximum transformation rate, lag phase and the correlation coefficient using the modified Gompertz model at different initial DBP concentrations.

Initial concentration (mg/l)	t_0 (h)	R_m (mg/l/h)	R^2
100	6.44	8.93	0.980
200	9.56	16.18	0.984
300	14.66	22.63	0.989
500	16.54	26.53	0.987
750	17.51	35.87	0.990

to 17.51 h with the increase in DBP concentrations. The maximum transformation rate (R_m) followed the same trend as the lag phase, increasing from 8.93 mg/l/h to 35.87 mg/l/h. Based on these data, the degradation rate of DBP was dose-dependent and the initial concentration of DBP may play an important role in the degradability of DBP by *Gordonia* sp. QH-11.

3.4. Biodegradation of DBP and PA by Gordonia sp. QH-11

Fig. 3 shows that 500 mg/l of PA could be rapidly degraded by QH-11 in the 10 h following a lag phase of 20 h. In addition, the amount of PA accumulated increased with the DBP degradation (500 mg/l). The maximum PA concentration peaked at 30 h. However, no PA was detected at 40 h. Based on these results, it could be concluded that the PA was transformed very quickly once the respective enzyme was activated.

3.5. Molecular detection and quantification of phthalate 3,4-dioxygenase gene expression in strain QH-11

It is generally accepted that phthalate 3,4-dioxygenase is responsible for PA transformation in Gram-positive bacteria. In our study, a fragment of 894 bp of the phthalate dioxygenase gene was cloned from strain QH-11. The sequence analysis showed that the obtained fragment was most homologous with the nucleotide sequences of phthalate 3,4-dioxygenase, the similarity between strain QH-11 and reference strains for primers designed was from 80% to 84%. A phylogenetic tree (Fig. 4) was constructed based on deduced amino acid sequence with a close similarity. As shown in Fig. 4, strain QH-11 is most closely clustered with the large subunit of the phthalate dioxygenase of *A. keyseri* (84% identity).

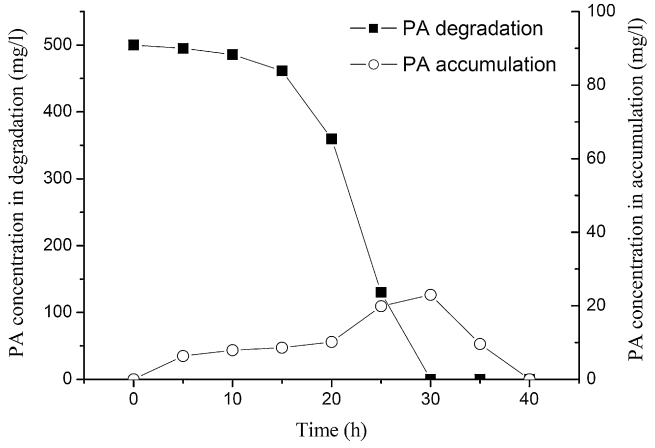

Fig. 3. PA degradation and accumulation during DBP degradation by QH-11.

In order to test whether or not phthalate 3,4-dioxygenase is induced in *Gordonia* sp. QH-11, strain QH-11 was cultivated in MSM containing DBP (500 mg/l), PA (500 mg/l) and glucose (1000 mg/l). The phthalate 3,4-dioxygenase expression levels at the logarithmic phase were compared, and the results are shown in Table 3. When cells grow on DBP and PA, the mRNA expression level of phthalate 3,4-dioxygenase were 43-fold and 248-fold higher than that of glucose, respectively. As PA is a major metabolic product of DBP degradation, this result strongly suggests that phthalate 3,4-dioxygenase expression level could be significantly enhanced after PA stimulation.

3.6. Identification of metabolites

To determine the metabolites or intermediates formed by *Gordonia* sp. QH-11, the concentrated samples were analyzed by GC/MS. Two intermediate products, MBP and PA were detected by comparing their mass spectra with the published mass spectra from NIST (National Institute of Standards and Technology) database. The major mass spectrometry of DBP and its two intermediate products can be found in Table 4. The biochemical pathway of DBP degradation was proposed to be through MBP and PA before cleavage of the aromatic ring structure.

4. Discussion

Environmental pollution is a major problem for the global community. Many of the microorganisms that have been identified as capable of metabolizing pollutants dwell in soils, sediments and waters. Bioremediation has become a major route in reducing environmental pollution. Among these bacterial strains, species of the genus *Gordonia* are known as degraders of recalcitrant pollutants. These bacteria can grow in unfavorable environments, making them capable of living in a wide range of environmental niches. The members of the taxa have been proposed as tools for biotechnical applications such as bioremediation and biosynthesis [21]. At the time of this study, the genus *Gordonia* comprised 29 species, and most species were isolated due to their abilities to degrade environmental pollutants [11,22]. In the present study, a bacterial strain capable of utilizing PAEs as the sole source of carbon and energy was isolated from activated sludge sample, and then was identified to the genus *Gordonia* based on 16S rRNA and *gyrb* gene sequence analysis. 16S rRNA gene sequence analysis of strain QH-11 showed highest similarity (99%) with that of *G. hydrophobica*. However, *gyrb* gene showed a low similarity between strain QH-11 and *G. hydrophobica* (89%). Previous studies have revealed that many *Gordonia* type strains with more than 97% similarity over the 16S rRNA gene yield DNA reassociation values of less than 60% [11]. This indicates that strain QH-11 is possibly a new species of the genus *Gordonia*. To our knowledge, there is no reported information on DBP degradation by *Gordonia* strain from activated sludge. And the optimum pH and temperature for DBP degradation by strain QH-11 are quite similar to many reported strains [15,23].

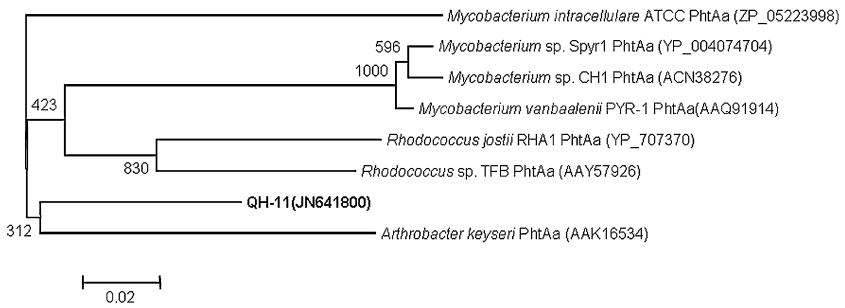

Fig. 4. Phylogenetic tree of phthalate 3,4-dioxygenase of *Gordonia* sp. QH-11.

Table 3
Relative quantitation using the comparative C_T method.

Substrate	Target average Ct	16S rRNA average Ct	ΔC_T target-16S rRNA	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T,-A}$	Rel. to glucose
Glucose	35.42	14.76	20.66	0.00	1
DBP	25.56	10.30	15.26	−5.40	43.1
PA	23.95	11.26	12.69	−7.97	248.0

There are also other studies on biodegradation of DBP by pure bacterial cultures, and kinetic model has been developed to describe the biodegradation process for some of these strains. For instance, Li et al. [23] reported that the DBP degradation by *Rhodococcus rubber* can be described as a first-order kinetics model when the initial DBP concentration was lower than 50 mg/kg, Xu et al. [24] also reported degradation of DBP by *Pseudomonas fluorescens* B-1 conformed to the first-order kinetic model. Recently, DBP degradation data by *Rhodococcus* sp. L4 was found to fit well to the same exponential model at concentrations varying 150–600 mg/l [2]. Fang et al. [8] reported DBP degradation profiles by *Enterobacter* sp. T5 also followed the first-order kinetic model. Wu et al. [14] used four *Gordonia* strains to degrade DBP. The degradation kinetics from these strains can be assumed to conform to the first-order kinetic equation. Similar results have also been shown by several other researchers [25,26]. In the present work, however, we found that DBP depletion curves by strain QH-11 fitted well to the modified Gompertz model, which is often used to estimate degradation curves for other PAEs [27,28]. This observation is in contrast to the results from all previous studies which reported DBP-degrading strains, including four strains of *Gordonia*. Therefore, it seems that the kinetic model of individual PAEs biodegradation were not associated with the compound structure, but a function of the strain's character.

It is well known that that the primary biodegradation of phthalates entails the sequential hydrolysis of the ester linkage, involving the formation of a monoester followed by PA. PA is a central intermediate in the biodegradation of all phthalate esters, where the key step is the hydroxylation of the aromatic ring by a ring-hydroxylating dioxygenase. In the case of Gram-positive bacteria, PA is degraded via 3,4-dihydroxyphthalate and protocatechuate by phthalate 3,4-dioxygenase [1]. Meanwhile, there are also some reports describe complete degradation of PAEs require more than one bacterial species, In one such report by Li and Gu [29], dimethyl isophthalate (DMI) was mineralized only in the presence of both *Klebsiella oxytoca* Sc and *Methylobacterium mesophilium* Sr, and all of reported *Gordonia* strains could not utilize PA as a carbon source. The first strain belonging to genus *Gordonia* (strain MTCC 4818) with ability to degrade butyl benzyl phthalate (BBP) was isolated by Chatterjee and Tapan [13]. The pathway of degradation of BBP by the strain MTCC 4818 was investigated and the result demonstrated that PA was a dead-end product of BBP degradation. Another study showed that a co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY could complete degradation of BBP [30]. Wu et al. [12] found *Gordonia* sp. strain JDC-2 can rapidly degrade DBP into PA, which accumulated in the culture medium. Altogether, Wu et al. [14] had compared degradation efficiency of DBP of four *Gordonia* sp. strains and cloned the 3,4-phthalate dioxygenase gene from these strains. It is interesting to note that all the four *Gordonia* strains cannot utilize PA as sole carbon and energy source [12,31]. In this study, we proved that strain QH-11 can completely degrade 500 mg/l of PA within 30 h, and phthalate 3,4-dioxygenase was also successfully obtained using a set of degenerate primers. Furthermore, compared with the untreated control (glucose), the expression level of phthalate 3,4-dioxygenase was significantly elevated after DBP and PA induced, suggesting that phthalate 3,4-dioxygenase may not be constitutively expressed in strain QH-11. Although only MBP and PA were detected as metabolites of DBP degradation in this study, the mRNA expression of phthalate 3,4-dioxygenase inferred that protocatechuate would be formed after PA degradation. Thus, the pathway of DBP biodegradation can be proposed as follows: (1) firstly, DBP is hydrolyzed to form MBP and then to PA; (2), PA is fast metabolized to PCA in conjunction with the activity of phthalate 3,4-dioxygenase, which enters the tricarboxylic acid (TCA) cycle, and then oxidized to carbon dioxide and water. This result is consistent with the conclusion reported by many previous researchers.

In summary, strain QH-11 was isolated from activated sludge and shown to be capable of degrading DBP. The optimum pH and temperature for DBP degradation by *Gordonia* sp. QH-11 are 7.0 and 30 °C, respectively. Unlike all of the previously reported DBP-degrading strains, the kinetics of DBP biodegradation by strain QH-11 could be described using modified Gompertz model. Moreover, the ability to degrade PA as sole carbon and energy source makes strain QH-11 different from all of PAEs-degrading *Gordonia* strains. This property highlights the potential of using this isolate for bioremediation of phthalate-contaminated environments. In addition, a gene encoding phthalate 3,4-dioxygenase was cloned, and RT-qPCR analysis demonstrated its expression can be enhanced after induced with PA. Further studies on the genetic and

Table 4
Degradation products of DBP by strain QH-11.

Compound	Molecular ion (m/z)	Retention time (min)	Major peaks (m/z)
PA	166	12.22	76, 104, 148, 207
MBP	222	19.20	105, 122, 149, 207
DBP	278	21.59	104, 121, 149, 278

biochemical characterization of dioxygenase involved in the oxygenation of PA from this bacterium may allow us to better understand the degradation mechanism of PAEs.

Acknowledgments

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