J. Microbiol. Biotechnol. (2010), **20**(10), 1440–1445
doi: 10.4014/jmb.1004.04034
First published online 6 August 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Biodegradation of Di-*n*-Butyl Phthalate by *Rhodococcus* sp. JDC-11 and Molecular Detection of 3,4-Phthalate Dioxygenase Gene

Jin, De-Cai[1†], Ren-Xing Liang[1†], Qin-Yun Dai[1], Rui-Yong Zhang[1], Xue-Ling Wu[1*], and Wei-Liang Chao[1,2*]

[1]*Department of Bioengineering, School of Minerals Processing and Bioengineering, Central South University, Changsha 410083, China*
[2]*Department of Microbiology, Soochow University, Taipei 111, Taiwan*

Received: April 22, 2010 / Revised: June 22, 2010 / Accepted: July 6, 2010

***Rhodococcus* sp. JDC-11, capable of utilizing di-*n*-butyl phthalate (DBP) as the sole source of carbon and energy, was isolated from sewage sludge and confirmed mainly based on 16S rRNA gene sequence analysis. The optimum pH, temperature, and agitation rate for DBP degradation by *Rhodococcus* sp. JDC-11 were 8.0, 30°C, and 175 rpm, respectively. In addition, low concentrations of glucose were found to inhibit the degradation of DBP, whereas high concentrations of glucose increased its degradation. Meanwhile, a substrate utilization test showed that JDC-11 was also able to utilize other phthalates. The major metabolites of DBP degradation were identified as mono-butyl phthalate and phthalic acid by gas chromatography–mass spectrometry, allowing speculation on the tentative metabolic pathway of DBP degradation by *Rhodococcus* sp. JDC-11. Using a set of new degenerate primers, a partial sequence of the 3,4-phthalate dioxygenase gene was obtained from JDC-11. Moreover, a sequence analysis revealed that the phthalate dioxygenase gene of JDC-11 was highly homologous to the large subunit of the phthalate dioxygenase from *Rhodococcus coprophilus* strain G9.**

Keywords: Isolation, DBP degradation, phthalate dioxygenase gene, metabolic pathway

Widely used in the manufacturing of plastics, phthalate acid esters (PAEs) are a group of synthetic chemicals, among which di-*n*-butyl phthalate (DBP) is important in the production of food wraps, plastic tubing, furniture, toys, shower curtains, and cosmetics [3, 7]. However, the extensive production and widespread use of DBP have resulted in its frequent detection in diverse environments [8], which is alarming, as DBP and certain other phthalates are suspected of being mutagens, hepatotoxic agents, and carcinogens [15]. Therefore, this pervasive exposure to such compounds *via* ingestion, inhalation, and dermal exposure on a daily basis [10] has resulted in DBP and other phthalates being classified as a top priority pollutant for risk assessment by several regulatory bodies [22].

As the rates of hydrolysis and photolysis of PAEs are very slow, the metabolic breakdown of these compounds by microorganisms is considered to be a major route for the environmental degradation of this widespread pollutant [13]. To date, a number of microorganisms from the genera *Delfia*, *Burkholderia*, *Rhodococcus*, and *Pseudomonas* have been isolated and characterized for the biodegradation of DBP [2, 13, 17, 19, 23]. Moreover, the metabolic pathways of DBP degradation using pure cultures have also been investigated [13, 17, 22]. However, very little information is yet available regarding the biodegradation pathway of DBP by microorganisms from the genus *Rhodococcus*.

It is well known that phthalic acid (PA) is a central intermediate in the biodegradation of phthalates, 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 3,4-phthalate dioxygenase [13]. Yet, few reports have been published about cloning the gene from Gram-positive phathalate-degrading bacteria, owing to limited sequences in GenBank [4, 5, 7, 20]. Thus, a PCR analysis using degenerated primer sets can provide a robust tool to determine the catabolic gene diversity.

*Corresponding author
X.-L.W.
Phone: +86-731-88804873; Fax: +86-731- 88710804;
E-mail: xueling0714@yahoo.com.cn
X.-L.C.
Phone: +86-731-88804873; Fax: +86-731- 88710804;
E-mail: wlchao@mail.scu.edu.tw

†These authors contributed equally to this work.

In the present study, a pure culture capable of using DBP as the sole carbon and energy source was isolated from sewage sludge, and the effects of pH, temperature, agitation rate, and glucose concentration on DBP biodegradation were investigated. A preliminary metabolic pathway for DBP is proposed, based mainly on the metabolites identified by gas chromatography–mass spectrometry (GC/MS). Moreover, PCR using a degenerated primer set was used to detect the phthalate dioxygenase gene involved in DBP degradation.

MATERIALS AND METHODS

Chemicals

The dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), di-*n*-octyl phthalate (DOP), and diisooctyl phthalate (DIOP), all at 99.5% purity, were obtained from the China National Medicine Group. The methanol was of high-performance liquid chromatography (HPLC) grade (Sigma, U.S.A.), and all the other chemicals and solvents were of analytical reagent grade.

Enrichment Culture and Isolation of Bacteria

The microorganisms were isolated using an enrichment-culture technique with a mixture of PAEs (DMP, DEP, DBP, and DOP) as the sole source of carbon and energy. The inoculum was sewage sludge obtained from Pingding County in Shanxi Province, China. Five g of sludge was added to a mineral salt medium (MSM) [3] containing 200 mg/l of PAEs (50 mg/l each of DMP, DEP, DBP, and DOP), 5.8 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 2.0 g/l $(NH_4)_2SO_4$, 0.16 g/l $MgCl_2$, 0.02 g/l $CaCl_2$, 0.0024 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.0018 g/l $FeCl_3$, and 0.0015 g/l $MnCl_2 \cdot 2H_2O$ in double-distilled water. The suspension was incubated for 7 days at 30°C on a rotary shaker at 175 rpm. Then, 2 ml of the enrichment culture was transferred to a fresh medium with a higher concentration of PAEs. The final enrichment was then streaked onto MSM agar (18 g/l) plates supplemented with a mixture of PAEs (500 mg/l). Visible colonies appeared after incubation for a week. The colonies were then transferred to fresh plates, and the incubation process was repeated until pure cultures were obtained.

Substrate Utilization Tests

Liquid MSM was supplemented with one of the following substrates (DMP, DBP, DEP, DOP, DIOP, phthalic acid, protocatechuic acid, naphthalene, diphenylamine, catechol, toluene, and salicylic acid; 200 mg/l) as the carbon source to examine the ability of the PAE-degrading bacterial strains to utilize these compounds. The substrate utilization was based on the microbial growth, which was determined by measuring the increase of the biomass (OD_{600}).

16S rRNA Gene Amplification and Phylogenetic Analysis

The genomic DNA was extracted using an EZ-10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc.) according to the manufacturer's instructions. For the 16S rRNA gene amplification, the genomic DNA was amplified using the bacterial universal primers F27 and R1492. The PCR mixture consisted of 2.5 µl of 10× PCR buffer, 2.5 µl of Mg_{2+} (25 mmol/l), 1 µl of dNTP mixture (each NTP, 2.5 mmol/l), 1 µl of each primer (62.5 pmol/l), 1 µl of the template DNA (100 ng/µl), and 0.5 µl of *Taq* DNA polymerase (5 U/µl; MBI Fermentas, Ontario, Canada) in a final volume of 50 µl. The PCR amplification parameters were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 10 min prior to cold storage at 4°C. The PCR products were then purified using an E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Inc.) and sequenced (by Sangon, Shanghai, China). Based on the similarities of the 16S rRNA gene sequences between strain JDC-11 and related species, a phylogenetic tree showing the relationship between the strains was constructed using Clustal X (1.8) and MEGA3.1 software.

Detection of Phthalate 3,4-Dioxygenase Gene

The phthalate 3,4-dioxygenase gene was amplified using the forward primer 5'-STTYAAYATGTGCCTSCAYC-3' (Y represents A or C, S represents G or C, R represents A or G, V represents A, C, or G) and reverse primer 5'-CCCARTTCTCVACRTCRTC-3', which were designed based on the conserved sequences of the phthalate dioxygenase reported by the National Center for Biotechnology Information (NCBI). The PCR mixture consisted of 5 µl of the PCR buffer (Mg^{2+} plus), 1 µl of dNTP (10 mmol), 1 µl of each primer, 0.5 µl of *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 were 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 products were purified using an E.Z.N.A. Gel Extraction Kit. The purified PCR products were ligated into the pGEM-T vector, and the plasmid was then transformed into *Escherichia coli* DH5α. After blue/white screening, white colonies were randomly selected and re-amplified using PCR. Representative positive clones were then selected for sequencing (by Sangon, Shanghai, China).

Biodegradation of DBP by Isolated Strain

The microorganisms were 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 (175 rpm), harvested, and washed three times with 0.02 mol/l phosphate buffer (pH 7.0). The washed cells were then 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 5 ml of the stock solution to a 250-ml sterile flask, 50 ml of sterilized MSM was added until the methanol was completely volatilized. One ml of the prepared cell suspension was then inoculated into the medium for the biodegradation test. All the tests were conducted in triplicate.

Analysis of DBP and Its Metabolites

The DBP residues were analyzed using a reverse-phase HPLC system. After adding 20 ml of ethyl acetate to the flask, a heavy emulsion was formed based on vibrating, and the aqueous and organic phases were separated by centrifugation at 12,000 rpm for 3 min. The aqueous samples were then extracted twice, while the ethyl acetate was evaporated to dryness, and redissolved in 10 ml of methanol. Approximately 0.5 ml of the DBP-containing methanol was passed through a 0.22-µm membrane filter, and 20 µl was injected into the Elite series HPLC system (Elite, China). A Hypersil ODS-C18 column (4.6 mm×200 mm×5 µm) was used for the separation, where the mobile phase consisted of a methanol:water solution [90:10 (v/v)] and the flow rate was 0.5 ml/min.

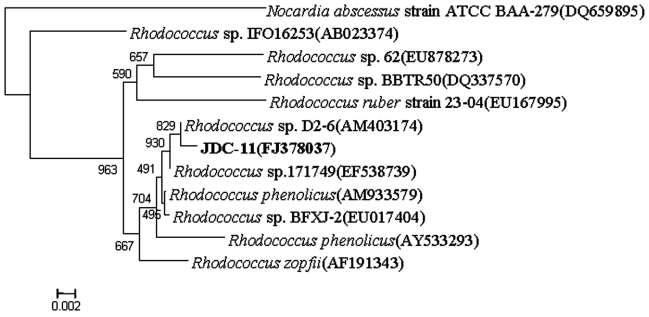

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence of JDC-11 and sequences of related species.
Distances were calculated using the neighbor-joining method. Numbers at branch points are bootstrap values (based on 1,000 samplings). GenBank accession numbers are included in brackets. *Nocardia abscessus* strain ATCC BAA-279 was used as the outgroup. Scale bars represent 0.002 substitutions per site.

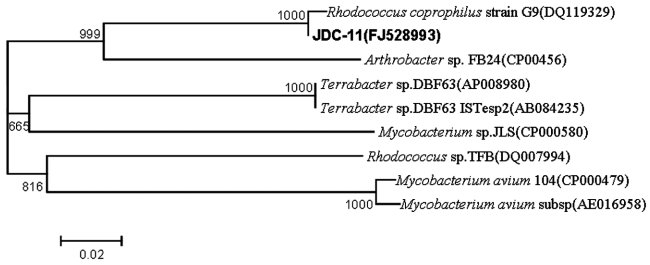

Fig. 2. Phylogenetic tree based on partial sequences of the phthalate dioxygenase gene (894 bp).
Distances were calculated using the neighbor-joining method. Numbers at branch points are bootstrap values (based on 1,000 samplings). GenBank accession numbers are included in brackets. Scale bars represent 0.02 substitutions per site.

The metabolites were analyzed using GC/MS (DSQ-Trace GC ULTRA) under the following conditions: DB-5MS (60 m×0.25 mm ×0.25 um) 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 50−650 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 (National Institutes of Standard and Technology, Gaithersburg, MD, U.S.A.).

Nucleotide Sequence Accession Numbers

The GenBank accession numbers for the sequences of the rRNA gene and phthalate 3,4-dioxygenase gene cloned in this study are FJ378037 and FJ528993, respectively.

Results

Microbial Identification and Phylogenetic Analysis

After four weeks of enrichment, a bacterial strain able to grow on PAEs as the sole source of carbon was successfully isolated. Designated as JDC-11, the strain was a Gram-positive, catalase-negative, nonsporulating, and nonpigmented bacterium without flagella. The colony that formed on the agar plates was white, irregular, wet, and convex after incubation at 30°C for a week. The phylogenetic relationships based on the 16S rRNA gene sequences are shown in Fig. 1. The sequence of JDC-11 was 99% similar to that of *Rhodococcus* sp. D2-6 (AM403174).

Substrate Utilization Test

The substrate utilization test indicated that the isolate had differing abilities as regards degrading phthalate esters (Table 1). Whereas it could rapidly degrade PAEs with shorter alkyl chains, such as DMP, DEP, and DBP, the PAEs with longer alkyl chains, such as DOP and DIOP, were poorly degraded. These results were similar to those reported in a previous study [1]. In addition, JDC-11 was also able to utilize the intermediate products of phthalic acid (PA) and protocatechuic acid (PCA), suggesting that JDC-11 may degrade DBP through PA and PCA as intermediates.

Cloning of Phthalate Dioxygenase Gene from the Strain

A fragment of 894 bp of the phthalate dioxygenase gene sequence was cloned from JDC-11 using a set of degenerate primers designed to detect the gene encoding the alpha subunit of phthalate dioxygenase. The sequence analysis showed that the obtained fragments of phthalate dioxygenase were highly homologous with the nucleotide sequences of phthalate 3,4-dioxygenase. A phylogenetic tree (Fig. 2) was constructed based on sequences with a close similarity. As shown in Fig. 2, strain JDC-11 was closely clustered with the large subunit of the phthalate dioxygenase of *Rhodococcus coprophilus* strain G9.

Table 1. Substrate utilization profile for strain JDC-11.

Substrate	Utilization	Substrate	Utilization	Substrate	Utilization
DMP	+[a]	DIOP	+	Salicylic acid	−
DEP	+	Phthalic acid	+	Naphthalene	−
DBP	+	Protocatechuic acid	+	Toluene	−
DOP	+	Catechol	+	Diphenylamine	−

[a]+, Positive; −, Negative.

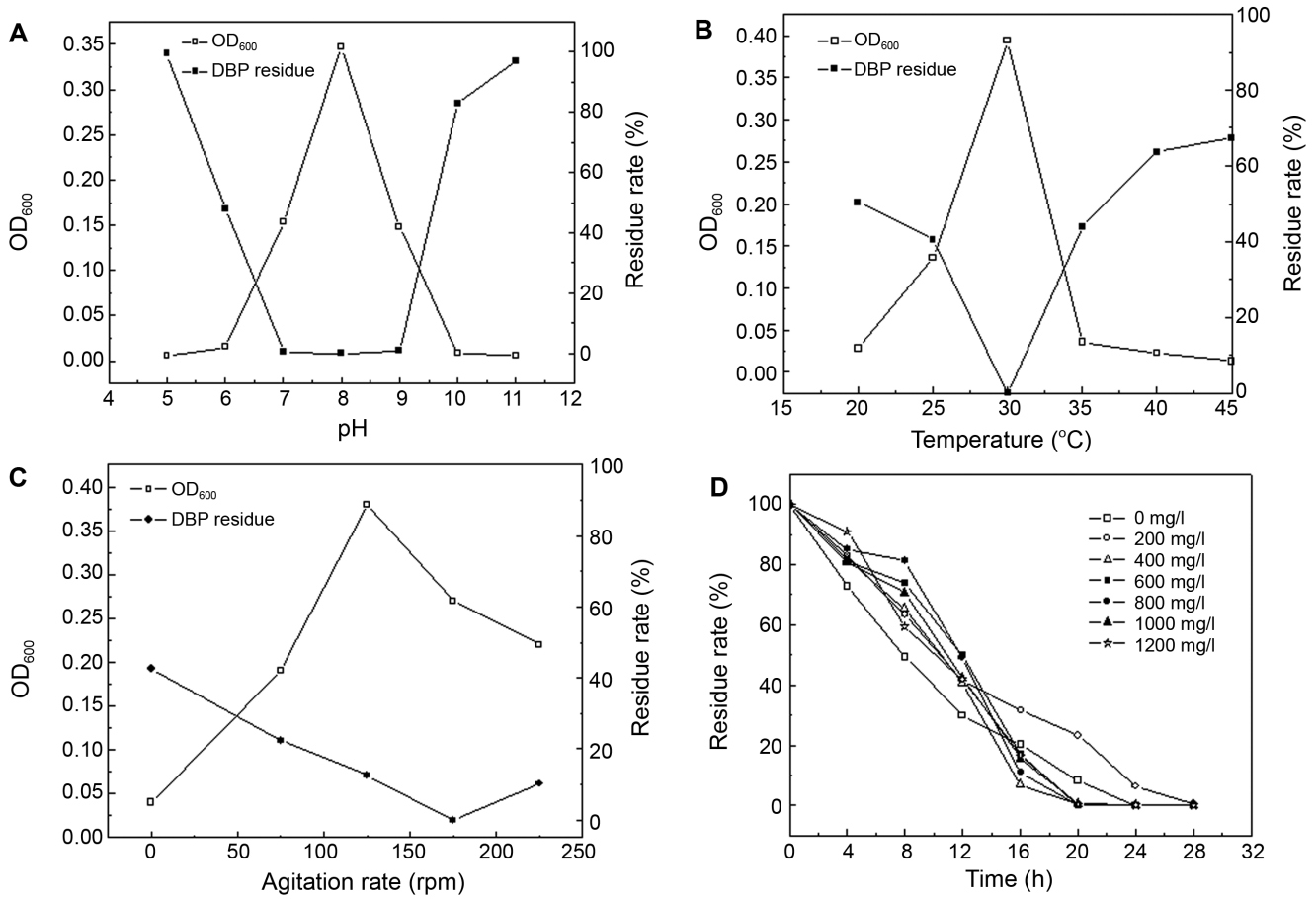

Fig. 3. Effects of pH (**A**), temperature (**B**), and agitation rate (**C**) on biomass and DBP biodegradation, and of glucose concentration (**D**) on DBP biodegradation under optimal conditions.
Data are averages of triplicate experiments.

Effects of Environmental Factors on Microbial Growth and DBP Degradation

The relationship between the residue rate (ratio between the residue in the medium and the total substrate added) and the pH was investigated (Fig. 3A). The concentration of DBP in the culture medium decreased when increasing the pH from 5.0 to 8.0, and no residual DBP was detected at pH 8.0 when the maximum biomass (OD_{600}) was achieved. The degradation of DBP also remained high at pH 7.0–9.0, indicating that JDC-11 preferred to grow and degrade DBP in a slightly alkaline environment. The optimum pH for DBP degradation and the microbial growth of JDC-11 was 8.0.

In addition, the effect of different temperature conditions (20, 25, 30, 35, 40, and 45°C) was assessed to determine the optimal temperature for DBP degradation by JDC-11 (Fig. 3B). The degradation rate and microbial biomass increased when raising the temperature from 20 to 30°C at which point the biomass reached its maximum value and the DBP was completely degraded. However, higher temperatures resulted in a decrease of the degradation rate. Therefore, the optimum temperature for DBP degradation was 30°C.

Furthermore, the effect of the dissolved oxygen content on DBP degradation was evaluated under different agitation conditions (Fig. 3C). As shown in Fig. 3C, the residue rate was 42.6% under resting conditions and decreased when increasing the agitation rate. No DBP was detected when the agitation rate was increased to 175 rpm. However, the residue rate increased when the agitation rate was greater than 175 rpm. Therefore, an agitation rate of 175 rpm was used for all the subsequent experiments.

Effect of Glucose Concentration on DBP Biodegradation

The effect of different concentrations of glucose, ranging from 0 to 1,200 mg/l, on the DBP degradation by JDC-11 was also investigated. As shown in Fig. 3D, the presence of glucose had a marked effect on the DBP degradation by strain JDC-11. During the initial 12 h of incubation, the existence of glucose inhibited the degradation of DBP, irrespective of the glucose concentration. However, after 12 h of incubation, the presence of glucose enhanced the

Table 2. Degradation products of DBP by strain JDC-11 determined by GC/MS analysis.

Compound[a]	Molecular ion (*m/z*)	Retention time	Major peaks
PA	166	15.13	76,104,148,196
MBP	222	21.02	93,121,149,167
DBP	278	22.63	76,104,149, 223

[a]MBP, Mono-butyl phthalate; PA, Phthalic acid.

DBP degradation rate, except for 200 mg/l of glucose, which inhibited DBP degradation.

GC/MS Analysis of Degradation Products of DBP by JDC-11

The degradation products of DBP by JDC-11 were determined using GC/MS in an attempt to elucidate the metabolic pathway. When comparing the mass spectra at a particular retention time with a mass spectra library, DBP and two metabolites were confirmed with reference to authentic standards (Table 2).

Discussion

The genus *Rhodococcus* encompasses diverse bacterial species inhabiting a variety of environmental niches, ranging from polluted soils to plants and animals [14]. Bacterial strains from the genus *Rhodococcus* are also acknowledged to play a key role in nature by decomposing persistent xenobiotics. To date, several studies have shown that different PAEs, including diethyl terephthalate (DETP) [8], di(2-ethylhexyl) phthalate (DEHP) [11], DOP [16], and DBP [13], undergo rapid biodegradation by bacterial strains isolated from various environments. In the present study, a bacterial strain (JDC-11) highly efficient in degrading DBP was isolated and identified as belonging to the genus *Rhodococcus* with 99% similarity based on 16S rRNA gene sequence analysis. In literature, several strains from other genera have also been isolated for the biodegradation of DBP. For example, Chao *et al*. [3] reported that soil bacteria took 1–3 days to completely degrade 100 mg/l of DBP, whereas another study reported that strain *P. fluorescens* B-1 took 144 h to completely degrade 100 mg/l of DBP [22]. In contrast, JDC-11 completely degraded 1,000 mg/l DBP within 24 h under optimal conditions (Fig. 3D). To the best of our knowledge, there has been no other report of complete DBP biodegradation at such a high concentration by a pure bacterial strain within such a short time. Therefore, strain JDC-11 would appear to be more efficient in degrading DBP than the results in previous reports, thereby offering great potential for the bioremediation of DBP from contaminated sites.

Previous studies have indicated that carbon and nitrate sources, phosphorous, and organic salt are all critical for the growth of microbes, as they can be used as electron acceptors to enhance the respiratory rate of the microorganism [6]. This study also investigated the effect of the glucose concentration on microbial growth and DBP biodegradation, and found that low concentrations of glucose inhibited the DBP degradation, whereas high concentrations of glucose increased its degradation. One explanation for these findings is that glucose and DBP compete as substrates to support bacterial growth. In this study, the bacteria appeared to give priority to glucose utilization, thereby inhibiting the DBP degradation at low concentrations of glucose, whereas high concentrations of glucose greatly enhanced the biomass, while also increasing the DBP degradation rate. Therefore, various factors should be considered when using bacteria for the bioremediation of phthalate-contaminated environments.

Several studies have already reported that the primary biodegradation of phthalates entails the sequential hydrolysis of the ester linkage, involving the formation of a monoester followed by phthalic acid. According to the metabolic intermediates produced by strain JDC-11 in the present study (Table 2), DBP was hydrolyzed to monobutyl phthalate and PA by the action of esterase. It is then generally supposed that the secondary biodegradation involves the mineralization of the PA and the formation of the corresponding alcohols [19, 21]. Under aerobic conditions, PA is degraded by two dioxygenase-catalyzed pathways to form the common intermediate protocatechuate [13]. PA is degraded to PCA either through a 4,5-dihydroxyphthalate intermediate in the case of Gram-negative bacteria [3] or 3,4-dihydroxyphthalate in the case of Gram-positive bacteria [3, 5]. PCA is then ultimately transformed into CO_2 and H_2O *via* meta or ortho cleavage pathways [13]. In the above biochemical pathway, phthalate dioxygenase plays a key role in the degradation of phthalate esters. For Gram-positive bacteria, phthalate 3,4-dioxygenase possesses large and small oxygenase subunits, including ferridoxin and ferridoxin reductase subunits [5, 20]. In the present study, a set of newly designed degenerate primers successfully amplified the phthalate 3,4-dioxygenase gene from JDC-11, indicating that strain JDC-11 harbors the phthalate 3,4-dioxygenase gene and may hydroxylate the phthalate ring at positions 3 and 4 for the degradation of DBP. Although only MBP and PA were detected as metabolites of degradation (Table 2), it should be noted that both PA and PCA were readily metabolized by JDC-11 under aerobic conditions (Table 1). Therefore, based on the above results, a tentative pathway for the degradation of DBP by JDC-11 is proposed (Fig. 4). In addition, the results of the present study indicated that the metabolic pathway used by JDC-11 was in accordance with the previously reported metabolic pathways of DBP degradation by most Gram-positive bacteria [4, 5, 7, 20].

In conclusion, 1,000 mg/l of DBP was completely degraded within 24 h by *Rhodococcus* sp. JDC-11 isolated from

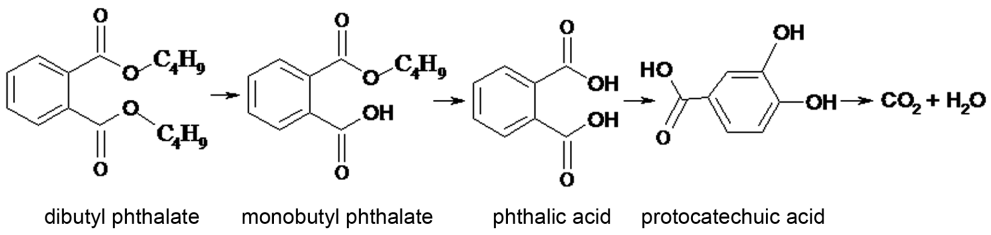

Fig. 4. Proposed metabolic pathway for DBP degradation by *Rhodococcus* sp. JDC-11.

sewage sludge. The optimum pH, temperature, and agitation rate for DBP degradation by JDC-11 were 8.0, 30°C, and 175 rpm, respectively. The results of this study also showed that the presence of glucose had a remarkable effect on DBP degradation. Furthermore, the degradation of DBP by JDC-11 would seem to proceed through MBP and PA before cleavage of the aromatic ring. The successful cloning of the phthalate 3,4-dioxygenase gene from JDC-11 will also help in furthering the present understanding of the genetic mechanism of DBP degradation.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant No. 30770388). We would like to thank Yuanli Xie for his technical support.

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