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# Genome sequencing and biodegradation characteristics of the *n*-butyl benzyl phthalate degrading bacterium-*Rhodococcus* sp. HS-D2

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ABSTRACT

The *n*-butyl benzyl phthalate (BBP) is an environmental pollutant used extensively in the manufacturing of plastics. For the bioremediation of phthalic acid ester pollutants in water, sediment, and soil, a BBP degrading bacterium *Rhodococcus* sp. HS-D2 was isolated from contaminated river sediment and characterized. The HS-D2 strain is capable of utilizing BBP as the sole source of carbon. A shaken culture containing 500 $mgL^{-1}$ of BBP produced complete degradation in 96 h. To study the metabolic characteristics and potential strategies for enhancing the biodegradation rates of BBP, a genome-scale metabolic model (GEM) of *Rhodococcus* sp. iYZ1601 was reconstructed based on the genome sequence of strain HS-D2. It included several sequential transporters and hydrolases were involved in the biodegradation process of BBP. Monoethylhexylphthalate (MEHP) and phthalic acid ester (PAE) hydrolases were confirmed as the key enzymes in phthalate degradation. The MEHP and PAE hydrolases catalyzed the conversion of BBP to butanal, phenylcarbinol, and phthalate as verified by HPLC analysis. The growth rate of HS-D2 and BBP consumption rate were analyzed *in silico* simulation, and were found to be consistent with the rates of HS-D2 growth and BBP consumption the *in vitro* experiment. In summary, *Rhodococcus* sp. HS-D2 biodegrades BBP, but the metabolic pathway of this bacterium needs further exploration to improve the biodegradation efficiency.



## 1. Introduction

Phthalic acid ester (PAE or phthalate) is an important group of plasticizers (Chatterjee and Karlovsky, 2010). The *n*-butyl benzyl phthalate (BBP) is used as additives to many materials (Navacharoen and Vangnai, 2011; Xu et al., 2005, 2007) such as vinyl foams. BBP has been detected in sediments, waters, soils, and human tissues due to their wide use and tendency to leach from products (Julinová and Slavík, 2012; Luo et al., 2015; Stales et al., 1997). In addition to the concern about their release into the environment, BBP is also difficult to degrade. Due to their increasing application and consumption, BBP has become widespread organic contaminant (Chatterjee and Dutta, 2008). They are now listed as priority pollutants by both European Commission and the US Environmental Protection Agency (Jianlong et al., 1995).

Microbial degradation is considered to be the most promising means of removing PAE from aquatic and terrestrial systems (Fang et al., 2010; Patil et al., 2006; Stales et al., 1997; Wang et al., 2004; Xu et al., 2005). At present, several publications report PAE biodegradation (Li et al., 2005; Wu et al., 2013; Yang et al., 2014); however, only a few address biodegradation of *n*-butyl benzyl phthalate (BBP) (Chang et al., 2004, 2007; Chatterjee and Dutta, 2003, 2008; Navacharoen and Vangnai, 2011; Xu et al., 2007; Yang et al., 2013). Microbial degradation of BBP is complex and difficult because BBP is an alkyl aryl phthalate (Yang et al., 2013). For these reasons, it is urgent to find potential bacterium species that have wide availability, high environmental endurance and efficient biodegradation capacity for BBP.

In this paper, a BBP-degrading strain HS-D2 was isolated from river sediment. It has been identified as *Rhodococcus* sp., and a preliminary genome was described. The genes associated with BBP degradation were identified based on genome sequencing. According to the results of genome annotation, iYZ1601, a GEM of HS-D2 was reconstructed. The growth rate of HS-D2 and BBP

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consumption rate in different conditions were calculated by this model. It could provide further insight into the BBP-degradation mechanism.

## 2. Materials and methods

### 2.1. Substrate preparation and the method of bacterial screening

The detailed list of substrates and the culture medium formulation are provided in Supplementary Materials S1. A 5 g (moist) of contaminated river sediment (Fu River, Wuhan, China.) were inoculated into 100 mL minimal salt medium (MSM) which contained 100 mg $L^{-1}$ BBP. The culture conditions were 150 rpm at 30 °C in shaker without light for 5 days. Every 5 days after this initial culturing, 10 mL of culture medium was transferred to fresh MSM with 100, 250, 300, 400, or 500 mg $L^{-1}$ BBP. Bacteria that degraded BBP in the enrichment culture were isolated and purified following this procedure (Yang et al., 2013). A strain, named HS-D2, was found that could effectively degrade BBP.

### 2.2. Strain identification

The isolated BBP-biodegrading strain was subjected to phylogenetical identification. The DNA of 16S rRNA was amplified with the universal primers 27f/1492r (Lane, 1991). The sequence of 16S rRNA was submitted to GenBank (accession number is KX500189.1). The amplified DNA products were sequenced and the sequence similarity determined by the BLAST program in GenBank. The closest references were downloaded for phylogenetic analysis using the MEGA version 6.0 program.

### 2.3. Substrate utilization and the optimal of pH, temperature on microbial growth and BBP biodegradation test

Strain HS-D2 was tested for its ability to grow on several carbon sources. It was inoculated into liquid MSM supplemented with Tween-80, Catechol, benzoicacid (BA), phthalic acid (PA), pyridine, DMP, DEP, DBP, DOP, or DEHP (100 $mgL^{-1}$) as carbon sources. Microbial growth was measured after 60 h of incubation by UV-3300 spectrophotometer (MAPADA, Shanghai, China).

Next HS-D2 on an agar plate was inoculated into MSM containing 500 $mgL^{-1}$ BBP and incubated in a shaker at 180 rpm at 30 °C to the logarithmic phase. Then bacteria were collected by centrifugation and washed with 0.05 M PBS (pH 7.5). The bacterial pellet was resuspended in the same concentration PBS to an OD600 of 1.0. The BBP (500 $mgL^{-1}$) was added to 150 mL Erlenmeyer flasks and inoculated with 1 mL of suspension plus 50 mL of MSM. The utilization of BBP (500 $mg.L^{-1}$) by HS-D2 under different pH (5.0–10.0) and temperature (15–42 °C) conditions was examined to determine the optimal conditions for microbial growth and BBP biodegradation. After 60 h of incubation, the microbial growth was determined and BBP residue was examined by HPLC analyses. All the experiments were performed three times.

### 2.4. The whole-genome sequencing and annotation

Genomic DNA of strain HS-D2 was extracted using a Genome DNA extraction kit (Axygen, Suzhou, China). We measured the purity with a ND-1000 spectrophotometer (USA).The Illumina High-Seq 2000 system was used to sequence the genome of strain HS-D2. In this work, we used the RAST server(Aziz et al., 2008), KAAS server (Moriya et al., 2007), and the NCBI database (Pruitt et al., 2009) to annotate the HS-D2 genome. The genome of strain *Rhodococcus* sp. HS-D2 was submitted to GenBank (accession number is NZ_LUTX00000000.1).

### 2.5. Genome-scale metabolic network reconstruction and in silico analysis

We reconstructed the draft model by the Model Seed (Devoid et al., 2013). MATLAB (v8.0.0.783, R2012b, The MathWorks, Inc) was used to convert the generated Microsoft Excel file into a COBRA model and to execute all necessary scripts present in the COBRA Toolbox (v2.0.6) (Schellenberger et al., 2011), a glpkmex linear solver used on constraint-based algorithms.

### 2.6. The experimental verification of the in silico analysis

The BBP degradation kinetics of strain HS-D2 was studied at the optimal pH and temperature determined from the above incubations. Flasks with strain HS-D2 and different concentrations of BBP (100, 250, 500, 750, or 1000 $mgL^{-1}$) were prepared and incubated at the optimal conditions. Residual BBP samples were taken at 6 h intervals and stored at 4 °C for HPLC analysis. The suspension (1 mL) of strain HS-D2 was transferred into 50 mL of MSM containing BBP (500 $mgL^{-1}$) and Tween-80 at the selected concentrations (0, 55, 110, 165, or 220 $\mu molL^{-1}$) in 150 mL Erlenmeyer flasks. Considering the low solubility of BBP, we first made a stock solution of BBP (10 $g.L^{-1}$) with a methanol cosolvent and then diluted this stock solution to 500 $mgL^{-1}$ with water to produce a working solution. After incubating at 180 rpm, 30 °C for 60 h, the microbial growth was determined by light microscopic observation and cell-counting. The culture medium often appeared to be creamy white due to the emulsification of BBP by Tween-80. Because this opacity would interfere with the measurement of OD600, the cell-counting method was adopted that quantified cells as Colony-Forming Units (CFU). BBP residue was examined by HPLC (VARIAN, USA) analyses with an Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm) and UV–Vis detector (UV325).

## 3. Results

### 3.1. The result of bacterial screening and strain identification

One strain (HS-D2) demonstrated BBP utilization after 6 weeks of enrichment and was selected for further investigation. It was a Gram-positive, aerobic bacillus with a width of 0.5–0.6 μm and length of 1.0–3.5 μm. Further, it was a non-spore-forming bacterium without flagella that formed colonies on the LB agar plates that were light orange in color, round, wet, and convex after 72 h of incubation at 30 °C. Strain HS-D2 had a 99% similarity to *Rhodococcus pyridinivorans* R04 (AF459741) based on 16S rRNA gene sequence comparison (Supplementary Materials S2).

### 3.2. The optimal pH and temperature of HS-D2 growth and BBP degradation

Bacterial growth of *Rhodococcus* sp. HS-D2 is pH sensitive, especially under acidic conditions (Fig. 1A). The rates of cell density change and BBP degradation increased rapidly when pH increased from 5.0 to 7.0. The highest OD600 value (1.06 ± 0.11) and BBP degradation rate (50.8% ± 3.24%) for HS-D2 were achieved at pH 7.0. When pH exceeded 7.0, the OD600 value and degradation rate decreased gradually, and degradation rate decreased substantially at pH 10.0 (to 8.1% ± 1.4%). In addition, the effect of different temperature conditions (15, 20, 25, 30, 37, and 42 °C) was assessed to determine the optimal temperature for BBP degradation by *Rhodococcus* sp. HS-D2 (Fig. 1B) (Leven and Schnürer, 2005). The BBP degradation rate increased rapidly as temperature increased from 15 to 30 °C. The highest OD600 value (1.13 ± 0.14) and BBP degradation rate (52.5% ± 3.8%) for HS-D2 were both achieved at

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