Bioremediation of environmental endocrine disruptor di-n-butyl phthalate ester by *Rhodococcus ruber*

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Abstract

In this study DBP-degradation strain CQ0301 was isolated from rubbish landfill soil. According to the biophysical, biochemical characteristics and analysis of 16S rRNA, the strain was identified as *Rhodococcus ruber*. Three new protein bands could be fractioned after DBP-inducing, which were suspected to participate the process of DBP-degrading. Catechol was suspected to be an intermediate product of DBP and cleaving the benzene ring was catalyzed by catechol 1,2-dioxygenase, because a highly activity of catechol 1,2-dioxygenase could be detected after DBP-inducing. The results of this study also showed the optimal pH value, optimal temperature which influenced the degradation rate in soil: pH 7.0–8.0, 30–35 °C. Kinetics of degradation reaction had been performed at different initial concentration and different time. Analyzed with SPSS10.0 software, the DBP degradation can be described as the same exponential model when the initial DBP concentration was lower than 50 mg/kg. The kinetics equation was ln C = −0.1332t + A, with the degradation half-life of DBP in soil (5.20 d). Inoculating CQ0301 could relieve DBP content in plant. We also found that adding nutrient materials into soil was useful for decreasing the DBP content in plant. In summary, we isolated a bacterium capable of degrading DBP and decreasing DBP content in plant. We also explored the mechanism of biodegradation and characterized the environmental factors influencing the degradation process in contaminated soil. Based on this work, we hope that these findings can provide some information for applying of bioremediation of DBP contaminated soil.

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Keywords: DBP; Biodegradation; Catechol-dioxygenase; *Rhodococcus ruber*

1. Introduction

Phthalic acid esters (PAEs), classes of refractory organic compounds, which are widely used in the plastic, coatings, and cosmetics industries, have received extensive attention in recent years. They are distributed in sediment, natural water, wastewater and soils (International Programme on Chemical Safety, 1992). Releasing phthalates into the ecosystem or wastewater effluent occurs during the production phase and via leaching and volatilization from plastic products during their usage and after disposal (Psillakis et al., 2004). Even at very low concentration, they are suspected of interfering with reproductive systems and behaviors in humans and wildlife, through disturbance of the endocrine system (Jobling et al., 1995). In addition, some of them are suspected of teratogenic, mutagenic and carcinogenic (Huff and Kluwe, 1984). Several regulatory bodies, such as the US Environment Protection Agency (US EPA, 1991), the European Union (1993) and China National Environmental Monitoring Center (Wang et al., 1995) classified phthalate esters as a top priority environmental pollutant.

Di-n-butyl phthalate (DBP) belongs to the family of PAEs. It is ubiquitous environmental contaminant, as indicated by its presence in air, water, and soil worldwide (Huang et al., 1994). Examining urinary phthalate metabolites, Blount et al. found that the general population appears to be exposed to disproportionately higher amounts of DBP compared with other phthalates (Blount...
et al., 2000). A multigenerational study performed by the National Toxicology Program showed that DBP is a male reproductive toxicant which had effects on animals exposed pre and postnatal (National Toxicology Program, 1991; Wine et al., 1997). Furthermore, studies by Mylchreest et al. showed that DBP exposure during gestation had the ability to affect the developing male reproductive tract (Mylchreest et al., 1999). Recently, Christopher et al. reported that there were gene expression changes in the fetal testis following DBP exposure, such as Star (steroidogenic acute regulatory protein), Scarb1 (scavenger receptor class B, 34 member 1, also known as Sr-b1), Cyp11a1 (cytochrome P450, family 11, subfamily a, 35 polypeptide 1, also known as P450 SCC), and Cyp17a1 (cytochrome P450 family 17, subfamily a, 36 polypeptide 1, also known as Cyp17) (Christopher et al., 2005). As a result, all of the information provided strong evidences that DBP is a potential toxic pollutant.

Metabolic breakdown of DBP by microorganisms is considered one of the major routes of environmental degradation for this widespread pollutant due to its low rate of the hydrolysis and the photolysis (Wolf et al., 1980). As the application of DBP contained PVC plastic films on agriculture dramatically increases, and a large quantity of wastewater, municipal and industrial sewage sludge are deposited on cultivated land each year, DBP is the most identified phthalate ester in agricultural soils (Kampe et al., 1988; Tao et al., 1993; Pang et al., 1995; Meng et al., 1996). Further studies showed that DBP played the toxic effects on crops and vegetable, so it could threaten the health of human (Chen et al., 1998). In the past few years, considerable attention has been paid to the analysis of environmental fate, general toxicity and biological degradability of DBP (Wang et al., 1997). However, little is known about the mechanism of the biodegradation and the effect of bioremediation by using degradation bacteria to contaminated soil.

The objective of this investigation was to isolate microorganisms capable of degrading DBP, explore the mechanism of biodegradation and characterize the environmental factors influencing the degradation process in contaminated soil.

2. Materials and methods

2.1. Chemicals

DBP with 99.0% analytical standards was obtained from Beijing Chemical Reagent Factory (China). Chemicals used for diluting and extracting DBP were analytical grade and were redistilled. Other chemicals used in this study were also of analytical grade. Glassware was meticulously cleaned to reduce any background contamination of phthalates. All chromic acid washed glassware was placed in a 300 °C oven overnight. After cooling, the glassware was rinsed twice with acetone and petroleum ether and air-dried ready for use.

2.2. Identification of the DBP-degrading bacterium

The microorganism was originally isolated from rubbish landfill soil by selective enrichment using DBP as the sole source of carbon. The concentration of DBP was varied from 10 to 2000 mg/l. After 3 months, the bacterium was isolated and purified.

The primers which were used to amplify 16S rRNA gene were Pf: 5‘-AGGGTTGTATCCTGGCTCAG-3’ and Pr: 5‘-ACGGCTACCTTGTACGACT-3’ corresponding to 8–27 and 1495–1514 bases of coli 16S rRNA gene respectively. PCR amplification conditions as following: each PCR mixture (25 μl) was composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.25 μM, template DNA, and 0.45 U of Taq DNA polymerase. The amplification program consisted of one cycle of 94 °C for 5 min; 38 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 100 s; and finally one cycle of 72 °C for 10 min. The amplified products were subjected to gel electrophoresis in 1% agarose, followed by ethidium bromide staining. Shanghai Shenyou Cooperation provided all the reagents and service of sequencing.

2.3. Detection of activity of catechol-dioxygenase and PAGE-SDS electrophoresis for whole cell protein

The method for detection of catechol-dioxygenase activity (Haysish, 1957; Sala-Trepat and Evams, 1971) was adopted.

Polyacrylamide gel electrophoresis (PAGE)-SDS electrophoresis was used to study strain whole cell protein. After 48 h cultivation, the bacterium body was collected and boiled for 5–8 min, and then centrifuged for 2 min. The supernatant was used for electrophoresis. In PAGE-SDS, the concentration of acrylamide was 4.4% with a pH of 6.8 in the upper gel and 10.0% with a pH of 8.8 in the lower gel.

2.4. Soil treatment

The soil sample was taken from Gaotanyan town, Chongqing, China. The physical and chemical properties of the soil were shown in Table 1.

The soil was grated and sifted carefully before DBP was sprayed. Stock solutions with concentration of 5000 mg/l were prepared by diluting DBP with petroleum. An appropriated volume of the stock solution was mixed with 5 ml

<table>
<thead>
<tr>
<th>Physical and chemical property of soil sample</th>
<th>pH</th>
<th>Organic matter (g/kg)</th>
<th>Total N (g/kg)</th>
<th>Pb (mg/kg)</th>
<th>Hg (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>7.10 ± 0.05</td>
<td>6.520 ± 0.181</td>
<td>0.035 ± 0.006</td>
<td>2.00 ± 0.16</td>
<td>0.255 ± 0.034</td>
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</table>
of Tween-80, 5 ml of acetone, 3000 ml of water, stirred violently, and then sprayed into the soil. The concentration of DBP which was used to study plants contamination was 50 mg/kg dried soil.

2.5. Determination of DBP in soil

Twenty grams dry soil was added to 40 ml petroleum/acetone (V/V: 3/1) and 3 g of anhydrous magnesium sulfate. The Erlenmeyer flasks were kept static overnight and then vibrated for 4 h. The extract was filtered from the soil by suction filtration through Whatman GFA glass microfibre filters. The flask and soil was rinsed three times with 50 ml of the solvent mixture, which was filtered. The combined filtrate was transferred to a separator funnel, which was then supplemented with 100 ml of 6% sodium sulfate solution. The funnel was vibrated for 5 min, and the water layer was removed, while the organic layer was transferred to a glass test-tube. The elution was evaporated under pure nitrogen gas to dryness. Finally, the residue was dissolved in 0.5 ml methanol prior to quantification by high-performance liquid chromatography (HPLC) with a ultra-violet detector (DAD, Agilent, USA).

The Agilent 1100 series HPLC consisting of a G1322A degasser, a G1311A QuatPump, a G1316A COLCOM and a G1315B diode array detector set at 210 nm, was used for the quantification of DBP concentration. A personal computer equipped with HP ChemStation was used to acquire and process chromatographic data. The mobile phase was a methanol:water:50 mM phosphoric acid mixture (70:25:5, V/V) at pH 2.35 and the flow rate was 1 ml/min. Under these chromatographic conditions, DBP could be separated finely. The recovery percentage of DBP was 96.4%. All tests were conducted in triplicate.

2.6. Determination of DBP in plant

Plant samples were oven-dried at 55–60 °C, ground in mortar and sieved to less than 1 mm. For each sample, 1.0 g was added to a 100-ml Erlenmeyer flask with ground-glass stopper, which was then supplemented with 20 ml petroleum/acetone (V/V: 3/1) and 3 g of anhydrous magnesium sulfate. The Erlenmeyer flasks were kept static overnight and then vibrated for 4 h. The extract was filtered from the soil by suction filtration through Whatman GFA glass microfibre filters. The flask and soil was rinsed three times with 50 ml of the solvent mixture, which was filtered. The combined filtrate was transferred to a separator funnel, which was then supplemented with 100 ml of 6% sodium sulfate solution. The funnel was vibrated for 5 min, and the water layer was removed, while the organic layer was transferred to a glass test-tube. These samples were then analyzed by gas chromatography equipped with an electron capture detector (SC-2000, China).

A 200 cm × 4 mm stainless steel column packed with chromsorp-WHP 80/100 mesh coated with 10% SE-30 was used under the following operating conditions: nitrogen carrier gas, 40 ml/min; column oven, 240 °C; and detector, 300 °C. Under these conditions, DBP in plant could be finely separated and exactly quantified. The recovery percentage of DBP was 95.6%. All tests were conducted in triplicate.

3. Results and discussion

3.1. Isolation and identification of the DBP-degrading bacterium

The bacterium capable of utilizing DBP as the sole source of carbon and energy was isolated from rubbish landfill soil. The initial concentration of DBP was 10 mg/l. After 3 months, one strain that could survive in the concentration of 2000 mg/l was studied in the following experiments. We named it CQ0301.

The diameter of CQ0301 colony was 0.05–0.15 cm. The colony showed round morphology, low prominence, smooth and glossy surface, salmon pink. Oxidase and catalase were positive. The morphology of CQ0301 was showed in Figs. 1 and 2.

By comparison, the sequence of CQ0301 16S rRNA with Rhodococcus ruber, the results showed that the similarity was 98.8%. Combined with analysis of morphology, physio-biochemical character and genetic specificity, CQ0301 was identified as R. ruber.

3.2. Detection of activity of catechol-dioxygenase

Catechol is the intermediate product of aromatic hydrocarbon compounds and cleaving benzene ring is the common pathway of aromatic hydrocarbon metabolism. Ring opening reaction could be catalyzed by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase (Strachan et al., 1998). As shown in Table 2, the activity of catechol 1,2-dioxygenase other than catechol 2,3-dioxygenase could be Fig. 1. Transmission electron micrograph of strain CQ0301(40000×).
detects in the crude enzyme of CQ0301 strain. Moreover, the higher activity of catechol 1,2-dioxygenase was detected only after DBP-inducing. From what has been mentioned above, catechol was suspected to be an intermediate product of DBP and cleaving the benzene ring was catalyzed by catechol 1,2-dioxygenase.

In this study, catechol was used as substrate to determine the reaction velocity of catechol 1,2-dioxygenase. As shown in Fig. 3, double-reciprocal plot was analyzed to calculate the Michaelis constant $K_m$ and maximum reaction velocity. The Michaelis constant $K_m$ and maximum reaction velocity of CQ0301 catechol 1,2-dioxygenase were 25 $\mu$mol/l, and 16.8 $\mu$mol/mg min, respectively.

3.3. PAGE-SDS electrophoresis of CQ0301 total cell protein

In order to investigate the differences of total cell protein between before DBP-inducing and after DBP-inducing, PAGE-SDS electrophoresis of total cell protein was analyzed. Fig. 4 showed that three new protein bands could be fractioned in the after DBP-inducing total protein. The results indicated that these proteins were suspected to participate the process of DBP-degrading. The molecular weights of the three bands were about 116.0 kDa, 66.0 kDa, and 40.0 kDa.

3.4. Environmental factors influencing the degradation process in soil

The hydrogen ion concentration influences the bacterial growth since pH value limits activity of enzymes. Accumulation of degradation intermediates such as phthalic acid also acidifies the culture medium, thereby inhibiting the further bacterial degradation of intermediates (Wang et al., 2003). The relationship between the degradation rate constants and pH was shown in Fig. 5. The rate of DBP degradation increased quickly when pH value of the soil was increased from 6.0 to 7.0. A high rate was achieved when pH value was from 7.0 to 8.0. When pH value was $>8.0$, the degradation efficiency decreased. The optimal pH value for DBP degradation was from 7.0 to 8.0. The result matched those reported in our previous study on DBP degradation in liquid (Jun et al., 2005).

Bacterium growth is sensitive to environmental temperature. In order to determine the optimal temperature of DBP degradation for CQ0301, different temperature condi-

Table 2

<table>
<thead>
<tr>
<th>Activities of catechol-dioxygenase in CQ0301 (A—after DBP-inducing, B—before DBP-inducing)</th>
<th>Catechol 1,2-dioxygenase (U/mg)</th>
<th>Catechol 2,3-dioxygenase (U/mg)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>5.146 ± 0.195*</td>
<td>0.109 ± 0.006*</td>
</tr>
<tr>
<td>B</td>
<td>0.091 ± 0.039**</td>
<td>0.06 ± 0.003**</td>
</tr>
</tbody>
</table>

* $p < 0.01$.
** $p > 0.05$.

Fig. 3. Kinetics of catechol 1,2-dioxygenase in strain CQ0301. Catechol was used as substrate to determine the reaction velocity of catechol 1,2-dioxygenase by crude enzyme of CQ0301. Double-reciprocal plot was analyzed to calculate the Michaelis constant $K_m$ and maximum reaction velocity.

Fig. 4. PAGE-SDS electrophoresis of CQ0301 total cell protein. Polyacrylamide gel electrophoresis (PAGE)-SDS electrophoresis was used to study strain whole cell protein. There are differences of total cell protein between before DBP-inducing (A) and after DBP-inducing (B), three new protein bands could be fractioned in the after DBP-inducing. The molecular weight of the three bands were about 116.0 kDa, 66.0 kDa, and 40.0 kDa.
tions (20°C, 25°C, 30°C, 35°C, 40°C, 45°C) were assessed. As shown in Fig. 6, the degradation rate increased with the increase of temperature between 20°C and 30°C. High rate was achieved for CQ0301 when the temperature sustained between 30°C and 35°C. Higher temperature resulted in the lowering of the degradation rate. The result indicated that the optimum temperature was 30–35°C, at which the degradation related enzyme reached the highest activity.

Carbon source, nitrate source, phosphor and organic salt are critical for the growth of microbial, because they can be used as electron acceptor to enhance the respiratory rate of microbial (Fuller and Manning, 2004). To determine the effect of adding nutrient on degradation rate, the strain was inoculated with kalium nitricum (1 mol/l) 2 ml and peptone 2 g in 100 g contaminated soil in B group. The different degradation rates were detected between adding nutrient and none-adding nutrient (p value <0.05).

3.5. Characteristics of DBP-degradation kinetics in soil

In order to investigate the effect of initial DBP concentration on degrading activity, the degradation kinetics character in soil was studied. Different initial DBP concentration (10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg) was assessed. As shown in Fig. 8, the time course of degradation rate was recorded.

The DBP biodegradation data we collected fit well with exponential model, \( C = b_0 \cdot e^{(-kt)} \). A first-order kinetics model, \( \ln C = -kt + A \) could be constructed by logarithmic transformation, where \( C \) is the initial concentration (mg/kg), \( k \) is the biodegradation rate constant, \( t \) is the time period, \( A \) is the constant, \( t_{1/2} = \ln 2 / k \) is the half life.

Table 3 shows the different DBP degradation kinetics equations at different DBP initial concentration. The results showed that when DBP initial concentration <50 mg/kg, DBP biodegradation reaction fit well with the first-order kinetics. Analyzed by SPSS10.0, DBP degradation kinetics equation in soil by CQ0301 was \( \ln C = 0.1332t + A \), and the half life of DBP in soil was 5.20 d.
3.6. Decrease DBP in plants by inoculation of CQ0301

There has been a growing concern regarding the environmental behavior and potential toxicity to plants associated with DBP (Wang et al., 1997). Some reports deal with plant uptake and toxic effects of DBP on crop and vegetable growth (Cai et al., 1994). DBP could be detected in human urinary (Blount et al., 2000), so chain of transformers was suspected to be a pollution pathway to threaten human health. To decrease DBP in plants is important for human health.

To investigate the effect of decreasing DBP in plants by inoculation CQ0301, we designed the experiments as following: group A which be watered with clean water used as the control group, group B was inoculated CQ0301 at 1 d, 5 d, 10 d after planting, group C was inoculated at 1 d, 5 d, 10 d after planting and added nutrient to the contaminated soil simultaneously. After 2 months, DBP concentration in plants was determined.

Fig. 9 showed the decreasing of DBP in plants by inoculation CQ0301 and adding nutrients. Analyzed with one-way ANOVA and T test, the results showed that there were significant differences of comparison between A and B, and B and C. The difference in plant uptake of DBP was caused by inoculation CQ0301 and adding nutrient to contaminated soil. All the results indicated that inoculation of degrading bacterium was a useful way to decrease DBP in plants and adding nutrient to soil could enhance the effect.

4. Conclusions

In summary, a strain CQ0301 capable of degrading DBP was isolated from DBP contaminated rubbish landfill soil. Analyzed with morphology, physio-biochemical character and genetic specificity, CQ0301 was identified as R. ruber. We explored the mechanism of DBP degradation and the results showed that catechol was suspected to be an intermediate product of DBP and cleaving the benzene ring was catalyzed by catechol 1,2-dioxygenase. Three new protein bands, which were suspected to participate the process of DBP-degrading, could be fractioned in the total protein of bacterium after DBP-inducing. The results of this study also showed the optimal pH value, optimal temperature that influenced the degradation rate in soil. We found that DBP concentration in plants could be decreased by inoculating CQ0301 and adding nutrient to contaminated soil was a useful way to enhance the effect.

Based on these findings, future work will be attempted to define more factors that influence the degradation effects. We hope that these findings can provide some information for applying of bioremediation of DBP contaminated soil.

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References


Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumper, J.P., 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environmental Health Perspectives 103, 582–587.


Mylchreest, E., Sar, M., Cattley, R.C., Foster, P.M., 1999. Disruption of androgen regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. Toxicology Applied Pharmacology 156, 81–95.


