Biodegradation of diethyl phthalate by *Pseudomonas sp.* BZD-33 isolated from active sludge

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Abstract. Diethyl phthalate (DEP) is a common pollutant, which is widely used in industry. A strain of DEHP-degrading bacteria was isolated from activated sludge. According to the phylogeny of 16S rDNA sequence, BDZ-33 strain was identified as *pseudomonas sp.* Biodegradation of DEP by *Pseudomonas sp.* BZD-33 was investigated. The results show that the BDZ-33 degradation DEP optimal pH and temperature of 8.0 and 15 ℃ respectively. The degradation concentration ranged from 100-400 mgꞏL⁻¹. The degradation efficiency and growth rate of the strain were higher under alkaline conditions than under acidic conditions. The best temperature below 20 ℃. The degradation rate decreases with the increase of temperature. After 60 h of culture, the degradation rate of DEP wastewater with a concentration of 400 mg/L reached 90%. The results showed that BDZ-33 could efficiently degrade DEP, which might be a promising DEP repair pathway.

1. Introduction

Diethyl phthalate (DEP) is a kind of phthalate (PAEs), which is widely used as plasticizer, building material and additive in the production of plastics and cosmetics[1]. DEP is one of most common used PAEs in the world. Three types of phthalic acid esters, di-methyl phthalate (DMP), di-n-butyl phthalate (DBP) and di-n-octyl phthalate (DOP) have been listed as priority pollutants by China National Environmental Monitoring Center and the US Environmental protection Agency[2]. PAEs have attracted much attention in recent years due to its extensive production, use and disposal. As a result they are widespread in the environment. In addition, these compounds have been shown to interfere with the reproductive systems of humans and animals, and to be carcinogenic and teratogenic. Phthalates (PAEs) are not only environmental pollutants, but also important endocrine disrupting compounds. In addition, DEP can be absorbed by crops and thus enter the food supply chain system, possibly endangering aquatic organisms and human health[3, 4]. DEP is one member of paes.

For the past few years, many studies have been carried out to find better methods for DEP degradation. Previous studies showed that the degradation pathways of DEP and DBP in the natural environment were mainly hydrolysis, photodegradation and biodegradation[5-7]. Studies have shown that the rate of DEP hydrolysis and photolysis in nature is very low, and microbial degradation is the main way of DEP degradation. PAEs degrading strains of *Rhodococcus, SpHingomonas, Microbacterium, Pseudomonas* and *Gordonia* have been isolated from different environments [8, 9]. In this study, we isolated a bacterium that can efficiently degrade DEP from activated sludge, identified it with 16S rDNA sequence, and studied its biodegradation kinetics and the influence of different environmental factors on the degradation process, so as to provide theoretical basis for improving the
biodegradation efficiency of DEP.

2. Materials and methods

2.1 Chemicals and reagents

The DEP used in this experiment was purchased from Kelong chemical reagent co., ltd. (ChengDu, China) with a purity of 99.5%. The rest of the chemical reagents are of analytical grade, and all solvents purchased from tianjin comeo reagents co., ltd. are of high performance liquid chromatography grade. The MM contained (1 L): FeCl₃ 0.0018 g, MgSO₄·7H₂O 0.5 g, (NH₄)₂SO₄ 1.0 g, K₂HPO₄ 1.70 g, Na₃MoO₄·0.0024 g, FeSO₄·7H₂O 0.05 g, NaNO₃ 0.5 g, CaCl₂·2H₂O 0.04 g.

The beef extract 3 g, peptone 5 g, NaCl 5 g, and pH 7.2 constituted the bacterial enriched nutrient solution (NB). Nutrient AGAR plates were prepared with NB with 2% AGAR.

2.2 Enrichment and isolation of DEP degrading bacteria

According to Wu[10] enrichment method, we made some modifications. Firstly, 5.0 g sludge and 200 ml MM solution of 100 mg/l DEP were added to 500 ml Erlenmeyer flask. Suspension at 25 ℃ rears six days of darkness. The rotary vibrating screen (rotating speed 140 RPM) was used for preliminary test. Then transfer 2 ml of rich bacteria into fresh medium cultured under the same conditions, and repeat culture for 5 times. During the transfer process, high concentration of DEP (200-500 mg/l) was contained each time. Finally add richment bands in the DEP mixture (500 mg/l) of the MM on the AGAR plate, at 25 ℃ incubation 1 week. The assumed colonies were modified and managed on re-streaked mm AGAR plates on the basis of colony morphology and color differences. The bacterial isolates were further purified by streaking on LB Nutrient Agar plates and then re-streaked onto MM agar plates with and without DEP to confirm their degradation abilities. The isolates can grow in the presence of DEP, but not in the absence of DEP.

2.3 Degradation experiment of BZD-33 strain

Through the analysis of the following environmental factors, the effects of them on the degradation of DEP in culture at the oscillation rate of 140 RPM for 60 h were studied. Temperature for 15, 20, 25, 30, 35, 40 and 45 ℃; Initial pH value for 4.0, 5.0, 6.0, 7.0, 8.0, 9.0; The initial concentration of DEP is 100 mg/l, 200 mg/l, 300 mg/l, 400 mg/l, 500 mg/l.

2.4 Determination of dep content

High performance liquid chromatography (HPLC) was used to determine the concentration of by-products in the supernatant (Aglient 1200 series). Column temperature was set to 40 ℃. Inject 40 l sample; Chromatography column(Inertsil ODS-2151-K.) was 6×150 mm.

2.5 Amplification of 16S rDNA

Bacterial genomic DNA was extracted into the extraction kit according to the manufacturer's instructions (Sangon Corporation, Shanghai, China). 16S rDNA gene sequencing was used for identification. Bacterial universal primers 27F (50-AGAGTTTTGATCCTGGCTCAG-30) and 1492R (50-GGCTACCTTGTAGACTT-30) PCR were used to amplify 16S rRNA with a length of about 1500 bp. PCR was performed(Bio-Rad USA) under the following conditions: preheated at 95 ℃ for 2 min; And then modified 1 min under 94 ℃, under 56 ℃ annealing 1 min, extends 3 min under 72 ℃, and 30 times, finally extended for 8 min in 72 ℃.

2.6 Sequence analysis of strain

Purified PCR products were sequenced directly. The sequence data of the closest relatives was retrieved in the NCBI database and aligned with CLUSTALW. The phylogenetic tree was constructed by using
neighborhood linkage method with MEGA 5.0. The trees were validated with 1000 repeated boot analyses.

3. Results and discussion

3.1 Isolation and identification of the DEP-degrading bacterium

After 35 days of enrichment, a strain with high biomass and high DEP degradation efficiency was isolated from the activated sludge for further study. According to Fig. 1, phylogenetic of the 16Sr RNA gene revealed strain BZD-33 clustered with members of the genus Pseudomonas. Its sequence similarity with Pseudomonas amygdale was 100%.

Figure 1. Phylogenetic trees are derived from BDZ-33 16S rRNA gene sequences and related species sequences. The distance is calculated by adjacency method. The number on the branch node is based on the bootstrap value of 1000 resamples. The scale indicates 0.002 replacements per site.

3.2 Effect of temperature on biodegradation of DEP

Based on pre-experiment, BDZ-33 was cultivated in condition of 25°C, pH 8, 500 mg/l, at a 140 rpm shaking rate. In the culture medium after 60 h, test the temperature influence on DEP degradation, the results showed that the optimum temperature range for the degradation of 15 °C to 25 °C. The temperature are not consistent with previous reports[11, 12]. At the first two experiment periods, the degradation was slow and increased after 36 h incubation. The degradation became slow after 60 h. There is no significant difference between 60 h incubation and 70 h incubation (P<0.05). In addition, 15°C was the optimal temperature for DEP degradation.
3.3 Effect of initial pH on biodegradation of DEP

To explore the effect of pH on strain degradation, we set different pH values. As shown in Fig. 3, the Effects of pH (4.0–9.0) on DEP biodegradation at an initial concentration of 400 mg/L. We observed that the consortium exhibited higher degradation efficiencies and growth rates in alkalinity than in acidity. The highest DEP degradation rate (approximately 89%) was achieved at pH 8.0. According to the report, the optimal pH value of other organic pollutants is between 7.0 and 8.0 [13, 14]. With the decrease of pH value from 8.0 to 4.0, the degradation rate of DEP decreased rapidly. When pH exceeded 9.0, the DEP degradation rate decreased slightly. The results showed that the optimal pH value for DEP degradation was 8.0, while 9.0 was the optimal pH value for BDZ-33 growth. The lower degradation rate and biomass under acidic conditions may be due to incomplete degradation of DEP by BDZ-33.
3.4 Effect of initial concentration on biodegradation of DEP
To determine the effect of initial DEP concentration on the degradation efficiency of bzd-33. Five different DEP concentrations were set in the experiment, namely 100, 200, 300 and 500 mg/l. Bacterial growth is sensitive to concentration. Fig. 4 showed, the DEP degradation rate decreased rapidly as concentration increased from 400 to 500 mg/l. In general, the degradation rate of bacterial strains was higher at different concentrations of 400 mg/l.

3.5 Degradation of DEP under optimal conditions
Results by above knowable, BZD-33 degradation DEP best temperature of 15 °C, pH value of 8. The degradation effect of DEP under this condition is shown in figure 5. The result illustrates the DEP degradation rate by BZD-33 was up to 94% after 6 d incubation. Fig. 5 also shows a characteristic degradation for DEP degradation at concentration of 500 mg/l in MM. After the initial concentration of 500 mg/l DEP was incubated for 6d, the DEP degradation rate of BZD-33 was the highest (about 94%). Existing research shows that paes fully biodegradable degradation strains in 30 °C condition. Therefore, this strain has greater mineralization potential.

4. Conclusions
A DOP-degrading strain BZD-33 was isolated from activated sludge. After 16S rRNA sequence analysis, it was identified as pseudomonas almon. Study the DEP degradation in MM optimum pH value, initial
concentration and temperature, biodegradable optimal pH value of 8.0, the temperature of 15 ℃. The optimal concentration should not exceed 400mg/l. This study also showed that under the appropriate pH and temperature conditions, the degradation ability of strain BZD-33 to DEP was up to 94% at 6 d, suggesting the existence of pseudomonas amygdalae. BZD-33 is a potential candidate for DEP degradation.

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