Biodegradation of Diphenyl ortho-phthalate DPP by *Pseudomonas sp.*BET-7 isolated from active sludge

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Abstract: Diphenyl ortho-phthalate (DPP), as a prior pollutant for control, has been widely detected in environment. One bacterial strain was isolated from activated sludge using Diphenyl ortho-phthalate as the sole source of carbon and energy. According to the phylogeny of 16S rDNA sequence, the bacterial strain BET-7 was identified as *Pseudomonas sp.* Biodegradation of DPP by *Pseudomonas sp.* BET-7 was investigated. Results showed that the optimum pH and temperature for DPP degradation by BET-7 were 8.0 and 15 ℃.The concentration of degradation ranged from 100 mg.L^{-1}-400 mg.L^{-1}. The strain exhibited higher degradation efficiencies and growth rates in alkalinity than in acidity. The optimum temperature was lower than 20 ℃. The degradation rate decreased when temperature went up .The strain could degrade approximately 90% of 400 mg/L DPP after 60 h of cultivation. The results suggest that BET-7 may represent a promising application for DPP bioremediation.

1.Introduction

Diphenyl ortho-phthalate DPP is the member of phthalic acid esters (PAEs), which are widely used as plasticizers, building materials, additives in plastics and cosmetics production [1]. Diphenyl ortho-phthalate DPP is one of most common used PAEs. Three of the phthalic acid esters, namly, 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 received increasing attention in recent years due to their widespread produce, use and disposal. Moreover, these compounds are concerning because they have been shown to interfere with the reproductive system of human and animal. Phthalate esters (PAEs) are a prominent group of environmental pollutants and endocrine-disrupting compounds in many environmental. In addition, DPP can be taken up by crops and thus enter the food supply chain system, which may harm aquatic organisms and human health [3,4,5]. DPP is one member of PAEs.

In recent years, many researches have been conducted to look for better way to degrade DPP including physical and chemical methods. Previous studies have revealed that DPP and DBP can be removed by natural processes in natural environments, such as hydrolysis, photo degradation and biodegradation [6,7,8]. Due to the low rate of chemical hydrolysis and photolysis of DPP, metabolic breakdown of this widespread pollutant by microorganisms is considered to be the major route. Several PAEs-degrading bacterial strains belonging to the genera *Sphingomonas*, *Pseudomonas, Rhodococcus, Microbacterium* and *Gordonia* have been isolated from different environments, such as active sludge [7,8,9,10].

In the present paper, a DPP-degrading bacterium was isolated from active sludge and identified by 16S rDNA sequence. The biodegradation kinetics and different environmental factors affecting this

process were investigated. The result from this study is expected to improve current understanding of the bioremediation of DPP and find more highly effective DPP-dergading strains.

2. Materials and methods

2.1. Reagents and chemicals
DPP (99.5% purity) for the experiment was purchased from Chengdu Kelong Chemical Reagent Co., Ltd., All the chemical reagents were of analytical grade and all solvents(Ethyl acetate and methanol) were of HPLC grade purchased from Tianjing kemiou Reagent Co., Ltd.

The MM contained (1L): $MgSO_4$ $7H2O$ 0.5 g, K_2HPO_4 1.70 g, $FeSO_4 \cdot 7H_2O$ 0.05 g, and $NaNO_3$ 0.5 g, $(NH_4)_2SO_4$ 1.0 g, Na_2MoO_4 0.0024 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, $FeCl_3$ 0.0018 g. The nutrient broth (NB) for bacteria enrichment consisted of beef extract 3g, peptone 5 g, NaCl 5 g, pH 7.2.Nutrient agar plates were made using NB supplemented with 2% agar.

2.2 Enrichment and isolation of DPP Strains
The enrichment procedure was according to Wu [11] with some modifications. Initially, 5.0 g of sludge was added to a 500-ml Erlenmeyer flask containing 200 ml of MM solution amended with concentration of 100 mg/l DPP. The suspension was incubated for 6 days in the dark at 25℃according to pre-experiment on a rotary shaker operated at 140 rpm. Subsequently, 2ml of the enrichment culture was serially transferred five times to fresh medium incubated under the same conditions. Then the final enrichment was streaked onto MM agar plates supplemented with a mixture of DPP (500 mg/l) and incubated 1 week at 25 ℃. Presumptive colonies were picked on the basis of differences in colony morphology and coloration and re-streaked onto MM agar plates amended with DPP.

2.3 Degradation experiments of BET-7
The following environmental factors were assayed to investigate their effects on DPP degradation within 60h of cultivation at a 140 rpm shaking rate. Temperature (15, 20, 25, 30, 35, 40 and 45 ℃); Initial pH value (4.0, 5.0, 6.0, 7.0, 8.0, 9.0); Initial DPP concentration(100mg/l,200 mg/l,300 mg/l,400 mg/l,500 mg/l).

2.4 Detection of DPP
Concentration of DPP in the supernatant solution was performed using high performance liquid chromatography (HPLC) (Aglient 1200 series). The column temperature was 40℃. The volume of the injected samples was 40μl. Chromatography column was Inertsil ODS-2151-K. 6× 150 mm.

2.5 Amplification of 16S rDNA
Extraction kit (Sangon Corporation, Shanghai, China) was used for the extraction of bacterial genomic DNA according to the manufacturer's instructions. Further identification was performed by 16S rDNA gene sequencing. and then about 1500 bp length of 16S rRNA was amplified through PCR by using the bacterial universal primer 27F (50-AGAGTTTGATCCTGGCTCAG-30) and 1492R (50-GGCTACCTTGTTACGACTT-30). PCR was performed (Bio-Rad USA) under the following conditions: preheated at 95 ℃ for 2 min; then denatured at 94 ℃ for 1 min, annealing at 56 ℃ for 1 min, extended at 72 ℃ for 3min for 30 cycles, last extended at 72℃ for 8 min.

2.6 Sequence analysis of strain
Purified PCR product was directly sequenced. The sequence data of the closest relatives were retrieved from NCBI database and aligned with CLUSTALW with all parameters set at their default values. A phylogenetic tree was then constructed using the neighbor-joining method with MEGA 5.0 software. The trees were validated using bootstrap analysis performed with 1000 replicates.

3. Results and discussion

3.1. Isolation and identification of the DPP-degrading bacterium

Following 35 days enrichment, several DPP-degrading strains were isolated from the activated sludge. One strain showed high biomass and high degradation efficiency was selected for further investigation. Phylogenetic of the 16Sr RNA gene (Fig.1) revealed strain BET-7 clustered with members of the genus Pseudomonas, and had a 100% sequence similarity with *Pseudomonas amygdale.*
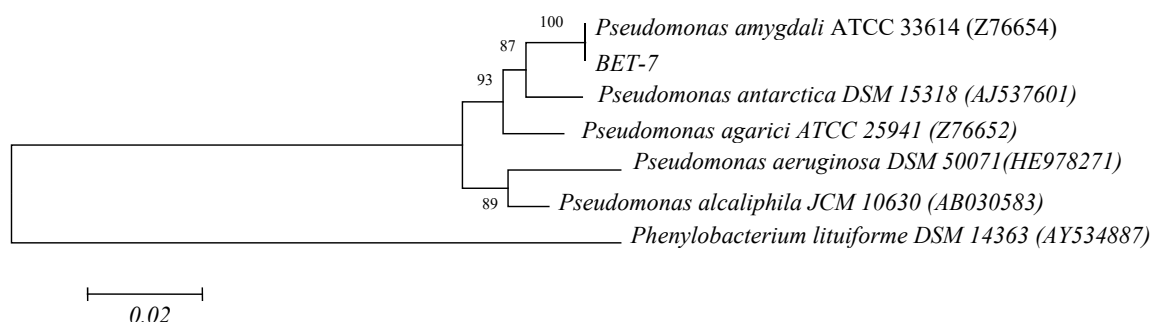

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence of BET-7 and sequences of related species.

3.2 Effects of temperature on DPP biodegradation

The strain was cultivated at condition of 25℃,500mg/l, pH8, at a 140 rpm shaking rate based on pre-experiment. The effects of temperature on the degradation of DPP in the culture medium were tested after incubation 60 h. The results showed that the optimal temperature for degradation ranged from 15 ℃ to 25 ℃. The temperature are not consistent with previous reports [13]. At the first two experiment periods, the degradation was slow and increased after 36h incubation. The degradation became slow after 60 h. There is no significant difference between 60 h incubation and 70h incubation ($P < 0.05$). The optimal temperature for DPP degradation was 15 ℃.
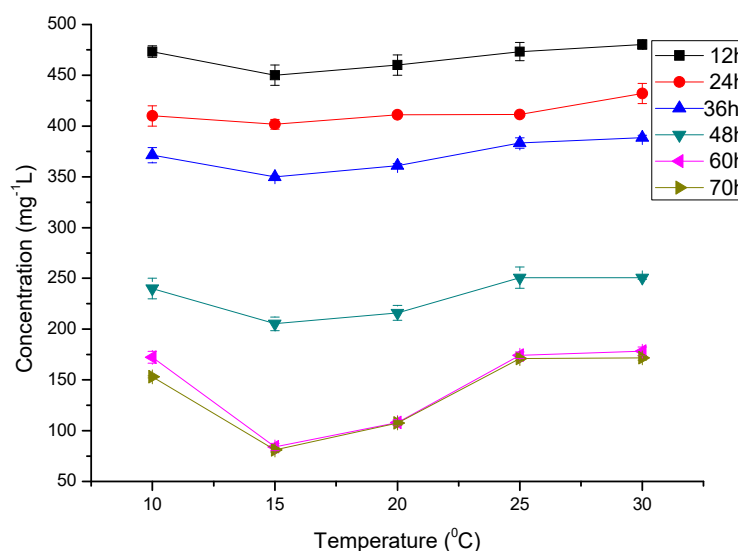

Fig. 2. Effect of temperature on degradation of DPP

3.3 Effects of initial pH on DPP biodegradation

In order to investigate the effect of pH on strain degradation, different pH value was setted.Fig.3.showed the Effects of pH (4.0–9.0) on DPP 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 DPP degradation rate (approximately 89%) was achieved at pH 8.0. The reported optimal pH values in degrading of various pollutants by other genus are ranging from 7.0 to 8.0 [12] [13].The degradation rate of DPP decreased rapidly when pH decreased from 8.0 to 4.0. When pH exceeded 9.0, the DPP degradation rate decreased slightly. The results indicate that pH of 8.0 and 9.0 are optimal for DPP degradation and growth.
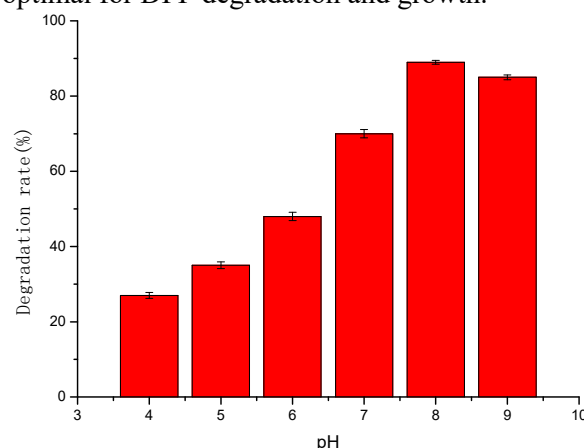

Fig. 3. Effect of pH and temperature on degradation of DPP

3.4. Effects of initial concentration on DPP biodegradation

In order to determine the effect of initial DPP concentrations on degrading efficiency by BET-7, experiment was conducted under DPP concentrations ranging 100, 200, 300,500 mg/l. Bacterial growth is concentration sensitive. As shown in Fig. 4 the DPP degradation rate decreased rapidly as concentration increased from 400 to 500mg/l.In general, the strain had a high degradation rate at the different concentration under 400 mg/l.
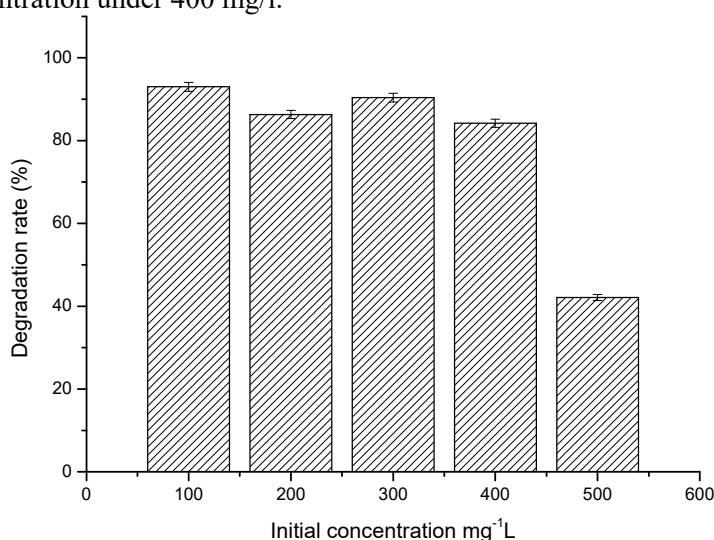

Fig. 4. Effect of initial concentration on degradation of DPP

3.5 Degradation of DPP at optimal condition

According to the above results, the temperature of 15℃ and pH of 8 are the optimum conditions for DPP degradation by BET-7. The effect of DPP degradation under this condition was showed in Fig.5.The result illustrates the DPP degradation rate by BET-7 was up to 94% after 6d incubation. Fig.

5 also displays a characteristic degradation for DPP degradation at concentration of 500mg/l in MM. After 6d incubation at an initial concentration of 500 mg/l DPP, the highest DPP degradation rate (approximately 94%) for BET-7 was achieved.
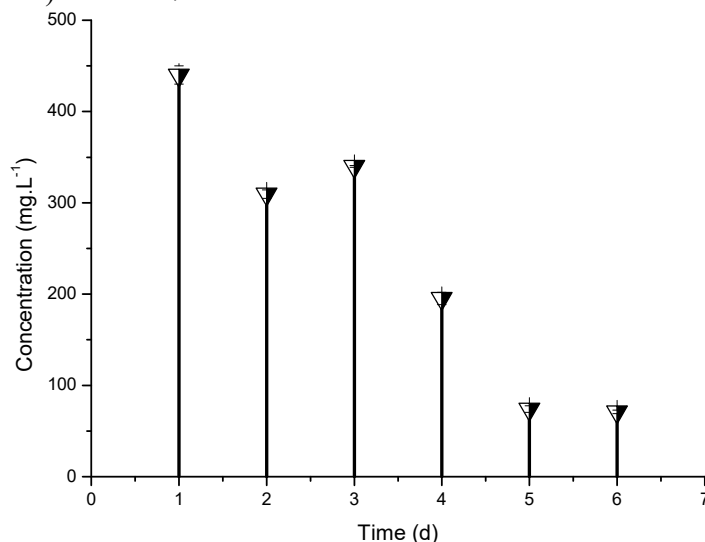

Fig.5. Degradation of DPP at optimal condition

4. Conclusions

A strain BET-7 that can degrade DPP was isolated from activated sludge. Based on 16S rRNA sequence analysis, the strain was identified as *Pseudomonas amygdali*. This study investigated the optimal pH, initial concentration and temperature for DPP degradation in MM. The optimum biodegradation pH and temperature was 8.0 and 15℃, respectively. The optimal concentration was no more than 400mg/l.The study also showed under optimal pH and temperature condition, strain BET-7 was capable of degrading DPP up to 94% in 6d incubation, which suggests that *Pseudomonas amygdali*. BET-7 is a potential candidate for DPP degradation.

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