

# Degradation of Di-N-Butyl Phthalate by Microbacterium Aoyamense Atpm-11 Isolated from Waste Water Treatment Plant

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**Abstract:** An efficient di-n-butyl phthalate bacterial strain ATPM-11 was isolated from activated sludge of waste water treatment plant (WWTP). Based on its morphological, physiobiochemical characteristics and 16S rRNA gene sequence, strain ATPM-11 was identified as *Microbacterium aoyamense* sp. The degradation characteristics were investigated under different environmental conditions. The results showed that the optimal temperature and pH for DBP degradation by ATPM-11 was 25 °C and 8.0, respectively. Under these conditions, ATPM-11 could effectively degraded more than 83% of DBP at 400 mg/L. The diversity of degradable substrates showed that strain ATPM-11 could degrade phthalate (DMP), DEP and DOP efficiently. Therefore, this bacterial strain has potential to be used in DBP bioremediation.

## 1 INTRODUCTION

Phthalic acid esters (PAEs), one of the synthetic organic compounds, are the widely used and a higher productivity as plasticizers, adhesive, additives, paint solvent and Printing inks in the world (Li et al., 2005). However, with the broad use of plastic products, the phthalic acid esters abound in the environment and they can migrate into the soil and rainwater, thus enter the water system, which may harm aquatic organisms and human health (Bai et al., 2012). 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 (Wang et al., 2008). PAEs can be degraded by chemical and physical methods, but microbial technology was regarded as the most efficient way due to its high efficiency and low toxicity (Wan, 2012). The hydrolysis and photolysis of DBP in the natural environment are very slow and are difficult to degrade. The physical method mainly consists of humic acid or activated carbon adsorption, relying on the strong pore structure and adsorption capacity of adsorbent to remove DBP in water (Li et al., 2013). The chemical method is mainly

photocatalytic degradation, which is the removal of DBP in water by ultraviolet light. Although physical and chemical methods have a good effect on the removal of DBP in water body, there are obvious defects, such as the final destination of DBP attached to the adsorbent. In comparison, the biological method is low cost and high efficient. (Guo et al., 2007; Ding, 2012; Zheng et al., 2007)

Presently, several PAEs-degrading bacterial strains belonging to the *Gordonia* sp. (Sarkar et al., 2013), *Enterobacter* sp. (Fang et al., 2010) and *Arthrobacter* sp. (Wen et al., 2014). They can be isolated from different environments, while their degrading efficiencies in other PAEs were low and far from meeting the actual pollution control requirements. Therefore, in order to improve the biodegradation rate of phthalate esters, it's necessary to isolate highly effective degradation bacteria (Li et al., 2014).

In the study, a DBP-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. And this study is expected to improve current understanding of the bioremediation of DBP and find higher effective DBP-degrading strains.

## 2 MATERIALS AND METHODS

### 2.1 Reagents and Chemicals

DBP (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 China. The minimal medium (MM) contained (1L): MgSO<sub>4</sub> 7H<sub>2</sub>O 0.5 g, K<sub>2</sub>HPO<sub>4</sub> 1.70 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.05 g, and NaNO<sub>3</sub> 0.5 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.0 g, Na<sub>2</sub>MoO<sub>4</sub> 0.0024 g, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.04 g, FeCl<sub>3</sub> 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 Nutrient Broth (NB) supplemented with 2% agar.

### 2.2 Enrichment and Isolation of Dbp Strains

The enrichment procedure was according to Wu (Wu et al., 2010) 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 DBP. The suspension was incubated for 6 days in the dark at 25 °C 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. At the same time, in the process of transfer, containing a higher concentration of DBP 200–500 mg/L each time. Then the final enrichment was streaked onto MM agar plates supplemented with a mixture of DBP (500 mg/L) and incubated 1 week at 25 °C. Presumptive colonies were picked on the basis of differences in colony morphology and coloration and re-streaked onto MM agar plates amended with DBP. The bacterial isolates were further purified by streaking on Nutrient Agar plates and then re-streaked onto MM agar plates with and without DBP to confirm their degradation abilities.

Isolates can grow in the presence of DBP but not in their absence were selected for further study.

### 2.3 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 (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). PCR was performed (Bio-Rad USA) under the following conditions: preheated at 95 °C for 2 min; then denatured at 94 °C for 1 min, annealing at 56 °C for 1 min, extended at 72 °C for 3min for 30 cycles, last extended at 72 °C for 8 min.

### 2.4 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 6.0 software. The trees were validated using bootstrap analysis performed with 1000 replicates.

### 2.5 Degradation Experiments of *Microbacterium Aoyamense* ATPM-11

The following environmental factors were assayed to investigate their effects on DBP degradation within 60 h of cultivation at a 140 rpm shaking rate. Temperature (10, 15, 20, 25 and 30 °C); Initial pH value (4.0, 5.0, 6.0, 7.0, 8.0, 9.0); Initial DBP concentration (100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L). Other PAEs (DOP, DEP, DMP, DEHP and DPP).

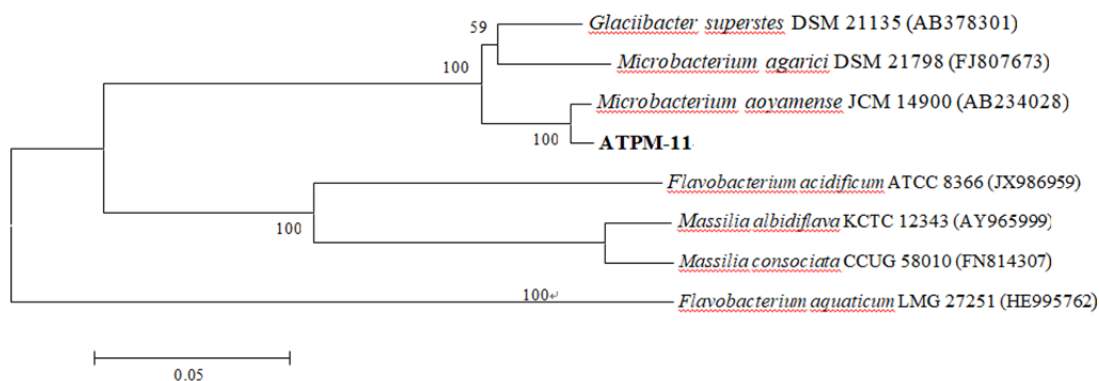


Figure 1: Phylogenetic tree derived from 16S rRNA gene sequence of ATPM-11 and sequences of related species. Distances were calculated using neighbor-joining method.

## 2.6 Analysis Method

Concentration of DBP in the supernatant solution was performed using high performance liquid chromatography (HPLC) (Aglient 1200 series). The column temperature was 40°C. The volume of the injected samples was 40µl; Chromatography column was Inertsil ODS-2151-K. 6× 150 mm. Ethyl acetate was added to each sample, which was vigorously shaken for 5 min, the aqueous and organic phases were separated by centrifugation at 8000 rpm for 5 min. The aqueous phase was extracted twice with equal volume of ethyl acetate. Ethyl acetate phase was dried over anhydrous sodium sulfate and evaporated, then dissolved in 10 ml of methanol.

## 3 RESULTS AND DISCUSSION

### 3.1 Isolation and Identification of the DBP-degrading Bacterium

After 35 days enrichment, one strain showed high biomass and high degradation efficiency was selected for further investigation. Phylogenetic tree of the 16Sr RNA gene revealed strain *Microbacterium aoyamense* ATPM-11 clustered with members of the genus *Microbacterium*, and had a 100% sequence similarity with *Microbacterium aoyamense* JCM 14900 (AB234028) (Figure 1).

### 3.2 Effects of Temperature on DBP Biodegradation

The strain was cultivated under condition of 25°C and pH 8. The DBP (500 mg/L) degradation efficiencies under 10, 15, 20,25and 30 °C were tested. The effects of temperature on the degradation of DBP in the culture medium were tested after incubation 60 h. The results indicate that the degradation is best at the temperature of 25 °C, and The degradation rate was 83% (Figure 2). The degradation rate was only 30%. There is no significant difference between 20 °C and 25 °C (P<0.05).

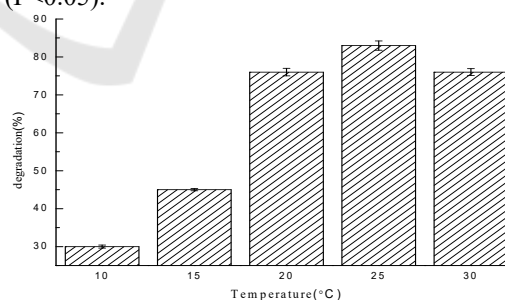


Figure 2: Effects of temperature on DBP biodegradation.

### 3.3 Effects of Initial pH on DBP Biodegradation

Figure 3 shows the results of pH (4.0–9.0) on DBP biodegradation at an initial concentration of DBP at 400 mg/L. Strain could effectively degraded DBP when pH ranged from 7.0 to 9.0. The optimal

degradation pH for DBP degradation by this bacterial strain were 8.0. The degradation rate could reach up to 93%. The degradation under acid conditions is poor. The degradation under acid conditions is poor. The optimal pH values in degrading of DBP are consistent with the other study, where the optimal pH for Di-n-butyl phthalate (DBP) Degradation by strain H-2 ranged from 7.0 to 9.0. (Lei et al., 2014).

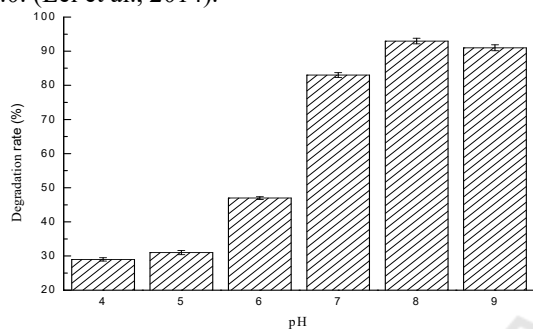


Figure 3: Effects of initial pH on DBP biodegradation.

### 3.4 Effects of Initial Concentration on DBP Biodegradation

The experiment was conducted under different DBP concentrations (100,200,300,400,500,600 mg/L) to investigate the influence of concentration on DBP degradation rate. As shown in Figure 4, when the initial concentration was 200 mg/L, *Microbacterium aoyamense* ATPM-11 had the highest degradation rate of DBP. The degradation rate was slightly lower than 200 mg/L when the concentration was 100mg/L. when the concentration gradually increased from 200mg/L to 600mg/L, the degradation rate reached the lowest value of 69%.

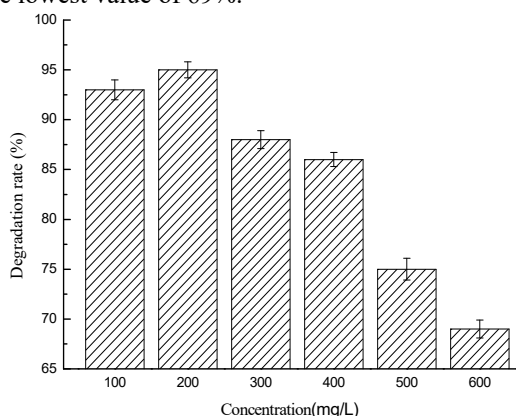


Figure 4: Effects of initial concentration on DBP biodegradation.

### 3.5 Degradation of Other PAEs by Bacteria

In order to investigate the degradation ability of the consortium to other commonly used PAEs in environment, the consortium was cultured in MSM supplemented with DBP, DOP, DEP, DMP, DEHP and DPP at 30 °C. Figure 5 shows that *Microbacterium aoyamense* ATPM-11 can also degrade the other APEs. The strain could degraded DEP and DMP with high efficiency up to 94 %.

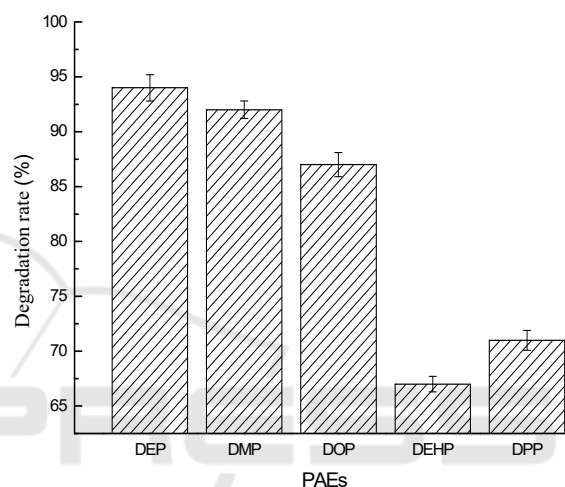


Figure 5: Degradation of other commonly used PAEs by bacteria.

## 4 CONCLUSIONS

A broad-spectrum and efficient di-n-butyl phthalate (DBP)-degrading bacterial strain *Microbacterium aoyamense* ATPM-11 was isolated from activated sludge of waste water treatment plant (WWTP). The strain *Microbacterium aoyamense* ATPM-11 could completely degrade DBP and the degradation rate was up to 93%. The temperature 25 °C and pH 8.0 are the optimal conditions for DBP degradation by strain ATPM-11. *Microbacterium aoyamense* ATPM-11 degrades DBP faster in alkalinity than in acidity. The strain *Microbacterium aoyamense* ATPM-11 could also degrade other commonly used phthalates like (DMP), DEP and DOP.

## REFERENCES

- Bai YH, Xu K and Zhao DB 2012 Exposure assessment of Di (2-ethylhexyl) phthalate of plastic food packaging materials. *Modern Food Science and Technology* **28(10)** 1423-1428
- Ding K 2012 Experimental research on carbon for the adsorption of DBP from water and dielectric barrier discharge [D]. *Nanchang: Nanchang University*
- Fang CR, Yao J, Zheng YG, Hu LF and Jiang CJ 2010 Dibutyl phthalate degradation by *Enterobacter* sp. T5 isolated from municipal solid waste inland fill bioreactor. *International Biodeterioration and Biodegradation* **64(6)** 442-446
- Guo D, Li Y, Zhao Y and Zhou J 2007 Activated carbon adsorption of di-n-butyl phthalate, nonyl phenol and bisphenol A in Yellow River water. *Water Wastewater Eng* **33(1)** 30-33
- Li JL, Shao XL, Liu SL, Yao K, Zhao Q, Hu XJ and Deng WQ 2014 Screening and identification of dibutyl phthalate degradation strains. *Modern Food Science and Technology* **30(10)** 10-113
- Li JX, Gu JD and Li P 2005 Transformation of dimethyl phthalate, dimethyl isophthalate and dimethyl terephthalate by *Rhodococcus ruber* Sa and modeling the process using the modified Gompertz model. *International Biodeterioration and Biodegradation* **55(3)** 223-232
- Li YH, Yi YC, Wu J, Wu J and Na W 2013 Isolation of a humus-reducing bacterium strain and its biodegradation of pollutants [J]. *Chin J Appl Environ Biol* **19(3)** 506-510
- Lei J, Chen Y, Long J, Guo YM, Yan ZY, Zhu Y and Gu PQ 2014 Isolation and Identification of a Di-n-butyl phthalate (DBP)-Degrading Strain H-2 and Its Degradation Characteristics. *Journal of Food Science* **35(15)** 203-206
- Sarkar J, Chowdhury P P and Dutta T K 2013 Complete degradation of di-n-octyl phthalate by *Gordonia* sp. strain Dop5 [J]. *Chemosphere* **90** 2571-2577
- Wan Y 2012 Study on Adsorption-desorption characteristics of dibutyl-phthalate on humic acid [D]. *Chongqing: Southwestern University*
- Wang P, Wang S and Fan CQ 2008 Atmospheric distribution of particulate- and gas-phase phthalic esters (PAEs) in a metropolitan city, Nanjing, East China, *Chemosphere* **12 (72)** 1567-1572
- Wen ZD, Gao DW and Wu WM 2014 Biodegradation and kinetic analysis of phthalates by an *Arthrobacter* strain isolated from constructed wetland soil. *Applied Microbiology and Biotechnology* 1-8
- Wu XL, Liang RX, Dai QY, Jin DC, Wang YY and Chao WL 2010 Complete degradation of di-n-octyl phthalate by biochemical cooperation between *Gordonia* sp. strain JDC-2 and *Arthrobacter* sp. strain JDC-32 isolated from activated sludge. *Journal of Hazardous Materials* **176** 262-268
- Zheng HH, Zhao LW and Tao J 2007 Photo degradation of dibutyl phthalate in flowing water. *Chin J Public Health* **23(2)** 210-211