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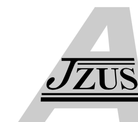

# Isolation and characteristics of *Arthrobacter* sp. strain CW-1 for biodegradation of PAEs

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**Abstract:** Isolation of new bacterial strains and recognition of their metabolic activities are highly desirable for sustainability of natural ecosystems. Biodegradation of dimethyl phthalate (DMP) under anoxic conditions has been shown to occur as a series of sequential steps using strain CW-1 isolated from digested sludge of Sibao Wastewater Treatment Plant in Hangzhou, China. The microbial colony on LB medium was yellowish, 3~5 mm in diameter, convex in the center, and embedded in mucous externally. The individual cells of strain CW-1 are irregular rods, measuring (0.6~0.7)×(0.9~1.0) μm, V-shaped, with clubbed ends, Gram positive and without any filaments. 16S rDNA (1438 bp) sequence analysis showed that the strain was related to *Arthrobacter* sp. CW-1 and can degrade PAEs utilizing nitrate as electron acceptor, but cannot mineralize DMP completely. The degradation pathway was recommended as: dimethyl phthalate (DMP)→monomethyl phthalate (MMP)→phthalic acid (PA). DMP biodegradation was a first order reaction with degradation rate constant of 0.3033 $d^{-1}$ and half-life 2.25 d. The DMP conversion to PA by CW-1 could be described by using sequential kinetic model.

**Key words:** Denitrifying bacteria, *Arthrobacter* sp. strain CW-1, Dimethyl phthalate (DMP), Phthalic acid (PA), DMP degradation pathway

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## INTRODUCTION

Phthalic Acid Esters (PAEs) are a class of refractory organic compounds that are widely used in manufacturing PVC to increase plasticity and intensity of the products. Because of the bulk application of the plastic products, phthalates (metabolites of PAEs) have been frequently detected in sediments, natural waters, soils, plants, aquatic organisms and human bodies (Atlas and Giam, 1981; Vikelsøe *et al*., 2002; Koch *et al*., 2003). Some of the phthalates and their metabolites are alleged to be carcinogenic, teratogenic and endocrine-disrupting substances (Booker, 2001; Wezel *et al*., 2000). PAEs have been listed as priority pollutants and endocrine-disrupting compounds by the China National Environmental Monitoring Center and the US Environmental Protection Agency. Therefore, phthalates have recently aroused much attention to be eliminated from the environment.

Microbial action has been assumed to be the principal mechanism for PAE degradation in both aquatic and terrestrial systems (Staples *et al*., 1997), and many investigations have shown biodegradation of PAEs in the soils, natural waters and wastewaters under aerobic and anaerobic conditions (Chatterjee and Dutta, 2003; Shen *et al*., 2004; Cartwright *et al*., 2000; Chang *et al*., 2005). Since nitrate exists in cropland (Li *et al*., 2002), so nitrate can be promising electron acceptor during biodegradation of PAEs. However, little information is available on biodegradation of PAEs in the presence of nitrate.

Thus PAEs biodegradation through nitrate reducing bacteria in soils can serve as a new biotechnology for abating PAEs pollution and can facilitate elimination of PAEs and nitrate from polluted environments. In the present study, we isolated a nitrate reducing bacterial strain to accomplish the biodeg-

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radation of PAEs, and proposed PAEs biodegradation pathway under denitrifying conditions.

## MATERIALS AND METHODS

### Enrichment and isolation of CW-1

The digested sludge used as inoculum for the present research was collected from Sibao Wastewater Treatment Plant in Hangzhou, China. The minimal medium used for isolation consisted of $CaCl_2·2H_2O$ (0.3 g/L), $Na_2HPO_4·2H_2O$ (0.065 g/L), $MgCl_2·6H_2O$ (0.165 g/L) and 1.25 ml/L trace element solutions I and II respectively (Qian and Min, 1986). The minimal medium used for enrichment contained 300 mg/L of $NO_3^-$ as electron acceptor and 100 mg/L of dimethyl phthalate (DMP) as the sole carbon and energy source. The sludge was put in a 4 L glass bottle and enrichment medium was applied on daily basis in draw and fill mode, and the culture was flushed with argon gas for 10 min before incubation at 30 °C each time to create anaerobic conditions. Enrichment was carried out for 6 months.

After enrichment, various dilutions of sludge were spread onto the solid minimal medium containing DMP and nitrate. The plates were incubated at 30 °C for 48 h under anoxic condition created by pyrogallic acid and sodium hydroxide (Zheng, 2005). The separated colonies were serially picked up and transferred onto the minimal medium repeatedly until pure isolate was obtained. After isolation, the CW-1 strain was transferred onto LB medium for preservation and further use.

LB culture medium containing beef yeast 30 g/L, peptone 50 g/L, NaCl 30 g/L, and 1.0 mol/L NaOH was used to adjust pH at 7.2.

### Phenotypic characterization

Selected colonies were singled out from LB agar plate into 1 ml distilled water. The bacterial suspension was stained by 1% to 2% solution of phosphotungstic acid (PTA) in a pH range of 6.5 to 7.0 for 15 to 30 s. By using a light microscope (TEM) (JEM1230, Japan), observations on cell morphology of CW-1 strain were carried out.

### 16S rDNA gene sequencing and phylogenetic analysis

16S rDNA analysis was carried out according to protocol developed by Ruan *et al*.(2005). DNA of the isolate was extracted by a slightly modified procedure of Wilson (1997). 16S rDNA genes were amplified using PCR with Taq polymerase (Boya) and the universal primer pair of 20F (5′-AGAGTTTGATCCT GGCTCAG-3′) and 1500R (5′-GGTTACCTTGTTA CGACTT-3′) described by Weisburg *et al*.(1991), and determined by the Shanghai Boya Biological Technique Company. Related sequences were obtained from the GenBank database of the NCBI (National Center for Biotechnological Information). Novel sequences were gapped, and unique sequences were subjected to a Blast search against Gene Bank. The final phylogenetic tree was constructed with the help of MegAlign software of DNASTAR based on the 16S rDNA sequences of 10 strains close to strain CW-1.

### Experiments for DMP biodegradation

One of the pure isolates (strain CW-1) was selected and pre-cultured in 100 ml sterile LB culture medium on a rotary shaker at 120 r/min under photophobic conditions. After 1 d, the cells were harvested by centrifugation at 10000 r/min for 5 min at 4 °C, and then washed with 10 ml of $K_2HPO_4$-$KH_2PO_4$ buffer solution (0.02 mol/L) under sterile conditions. Centrifugation and washing with buffer were repeated thrice. One-hundred ml cell suspension was prepared as inoculum by using $K_2HPO_4$-$KH_2PO_4$ solution.

The concentration of the minimal medium and $NO_3^-$ was the same as that used for enrichment of culture and transferred to the serum bottle. After autoclaving at 121 °C for 30 min, the filter sterilized DMP (100 mg/L) was allowed to cool down. Fifteen ml inoculation sample was added into the 150 ml capacity serum bottle. Then the culture was flushed with argon gas for 10 min to drive the oxygen out. Sterile conditions were ensured in carrying out all these steps to avoid contamination.

All experimental cultures were incubated at fixed temperature (30 °C) under photophobic conditions. All experiments were conducted in duplicate along with their uninoculated controls.

Aqueous samples were periodically withdrawn by sterile syringes, and centrifuged at 6000 r/min for 5 min. Aqueous solution was extracted through 0.22 μm membrane and was subsequently analyzed with high-pressure liquid chromatography (HPLC).

**Analytical procedures**

The samples were analyzed to determine the amounts of DMP through HPLC (Agilent 1100 Series, USA) equipped with Diotron array Detector and Zorbax SB C18 (4.6×150 mm, 5 μm) Chromatography column.

The mobile phase was methanol-0.5% of acetic acid solution (50:50, v/v) at flow rate of 1.00 ml/min. UV detector and detecting wavelength was set at 254 nm.

Liquid chromatography mass spectrometry (LC-MS) was performed by using Agilent 1100 SL machine. The mass spectrometry was operated with ESI ionization source, positive mode, mass scanner range of 100~300 amu, fragment at 100 V; drying gas flow was 13.0 L/min; nebulizer pressure was 60 Psig; drying gas temperature was 350 °C and capillary voltage was 4000 V. The rest of the analytical methods were identical to HPLC. The reaction kinetics was calculated according to (Bittrich *et al*., 1979).

## RESULTS AND DISCUSSIONS

**Isolation and identification of strain CW-1**

Colonies formed on agar plates of LB media (see "Materials and Methods") under anaerobic conditions were subjected to be studied genetically by means of PCR-amplified 16S rDNA fragments. The partial 16S rDNA sequence of the strain (comprising 1438 nucleotides) was determined. The sequence was submitted to the GenBank database where accession No. DQ234386 was allotted for the said sequence. A phylogenetic tree was constructed based on the 16S rDNA sequence as described in "Materials and Methods". Phylogenetic tree for strain CW-1 based on 16S rDNA sequences is shown in Fig.1.

Database search with the 16S rDNA sequences showed *Arthrobacter ardleyensis* to be the closest relative of strain CW-1, because it was clustered closely with it. The bacterial strain thus obtained was designated as *Arthrobacter* sp. CW-1.

**Characterization of isolate**

The cells of DMP-degrading *Arthrobacter* sp. strain CW-1 are irregular rods, measuring (0.6~0.7)×(0.9~1.0) μm. Individual cells are V-shaped, with clubbed ends, Gram positive and without any filaments. Some smaller rods with round shape (cocci) were also found in the culture (Fig.2), arranged singly, in pairs, or in irregular clusters. The morphological characteristics of strain CW-1 are shown in Fig.2. The colony on LB medium was yellowish, 3~5 mm in diameter, convex in the center having mucous on the surface.

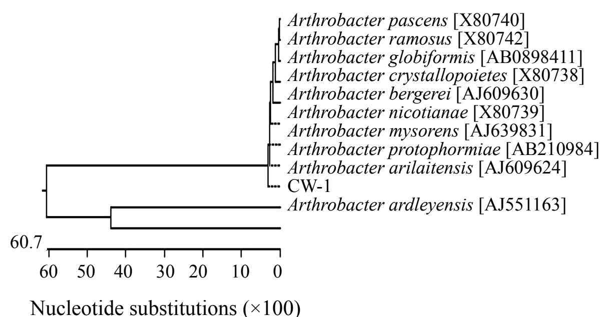


**Fig.1 Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain CW-1 in the Group 20, irregular, nonsporing Gram-positive rods. The scale indicates percent of substitutions per nucleotide position, and the numbers in parentheses are GenBank database accession numbers**

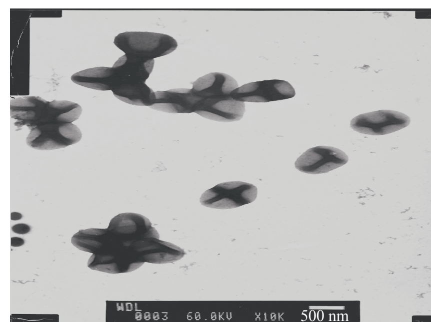


**Fig.2 Electron micrograph of strain CW-1**

**Biodegradation of DMP under anoxic conditions**

Comprehensive screening protocols, such as GC-MS or LC-MS of fractionated samples, can identify several hundreds of individual components in complex environmental samples, and combined with the potential of tandem mass fragmentation (MS) technique might be used to identify unknown compounds. In the present study, the components of culture liquid were analyzed through HPLC and LC-MS techniques. The results demonstrated that there were three noticeable peaks with retention times at 2.344, 2.986 and 4.881 min respectively in the MS (Fig.3).

Analysis by LC-MS showed that the phthalates could be predictably cleaved at the carbon-carbon bonds between the aromatic ring and the carbonyl

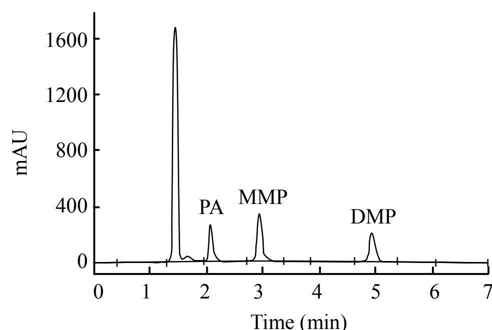


**Fig.3 HPLC chromatograms of DMP degradation by strain CW-1. Peaks show the presence of PA, DMP and MMP in minimal medium**

groups and the carbon-oxygen bonds linking the carboxyl groups to the alkyl chains. Cleavage of these bonds in DMP would yield fragments of molecular mass 166 and 180, and a phthalate possessing a methyl alkyl chain would produce all two fragments. LC-MS analysis of commercial standards confirmed the predicted fragmentation, with peaks 167.1 m/z and 181.1 m/z present in the DMP mass spectrometry profile, and other peaks detected in the DMP profile.

The peak at 4.881 min had the same retention time as that of the standard DMP sample; the MS displayed molecular ion peak (DMP+H) at 195.1 m/z. Other discernible peaks in the mass spectrum were 217.1 m/z (DMP+Na), 233.1 m/z (DMP+K), so the peaks were identified as DMP.

The retention time for the peak at 2.344 min is analogous to that for PA standard sample; the MS exhibited molecular ion peak (PA+H) at m/z 167.1. In this minimal culture liquid, other discernible peaks in the mass spectrum above 200 m/z [i.e. 205.1 m/z (PA+K), 206.1 m/z (PA+Ca), 227.1 m/z (PA+Na+K), 243.0 m/z (PA+K+Ca−2H), 245.0 m/z (PA+K+Ca)] were identified as PA.

For the peak at retention time of 2.986 min, the MS displayed molecular ion peak (MMP+H) at 181.1 m/z, while other discernible peaks in the mass spectrum were 203.1 m/z (MMP+Na), 219.1 m/z (MMP+K), 220.1 m/z (MMP+Ca), 241.1 m/z (MMP+Na+K), 257.0 m/z (MMP+K+Ca−2H), 259.0 m/z (MMP+K+Ca); these MS peaks indicated the presence of MMP.

DMP, MMP and PA were also detected in the culture liquid. It could be deduced that MMP and PA may be the first and second intermediates during DMP biodegradation under anoxic conditions.

Phthalate has the basic structure of an esterified benzene dicarboxylic acid with two alkyl chains. Commonly, primary biodegradation of the phthalate involves the sequential hydrolysis of the ester linkage between each alkyl chain and the aromatic ring, forming first the monoester and subsequently phthalic acid (PA). Wang *et al*.(1999) reported that under anaerobic conditions, mono-*n*-butyl phthalate (MBP) and PA were the ultimate products of di-butyl phthalate (DBP) hydrolysis in the presence of nitrate. Xia *et al*.(2002) also found that the PA was the intermediate compound during anaerobic biodegradation of PAEs.

In our present investigations, we obtained MMP and PA in minimal medium when DMP and nitrate were supplied in the minimal culture. It implies that *Arthrobacter* sp. CW-1 utilized DMP as electron donor along with nitrate as electron acceptor to hydrolyze DMP into MMP and PA under anoxic conditions.

**Variations in the DMP, MMP and PA concentrations and dynamics of DMP biodegradation**

*Arthrobacter* sp. CW-1 utilized DMP under anoxic conditions because its concentration progressively decreased from 0.539 mol/L to 0.053 mol/L within 8 d in the culture liquid (Fig.4). Simultaneously, MMP concentration gradually increased initially and then decreased after reaching its peak. Furthermore, the PA concentration steadily increased with the passage of time. The results have established the pathway for DMP biodegradation under anoxic conditions, and have also revealed that DMP could not be mineralized to $CO_2$ by *Acthrobacter* sp. strain CW-1.

The DMP biodegradation curve in Fig.4 fits well the equation for first order kinetic reaction. The result showed that the degradation rate constant is 0.3033 $d^{-1}$ and that the degradation half-life is 2.25 d. During DMP biodegradation, mono-methyl phthalate concentration increased up to the peak 0.216 mol/L, and then decreased to 0.048 mol/L after 3 d. PA concentration consistently increased to 0.484 mol/L within 8 d. The total concentration of DMP, MMP and PA remained steady during the experiment. It can be concluded that the DMP could only be hydrolyzed by *Arthrobacter* sp. strain CW-1 due to lack of enzyme for the biodegradation of PA. On the other hand, PA amassing affects the further biodegradation of DMP.

The research results of Shen *et al.*(2004) also proved that DMP could not be biodegraded further while severing as the sole carbon source for accumulating PA. The overall conversion from DMP to PA could be described with sequential kinetic model:

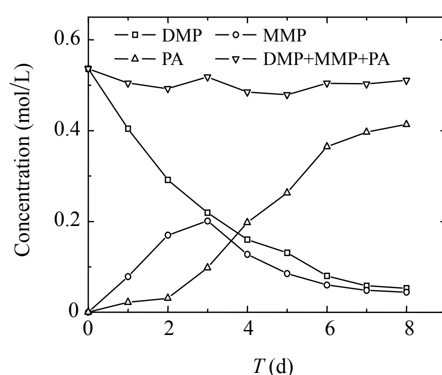


**Fig.4 Variation of DMP, MMP and PA in the presence of *Arthrobacter* sp. CW-1**

Differential rate equations for the sequential kinetic model can be constructed as follows:

$$-\mathrm{d}(DMP)/\mathrm{d}t=k_1C_{\mathrm{DMP}},$$
$$\mathrm{d}(MMP)/\mathrm{d}t=-k_1C_{\mathrm{DMP}}+k_2C_{\mathrm{MMP}},$$
$$\mathrm{d}(PA)/\mathrm{d}t=k_2C_{\mathrm{PA}},$$

where $C_{\mathrm{DMP}}$ is DMP concentration (mol/L); $C_{\mathrm{MMP}}$ is MMP concentration (mol/L); $C_{\mathrm{PA}}$ is PA concentration (mol/L); $k_1$, $k_2$ are biodegradation constants.

The differential rate equations yield the following integral rate equations:

$$C_{\mathrm{DMP}}=0.531\mathrm{e}^{-0.315t},$$
$$C_{\mathrm{MMP}}=0.531\mathrm{e}^{-0.315t}-0.531\mathrm{e}^{-0.63t},$$
$$C_{\mathrm{PA}}=0.531-1.062\mathrm{e}^{-0.315t}+0.531\mathrm{e}^{-0.63t},$$

where $t$ is biodegradation time.

## Variations in pH of the medium and growth of strain CW-1

Since DMP cannot be mineralized completely in the absence of anoxic conditions, hence resulted in the PA accumulation in higher amounts in culture medium. This resulted in decreased pH value of growth medium from 7.13 to 6.40. Conversely, DMP degradation in the presence of nitrate results in increased pH value due to PAEs mineralisation and the continual consumption of protons which contradicts the results of Chang *et al.*(2005). This variation in pH value might be due to incomplete biodegradation of DMP and the accumulation of PA.

The growth of a bacterial strain can be measured by using spectrophotometer at 600 nm absorbance, called as optical density or $OD_{600}$. Its initial $OD_{600}$ value was 0.138. The growth rate remained steady throughout the experiment. This constant growth rate during the experiment implies that *Arthrobacter* sp. strain CW-1 could only hydrolyze DMP to PA, but could not utilize DMP as energy and carbon source to support its growth. The pH variations and growth rate for *Arthrobacter* sp. strain CW-1 during the biodegradation of DMP are shown in Fig.5.

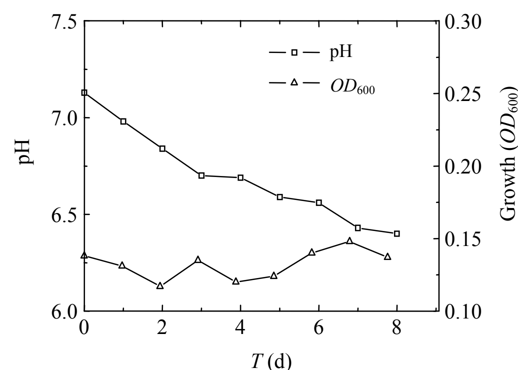


**Fig.5 Variations in the pH of growth medium and growth rate ($OD_{600}$)**

## DMP biodegradation pathways under anoxic conditions

Although results suggest that MMP and PA are the ultimate products after treatment of CW-1 colonies utilizing DMP and nitrate under anoxic conditions, strain CW-1 failed to attack PA in the absence of oxygen; the PA could not be further mineralized. When enriched sludge was used as inoculum in our research, DMP could be mineralized to $CO_2$ while PA did not accumulate in the culture medium. The degradation pathways are in consistence with the results of previous studies (Wang *et al.*, 1999; Xia *et al.*, 2002; Xu *et al.*, 2005; Levén and Schnürer, 2005). Based on the discussion above, the possible DMP degradation pathway proposed for strain CW-1 under anoxic conditions is shown in Fig.6.

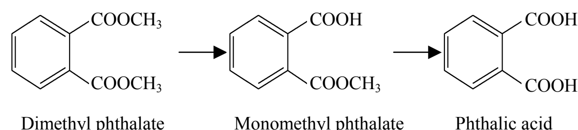


**Fig.6 Proposed pathway for DMP degradation under denitrifying conditions**

# CONCLUSION

Biodegradation of DMP under anoxic conditions has been shown to occur as a series of sequential steps using strain CW-1 isolated from digested sludge of Sibao Wastewater Treatment Plant in Hangzhou, China. 16Sr DNA (1438 bp) sequence analysis showed that the strain was related to *Arthrobacter* sp. CW-1 could degrade PAEs utilizing nitrate as electron acceptor, but could not mineralize DMP completely. Primary degradation of DMP to PA has been reported to involve the hydrolysis of each of the two dimethyl chains of the phthalate to produce the monoester MMP and then PA.

DMP concentration decreased from 0.539 mol/L to 0.053 mol/L within 8 d, and the biodegradation exhibited a first order kinetic reaction. Degradation rate constant is 0.3033 $d^{-1}$, with degradation half-life period of 2.25 d. The overall conversion from DMP to PA could be described as a sequential kinetic model. The variation in total DMP, MMP and PA concentrations demonstrated that DMP is not a source of carbon and energy and can be hydrolyzed by *Arthrobacter* sp. strain CW-1 only.

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