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Anoxic biodegradation of dimethyl phthalate (DMP) by activated sludge cultures under nitrate-reducing conditions

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Abstract

Worldwide extensive use of plasticized plastics has resulted in phthalates pollution in different environment. Nitrates from industry and agriculture are also widely disseminated in the soils, natural waters and wastewaters. Dimethyl phthalate (DMP) biodegradation by activated sludge cultures under nitrate-reducing conditions was investigated. Under one optimized condition, DMP was biodegraded from 102.20 mg/L to undetectable level in 56 h under anoxic conditions and its reaction fitted well with the first-order kinetics. Using the high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) analysis, mono-methyl phthalate (MMP) and phthalic acid (PA) were detected as the major intermediates of DMP biodegradation. When combined with the determination of chemical oxygen demand (COD_{Cr}) removal capacity and pH, DMP was found to be mineralized completely under anoxic conditions. The biodegradation pathway was proposed as DMP → MMP → PA → ... → CO_2 + H_2O. The molar ratio of DMP to nitrate consumed was found to be 9.0:1, which agrees well with the theoretical stoichiometric values of DMP biodegradation by nitrate-reducing bacteria. The results of the non-linear simulation showed that the optimum pH and temperature for the degradation were 7.56 and 31.4°C, respectively.

Key words: biodegradation; dimethyl phthalate; phthalic acid; nitrate reduction; mineralization

Introduction

Phthalic acid esters are synthetic organic compounds used predominantly as additives in plastics to improve mechanical properties of the plastic resin, particularly flexibility. In the absence of a plasticizer, the plastic becomes more brittle or inflexible (CEFIC, 1989). However, in order to provide the required flexibility, the phthalate plasticizer is not bound covalently to the resin and is able to migrate into the environment (Nilsson, 1994). Due to the global use of plasticized plastics, phthalates have been detected in every environment in which they have been sought (Giam, *et al*., 1984), with the highest concentrations detected in areas adjacent to phthalate production or plastics processing facilities.

The extensive use of PVC products has resulted in considerable release of phthalic acid esters (PAEs) into the soils, sediments and natural waters (Atlas and Giam, 1980; Jonsson *et al*., 2003; Ritsema *et al*., 1989). PAEs can enter human body via the food chains, affecting human health leading to cancers. PAEs have been listed as priority pollutants and endocrine-disrupting compounds by the US Environmental Protection Agency and China National Environmental Monitoring Center.

Microbial action has been confirmed to be the principal mechanism for PAEs degradation in both aquatic and terrestrial systems (Staples *et al*., 1997). It is reported that the PAEs in various environments can be biodegraded under anaerobic and aerobic conditions (Chang *et al*., 2005; Chatterjee and Dutta, 2003; Cartwright *et al*., 2000; Shen, *et al*., 2004). Some bacterial species like *Pseudomonas uorescens* B-1 (Xu *et al*., 2005) and *Pasteurella multocida* Sa (Li *et al*., 2006) using PAEs as sole carbon source sequentially hydrolyze the ester linkage between each alkyl chain and the aromatic ring, resulting in the formation of monoesters and phthalic acid (PA).

The complete mineralization of phthalate in the environment is restricted to microbiologically-mediated processes, and the biodegradation process presumably follows a series of stages that are common to all phthalate esters (Ribbons *et al*., 1984; Staples *et al*., 1997). Phthalate has the basic structure of an esterified benzenedicarboxylic acid with two alkyl chains, and its primary biodegradation involves the sequential hydrolysis of the ester linkage between each alkyl chain and the aromatic ring, resulting in the formation of the monoester and subsequently PA (Johnson *et al*., 1984). Its secondary biodegradation results in mineralization of the PA by a number of pathways (Pujar and Ribbons, 1985; Elder and Kelly, 1994; Eaton, 1980).

Since PAEs and nitrate can coexist in many environments and a number of bacterial species utilize PAEs as electron donors for the reduction of nitrate to gaseous nitrogen under anoxic conditions. Our present study aimed at

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investigating DMP biodegradation and its possible mechanism under anoxic conditions because of its ecological significance.

1 Materials and methods

1.1 Inoculum, culture medium and enrichment

The anaerobic digested sludge used as inoculum for the present research was obtained from Sibao wastewater treatment plant in Hangzhou, China. Sludge filtrate 2 L was put into a 4-L glass bottle and mixed with the enrichment medium. The culture system was then flushed with oxygen-free argon gas for 10 min and was incubated at 30°C. The minimal medium used for enrichment consisted of 300 mg/L of nitrate (as electron acceptor) and 20 mg/L of DMP (as source of carbon and energy). The minimal medium contained $CaCl_2{\cdot}2H_2O$ (0.3 g/L), $Na_2HPO_4{\cdot}2H_2O$ (0.065 g/L), $MgCl_2{\cdot}6H_2O$ (0.165 g/L) and 1.25 ml/L trace element solutions I and II respectively (Qian and Min, 1986). The minimal medium for DMP-degrading cultures was replaced with fresh medium at approximately 3-d intervals on the depletion of DMP. During enrichment the DMP concentration was gradually increased from 20 to 100 mg/L. The DMP-degrading enrichment medium was replaced more than 10 times before using it in the biodegradation experiments.

1.2 DMP biodegradation experiment

After the enrichment, digested sludge was washed three times with 0.02 mol/L K_2HPO_4-KH_2PO_4 buffer solution and transferred into 500 ml conical flasks. All the conical flasks contained 300 ml minimal medium with 40 ml washed sludge, 100 mg/L DMP and 300 mg/L nitrate. Medium pH was adjusted at 7.0 by using 1 mol/L NaOH solution. After flushing continuously with oxygen-free argon gas for 10 min, the flasks were made airtight by applying butyl rubber stoppers and were sealed with paraffin. The culture was then incubated at 30°C.

The following factors were manipulated to investigate their effects on the biodegradation of DMP: pH (5.0, 6.0, 7.0, 8.0, or 9.0); temperature (20, 25, 30, 35, or 40°C). The control experiment was also conducted using the same minimal medium without any nitrate added.

Aqueous samples were periodically collected by sterile syringes, and were centrifuged at 6000 r/min for 5 min. Each aqueous solution was then filtered through 0.22-μm membrane and was subsequently analyzed by HPLC.

1.3 Analysis of DMP and its metabolites

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

The mobile phase used was methanol-0.5% of acetic acid solution (50:50, v/v) with a flow rate of 1 ml/min. The sample 20 μl was injected into a Zorbax SB C18 (4.6 mm × 150 mm, 5 μm) chromatography column at 40°C. UV detector and detecting wavelength was set at 254 nm, 20 nm strip width 330 nm comparison wavelength and 50 nm strip width by the ration method.

DMP degradation metabolites were identified using Agilent 1100 System with SL Mass Spectrometer. The mass spectrometer was operated with ESI, a positive ion detector with a mass scanner range of 100–300 amu; fragment ion voltage 100 V; evaporate gas rate was 13.0 min^{-1} at vaporizer pressure of 60 psig; evaporation temperature of 35°C and capillary voltage was 4000 V. The rest of the analytical methods were identical to HPLC.

1.4 Other analytical procedures

Chemical oxygen demand (COD), ammonia, nitrite, nitrate were measured according to standard methods for the examination of water and wastewater (APHA, 1985).

2 Results and discussions

2.1 Variations in the DMP concentration

The variations in the DMP concentrations under denitrifying conditions were determined using external standard method (Fig.1). The control (CK) sample was treated by inoculum without nitrate. The DMP concentration in the culture medium under denitrifying conditions decreased from 102.20 mg/L to undetectable level within 56 h. In contrast, the DMP concentration in CK varied little (from 103.01 to 96.30 mg/L), implying that the nitrate-reducing bacteria did not function well in inoculum without nitrate due to the lack of nitrate as electron acceptors. However, the concentration of DMP still decreased about 6.5% during control experiment, probably because of the presence

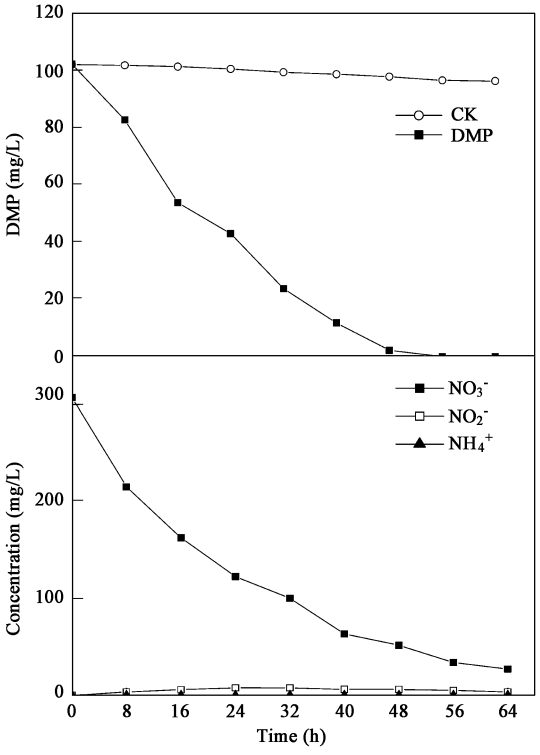

Fig. 1 Variation in the DMP and nitrogenous compound concentrations under denitrifying conditions.

of some other DMP degrading species present in the inoculum.

The following first-order kinetics equation was used to simulate the procedure of DMP biodegradation by nitrate-reducing bacteria.

$$C = C_0 e^{-kt} \quad (1)$$

$$C = 108.90\ e^{-0.047t} \qquad (R^2 = 0.9673) \quad (2)$$

where, C is the initial concentration of DMP; t is time; k is the first-order rate constant.

It demonstrated that the DMP biodegradation by nitrate-reducing bacteria fitted well with the first-order kinetics equation.

In this experiment, nitrate was used as the electron acceptor for the biodegradation of DMP. As DMP degraded, nitrate concentration also changed from 307.35 to 27.09 mg/L. Meanwhile, nitrite concentration initially increased up to 7.59 mg/L but then decreased to zero without any obvious accumulation. However, ammonia concentration did not change throughout the experiment. The variations in the concentration of nitrogenous compounds are shown in Fig.1.

Nitrate has two distinct transformation pathways under anaerobic and anoxic conditions: (1) replacing atmospheric O_2 to act as the electron acceptor during heterotrophic denitrification, forming the nitrite or dinitrogen, and (2) dissimilatory nitrate reduction during the anaerobic digestion producing nitrite and ammonia (Shen, 2002). Since the ammonia was not detected during the DMP biodegradation using nitrate, it could be concluded that the nitrate transformation was carried out through nitrate-reduction pathways (heterotrophic denitrification) instead of dissimilatory pathways.

2.2 Identification of intermediates of the DMP biodegradation

In present study, the samples taken at various intervals from the culture medium were analyzed by HPLC and LC-MS techniques. It is evident that there were three noticeable peaks in the mass fragmentation (MS) with retention times of 2.344, 2.986 and 4.881 min respectively (Fig.2).

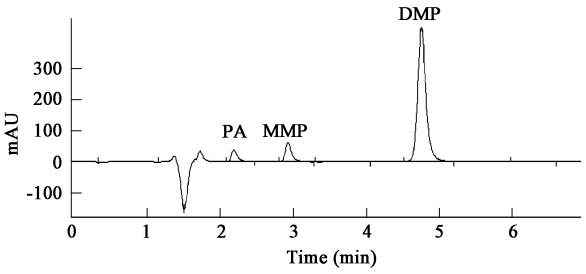

Fig. 2 HPLC chromatograms of DMP degradation.

Phthalate ester possesses the basic structure of an esterified benzene dicarboxylic acid with two alkyl chains. Commonly, the 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 PA. The hydrolysis of the DMP would yield fragments of molecular mass 166 and 180, and a phthalate possessing a methyl alkyl chain would produce 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 with standard sample of DMP, the MS displayed molecular ion peak (DMP+H) at 195.1 *m/z*. The other discernible peaks in the mass spectrum were 217.1 *m/z* (DMP+Na) and 233.1 *m/z* (DMP+K) etc., so these peaks were identified as DMP.

The retention time for peak at 2.344 min was analogous to that for standard sample of PA, 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* (Na+K), 243.0 *m/z* (PA+K+Ca–2H) and 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* (Ca+MMP), 241.1 *m/z* (MMP+Na+K), 257.0 *m/z* (MMP+K+Ca–2H) and 259.0 *m/z* (MMP+K+Ca), etc., these MS peaks indicated the presence of MMP.

Given the fact that DMP, MMP and PA were all detected in the culture medium, it could be deduced that MMP and PA might be the first and second intermediates respectively during DMP biodegradation under anoxic conditions.

The results coincide with the results from previous studies on the degradation of PAEs by microorganism isolated from activated sludge, mangrove sediment and deep ocean sediment (Shen *et al*., 2004; Li *et al*., 2006; Wang and Gu, 2006). They also found that the initial hydrolysis of the ester bonds was carried out by two different bacteria species indicating the high specificity of the enzymes to the substrates.

2.3 Variations in the concentration of MMP and PA

As compared to the determination of the standard sample, the variations in the MMP and PA concentrations are shown in Fig.3. During the degradation of DMP, the MMP concentration initially increased to 16.43 mg/L and then decreased to zero. While the PA concentration ranged from 0 to 2.08 mg/L without obvious accumulation. It is well

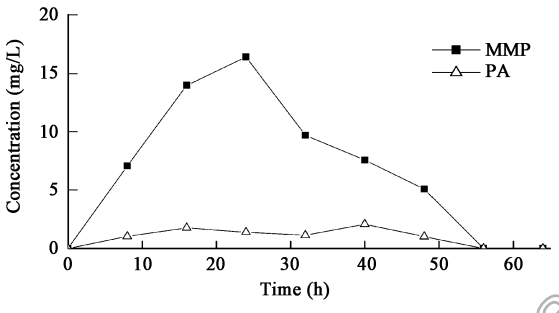

Fig. 3 Variations in the MMP and PA concentrations under denitrifying conditions.

established that DMP is relatively easier to be hydrolyzed compared with PA. In comparison to other results (Shen *et al*., 2004; Wu *et al*., 2007), the PA would accumulate in the culture medium when pure culture was used to biodegrade the DMP. While using nitrate-reducing bacterial mixed culture, the PA could be biodegraded completely and did not accumulate during the experiment.

2.4 Variations in the COD_{Cr} concentration and pH value

The COD_{Cr} variations with the passage of time are presented in Fig.4. It reveals that there exists a significantly ($p<0.05$) negative correlation (r = –0.9914) between COD_{Cr} concentration and time (h). Further, correlation between DMP and COD_{Cr} concentration was very high (correlation constant, i.e. $R^2 = 0.9828$) that reflects the total DMP biodegradation. The initial COD_{Cr} concentration in the culture liquid was 221.87 mg/L, and then decreased consistently to 27.73 mg/L during the incubation period, which is close to the background COD_{Cr} concentration of 20.43 mg/L, which suggests that DMP in the culture could be mineralized into carbon dioxide completely.

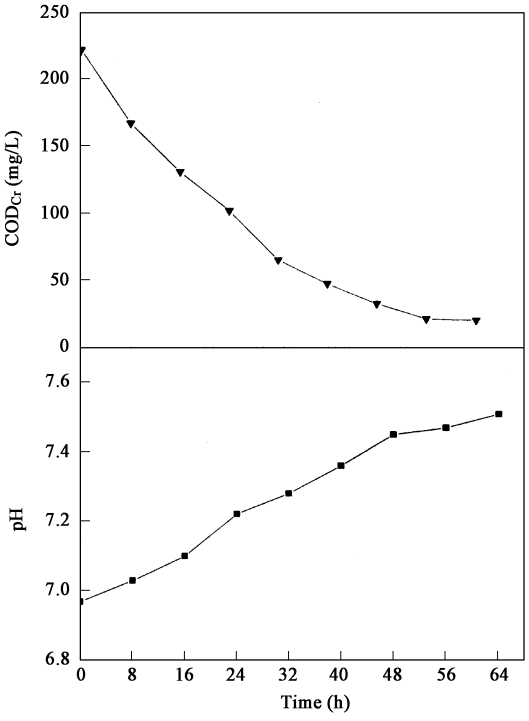

Fig. 4 Variations in the COD_{Cr} concentrations and pH value under denitrifying conditions.

The variations in pH values of the culture are shown in Fig.4. The pH of culture liquid increased from 6.97 to 7.51. The results were consistent with the theoretical values depicted in Eq. (3).

$$1/6NO_3^- + H^+ + 5/6e^- = 1/2N_2 + 1/2H_2O \quad (3)$$

The results are comparable to previous findings (Wang *et al*., 2003; Li *et al*., 2004; Wu *et al*., 2007), in which isophthalate and phthalic acid were found to be the ultimate products when the pH decreased, independent of the means of treatment by either mixed or pure culture. However, it was proved that the pH values were in range of 6.7 to 7.3 during the incubation period when diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) were mineralized by river sediment under anaerobic conditions, (Chang *et al*., 2005). From pH variations, it can be concluded that the DMP was mineralized by nitrate-reducing bacteria.

2.5 Proposed biodegradation pathway and stoichiometric relationship

Based on the variations in the concentrations of intermediates, COD_{Cr} and pH value, it was concluded that the DMP could be mineralized by nitrate-reducing bacteria. The pathways for DMP degradation under denitrifying conditions was suggested as follows (Fig.5).

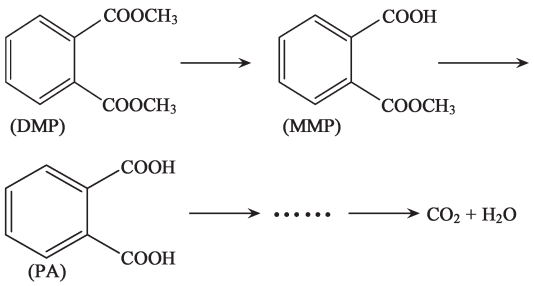

Fig. 5 Proposed pathways for DMP degradation under denitrifying condition.

If the cell production is neglected and DMP is assumed to be mineralized completely, following stoichiometric relationship can be formulated (Eq. (4)).

$$C_{10}H_{10}O_4 + 8.4NO_3^- + 8.4H^+ = 4.2N_2 + 10CO_2 + 9.2H_2O \quad (4)$$

For the biodegradation of 0.50 mmol/L DMP, the determined consumption of nitrate was found to be 4.52 mmol/L. So the calculated ratio of nitrate to DMP was 9.0:1, which matches well with the theoretical value (8.4:1) as shown in Eq.(3). We can infer that the main product of nitrate reduction was dinitrogen.

2.6 Effect of temperature and pH on DMP biodegradation

The relationship between the DMP biodegradation rate constant and pH value is shown in Fig.6a. The rate constant decreased when pH was higher than 9.0 or lower than 6.0. It was shown that the hydrogen ion concentration in the culture medium greatly influenced the denitrification activity. The results of Alvarez and Vogel (1995) and Xu (1994) show that the optimal pH for denitrification was in the range of 7.0–8.0. The activity of NO_x reductase would be restricted under low pH value resulting in a lower denitrification rate. The results of the non-linear simulation showed that the optimum pH for the biodegradation activity of nitrate-reducing bacteria was 7.56.

The temperature dependence of DMP biodegradation by nitrate-reducing bacteria was studied in the range of

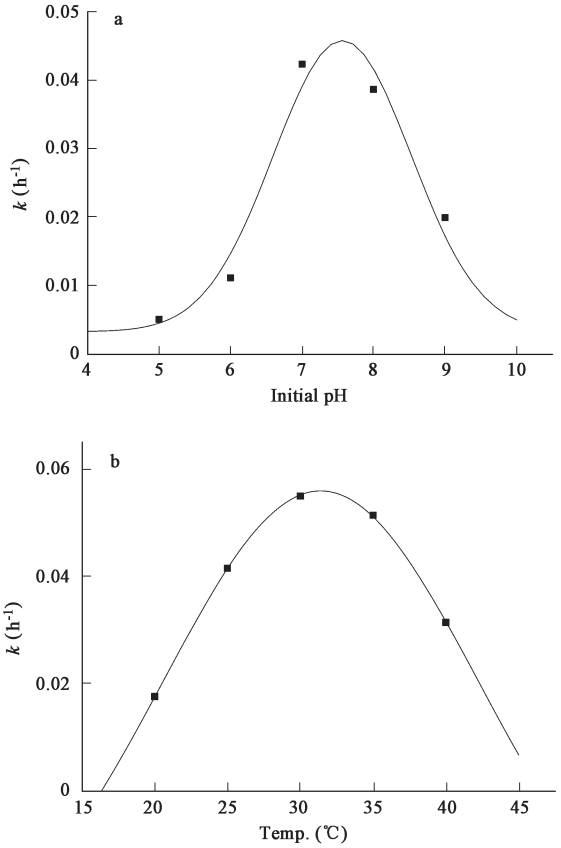

Fig. 6 Relationship between initial pH(a) and temperature (b) vs. *k*.

20–40°C (Fig.6b). The rate constant increased with the increase in temperature range of 20 to 32°C. The higher temperature resulted in the decrease of the degradation rate constant. The optimum temperature was found to be 31.4°C.

3 Conclusions

In this study, we investigated anoxic biodegradation of DMP by activated sludge cultures under nitrate-reducing conditions. Our results showed that DMP could be mineralized completely, i.e. converted into carbon dioxide and water by nitrate-reducing bacteria. Based on the HPLC and LC-MS analysis, DMP biodegradation pathway was proposed as DMP→MMP→PA→ ... →CO_2+H_2O. The molar ratio of consumed DMP to nitrate was determined to be 9.0:1, which was in great accordance with the theoretical stoichiometric value. The results of the non-linear simulation showed that the optimum pH and temperature for DMP degradation under anoxic conditions were 7.56 and 31.4°C respectively.

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