# Removal of Di-*n*-butyl Phthalate Using Immobilized Microbial Cells[*]

**SUN Xiaoran**(孙晓然)[a,b,**] and **SHAN Zhongjian**(单忠键)[a]
[a] School of Chemical and Environmental Engineering, China University of Mining and Technology, Beijing 100083, China
[a] Department of Chemical Engineering, Hebei Polytechnic University, Tangshan 063009, China

**Abstract**   The biodegradation of di-n-butyl phthalate (DBP) using immobilized microbial cells was carried out in an internal airlift loop reactor with ceramic honeycomb supports. A strain that is capable of degrading DBP was isolated from the activated sludge and identified as *Bacillus* sp. using 16S rDNA sequential analysis. *Bacillus* sp. could be rapidly attached onto the ceramic honeycomb supports. The immobilized cells could effectively degrade DBP in batch and continuous experiments. When the influent concentration of DBP was 50mg·L$^{-1}$, the effluent DBP reached less than 1mg·L$^{-1}$ with 6h hydraulic retention time (HRT) in continuous experiment. The immobilized microbial cells could grow and accumulate through the biodegradation of DBP, and the rate of degradation is accordingly increased. The possible pathway of DBP biodegradation using immobilized cells was tentatively proposed.
**Keywords**   biodegradation, phthalic acid ester, priority pollutant, bioreactor, immobilized cells

## 1   INTRODUCTION

Phthalic acid esters (PAEs) are a class of refractory organic compounds, which have been widely used as plasticizers. Three phthalic acid esters, namely, dimethyl phthalate (DMP), di-*n*-butyl phthalate (DBP), and di-*n*-octyl phthalate (DOP) have been listed as the priority pollutants by China National Environmental Monitoring Center and the U.S. Environmental Protection Agency[1,2].

PAEs are among the most common industrial chemicals and have become widespread in the environment, as they have been found in sediments, waters, and soils[3—8]. Some of them are suspected to be mutagents[3] and carcinogents[4]. Di-*n*-butyl phthalate (DBP) belongs to the family of phthalic acid esters, it is the most frequently identified PAE in diverse environmental samples including groundwater, river water, drinking water, open ocean water, soil humates, lake sediments, and marine sediments, and it is one of the most widely used plasticizer in China[9]. It has received increasing attention in recent years because of its widespread use and ubiquity in the environment.

Phthalic acid esters enter either directly or indirectly into the aquatic environment. As a result of their low water solubility and high octanol/water partition coefficients, they tend to accumulate in the soil or sediment and in the biota living in the waters. The level of accumulation depends on their solubility and partition coefficients and also on their rates of degradation through chemical or biological pathways.

Several studies have been performed on the biodegradation of several PAEs under aerobic conditions in soil and sediments[5—7], in river, lake or sea water[8,9], and wastewater[10—22], in activated sludge [13—16] and in acclimated sludge[17,18] and under anaerobic conditions[19—22]. These studies focused on the biodegradability of the different PAEs and the pathway of degradation. On the basis of these studies, it can be concluded that dimethyl (DMP), diethyl (DEP), di-*n*-butyl (DBP), and butyl-benzyl (BBP) degrade quickly to a large extent, whereas di-(2-ethylhexyl) (EDHP) and di-*n*-octyl (DOP) phthalate degrade at a slow rate to a small extent. Huang *et al.*[23] found that some of PAEs, such as DBP and DEHP, were part of the main refractory organic compounds in municipal wastewater, which are difficult to be degraded. In conventional activated sludge plants, most of the organic pollutants that are passed through the treatment facilities remain unaltered.

Immobilization of microbial cells shows good prospects for improving the efficiency of certain bioprocesses. Compared with the use of suspended cells, immobilization has several potential advantages: (1) simple and more reliable biomass retention, (2) increased volumetric biodegradation rate through the accumulation of high biomass, (3) high dilution rate without washout, and (4) enhanced retention and stability of slow-growing microorganisms, especially when growth rates are slowed down by inhibition[24]. Biodegradation using immobilized cells has been widely investigated for several toxic compounds, including 4-chlorophenol, quinoline, benzene derivatives, chlorobenzoates, and 2, 4-dichlorophenol (2,4-DCP)[25—29].

A novel type of bioreactor, *i.e.*, an internal airlift loop reactor with ceramic honeycomb supports, was developed by the research group of the authors of this study, and it was used for the biodegradation of chlorophenols, quinoline, and high-carbohydrate wastewaters. The objectives of this study were to characterize the biodegradation of DBP using immobilized microbial cells in this bioreactor.

## 2   MATERIALS AND METHODS
### 2.1   Bioreactor

Figure 1 is the schematic diagram of the bioreactor. A draft tube with a diameter of 100mm and length of 650mm can be seen at the center of the column, which had a top section with a diameter of 300mm
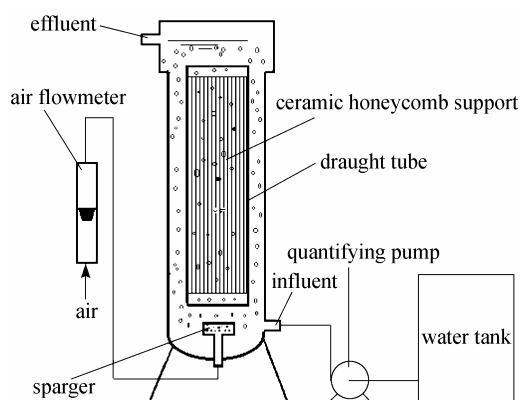
---

**Figure 1    Schematic diagram of the bioreactor**

and height of 150mm and a bottom section with a diameter of 200mm and height of 700mm. Inside the draft tube, there were 3 ceramic honeycomb supports with a diameter of 95mm and height of 200mm, and pore holes with 8mm×8mm cross-section. The influent was pumped into the reactor at the bottom and overflowed at the top. Compressed air was introduced at the bottom of the draft tube and was mainly flowed up through the draft tube. On the basis of the tracer studies, the circulation rate through the draft tube was found to be approximately $0.5m^3 \cdot h^{-1}$.

## 2.2    Microorganism

A strain, which is capable of degrading DBP was isolated from the activated sludge of the wastewater treatment plant enriched with DBP in shake culture at 30℃ and purified by successive streak transfers on agar-plate medium containing ($g \cdot L^{-1}$) tryptone 10; beef extract 5; agar 15, and NaCl 5; and pH was adjusted to 7.3—7.5. The strain was maintained in slant tryptone–glucose extract agar medium. The strain was identified as *Bacillus* sp. using16S rDNA sequential analysis at Institute of Microbiology, the Chinese Academy of Science, China.

## 2.3    Bacterial growth and liquid medium

The bacteria were cultivated in a shake flask for 48h in the following liquid medium containing ($g \cdot L^{-1}$): tryptone 10, glucose 5, sodium chloride 5, beef extract 5, yeast extract 2, ammonium sulfate 0.1, magnesium sulfate 0.5, potassium dihydrogen phosphate 0.5, and di-sodium hydrogen phosphate 0.5.

## 2.4    Immobilization

A total of approximately 10 liters of liquid with the bacteria cultivated in the shake flasks was added to the ceramic support in the bioreactor so that the microbial cells could be immobilized into the pores of the ceramic support via absorption. Finally, approximately 70% of the inoculated biomass would remain in the ceramic honeycomb support. The microorganisms were inoculated once, and then the different experiments were continuously carried out in the order presented below.

## 2.5    Batch degradation of DBP

The synthetic wastewater containing $20mg \cdot L^{-1}$ of DBP plus an inorganic culture medium contain $(NH_4)_2SO_4$ $0.1g \cdot L^{-1}$ as the nitrogen source, $KH_2PO_4$ $0.5g \cdot L^{-1}$ and $Na_2HPO_4$ $0.5g \cdot L^{-1}$ as the phosphorus source, $MgSO_4 \cdot 7H_2O$ $0.5g \cdot L^{-1}$ as the magnesium source, and yeast extract $0.02g \cdot L^{-1}$ as the source for trace minerals and vitamins. The DBP concentration was monitored over time.

## 2.6    Continuous degradation of DBP

The degradation of DBP was continuously carried out for 345h. The influent concentration of DBP was increased stepwise from approximately $15mg \cdot L^{-1}$ to $50mg \cdot L^{-1}$. The hydraulic retention time (HRT) was 6h. The samples were taken at regular time intervals for the analysis of DBP in the influent and effluent.

## 2.7    Analytical methods

The concentration of DBP was analyzed using gas chromatography (GC) system (Hewlett–Packard model 5890A with a flame ionization detector). The column temperature was 280℃ and the flow rate of nitrogen gas was $30ml \cdot min^{-1}$. The volume of the injected samples was 2μl, and the detection limit was 1ng. The mass spectra were recorded using a model 5972 mass spectrometer. The biodegraded products of DBP were analyzed using GC-MS.

## 2.8    Scanning electron microscopy

The biomass in the ceramic support was observed using scanning electron microscope (Model: HITACHI S-570, Japan). A sample of ceramic support with immobilized microbial cells was first removed by cutting it out with a saw. Then, each sample was successively dehydrated with 30%, 50%, 70%, 85%, and 95% ethanol for 20min and twice with 100% ethanol for 20min. The dehydrated sample was mounted on sample plate using an electrically conducting paster and sputter-coated using gold. The samples were analyzed using scanning electron microscope in vacuum.

## 3    RESULTS AND DISCUSSION
### 3.1    Microbial immobilization in the ceramic support

*Bacillus* sp. was immobilized well onto the ceramic support. Fig.2 shows the significant immobilization of the bacterial cells, which was taken shortly after the inoculation of the ceramic support. The rapid and strong immobilization of *Bacillus* sp. on the wall of the ceramic support facilitates good biomass retention and rapid start-up of the bioreactor.

### 3.2    Degradation of DBP in batch experiments

First, degradation of DBP in batch experiments was carried out in the bioreactor with immobilized *Bacillus* sp. Fig.3 shows that the biodegradation of DBP in the first test was completed within 33h, where the concentration was reduced from $18mg \cdot L^{-1}$ to approximately zero, whereas the same process in the

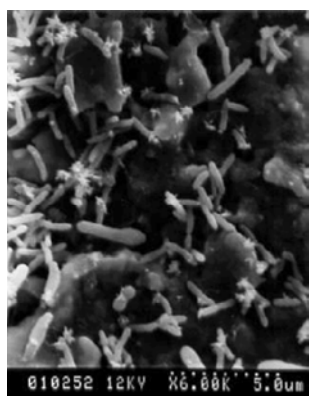**Figure 2** *Bacillus* **sp. immobilized onto ceramic support in the beginning**



**Figure 4   Degradation of DBP in continuous experiment**
○ influent;  ● effluent

second test was completed within 12h, where the concentration was reduced from 22mg·L$^{-1}$ to zero . Linear regression analysis yielded slopes that corresponded to DBP that ranged from $-0.57$mg·L$^{-1}$·h$^{-1}$ in the first test to $-1.8$mg·L$^{-1}$·h$^{-1}$ in the second test. The slope of the second test was three times of the slope in the first test, which indicated that immobilized *Bacillus* sp. rapidly adapted to DBP. The rapid adaptation to DBP can be attributed to the microbial growth and the accumulation of several biomass. This hypothesis is consistent with the results in Figs.2 and 3 and will be discussed in detail in the following sections.
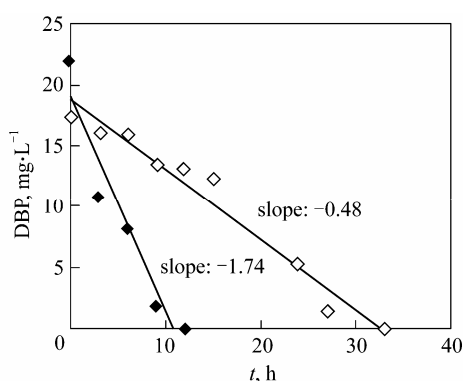


**Figure 3   Batch degradation of DBP using immobilized** *Bacillus* **sp.** [Linear regression gives these slopes in mg·L$^{-1}$·h$^{-1}$: $-0.48$ ($r^2=0.984$) for the first test and $-1.74$ ($r^2=0.945$) for the second test]
◇ first batch;  ◆ second batch

### 3.3   Degradation of DBP in continuous experiment

The continuous degradation of DBP was carried out, in which the hydraulic retention time was 6h and the DBP concentration of the influent increased stepwise. Fig.4 shows the variation of DBP concentrations of the influent and effluent with time. Degradation of the initial influent concentration (about 15mg·L$^{-1}$) steadily increased over the first 50h and approximately 100% of DBP was removed. The DBP concentration of the effluent remained low even though the DBP concentration of the influent increased stepwise, and the removal percentage was higher than 98%
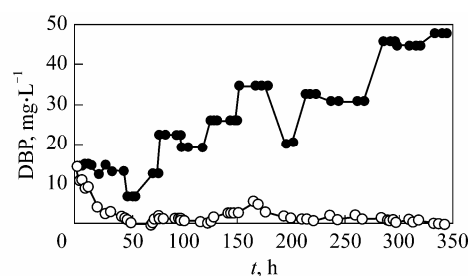
when the influent concentration finally increased to approximately 50mg·L$^{-1}$.

The degradation of DBP in the continuous experiment could be divided into three phases: 0 to 76h, 76 to 167h, and 167 to 345h. The average volumetric removal rates were 0.51mg·L$^{-1}$·h$^{-1}$, 1.5mg·L$^{-1}$·h$^{-1}$, and 2.4mg·L$^{-1}$·h$^{-1}$, respectively. These volumetric rates were similar to those of the batch tests, but the volumetric rate for the third phase was higher than that of the batch tests. The increase in volumetric rate, even though the DBP concentration was lower in the continuous experiments than that of the batch tests, suggested that the immobilized bacteria were continuously accumulated in the bioreactor.

At the end of the continuous experiments, the samples were observed using SEM. Fig.5 shows that the immobilized cells were more densely accumulated than those in Fig.2. The results of SEM observation supported the conclusion that the microbial cells could continue to grow and accumulate as it caused the biodegradation of DBP. Fig.3 also shows that some mineral deposits, probably inorganic salts in the medium were precipitated at the surface. From the comparisons of Figs.2 and 5, it can be seen that the bacteria on the surface were larger and denser after the continuous experiment compared with those immediately after the inoculation, and this suggested that the microbial cell could grow through the utilization of DBP.



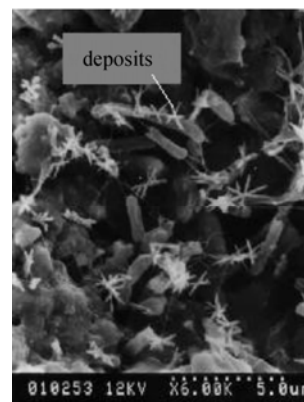**Figure 5** *Bacillus* **sp. immobilized onto ceramic support after continuous degradation experiments**

### 3.4   Identification of metabolites

To identify the metabolites, the intermediate products were extracted with diethyl ether, and analyzed

using GC and GC-MS. The results of GC analysis indicated that methylated intermediates from the biodegradation of DBP appeared as two peaks; and the retention time of one peak was assigned to methylated phthalic acid. But the two peaks were completely disappeared after further incubation and no novel peak was appeared after the completion of DBP biodegradation. When DBP was used as the carbon source, the isolated products were identified as methyl butyl phthalate and dimethyl phthalate (DMP), respectively. This result suggested the possible pathway of DBP biodegradation as follows: first, di-butyl phthalate was hydrolyzed to form mono-butyl phthalate and then to phthalic acid, which entered the tricaboxylic acid (TCA) cycle, and there it was oxidized to carbon dioxide and water.

The degradation pathway of DMP using immobilized cells can be tentatively proposed as follows:

di-$n$-butyl phthalate→mono-butyl phthalate→
phthalic acid→protocatechuic acid→
ring cleavage and mineralization
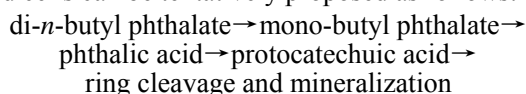
This was consistent with the conclusion reported by previous researchers using free cells. This result indicated that the immobilization of microbial cells did not change their original metabolic pathway[10].

## 4   CONCLUSIONS

The following conclusions may be drawn on the basis of this study:

(1) A strain, which is capable of degrading DBP was isolated from the activated sludge and identified as *Bacillus* sp. using 16S rDNA sequential analysis. This strain can use DBP as the sole source of carbon and energy.

(2) Microbial cells could be easily immobilized into the micropores within the ceramics, which supplied a effective microenvironment for the microorganisms to metabolize, proliferate, and degrade DBP.

(3) Immobilized microorganisms exhibited improved stability for the degradation of DBP both in batch and continuous experiments. When the influent concentration of DBP was $50 mg \cdot L^{-1}$, the effluent concentration of DBP could reach less than $1 mg \cdot L^{-1}$ with 6h hydraulic retention time (HRT) in continuous-flow tests.

(4) The GC-MS analysis reveled the possible pathway of DBP biodegradation as follows: first, di-butyl phthalate was hydrolyzed to form mono-butyl phthalate and then to phthalic acid, which entered the tricaboxylic acid (TCA) cycle, and there it was oxidized to carbon dioxide and water.

## REFERENCES

1   United States Environmental Protection Agency, "Fate of priority pollutants in publicly owned treatment works", Final Report, EPA 440/1-82/303.
2   Staple, A.C., Peterson, D.R., Parkerton, T.H., Adams, W.J., "The environmental fate of phthalic esters: A literature review", *Chemosphere*, **35**, 667—749(1997).
3   Kozumbo, W.J., Kroll, R., Rubin, R.J., "Assessment of mutagenicity of phthalate esters", *Environ. Health Perspect.*, **45**, 103—110(1982).
4   Gledhill, W.E., Kaley, R.G., Adams, W.J., Hicks, O., Michael, P.R., Saeger, V.W., Leblanc, G.A., "An environmental safety assessment of butyl benzyl phthalate", *Environ. Sci. Technol.*, **14**, 301—305(1980).
5   Shanker, R., Ramakrishna, C., Seth, P.K., "Degradation of some phthalic acid esters in soil", *Environ. Pollut.*, *Ser. A.*, **39**, 1—7(1985).
6   Wang, J.L., Liu, P., Shi, H.C., Qian, Y., "Biodegradation of phthalic acid esters in soil by indeginous and introduced microorganisms", *Chemsphere*, **35**, 1747—1754(1997).
7   Wang, J.L., Zhao, X., Wu, W.Z., "Biodegradation of phthalic acid esters (PAEs) in soil bioaugmented with acclimated activated sludge", *Process Biochem.*, **39**, 1837—1841(2004).
8   Walker, W.W., Cripe, C.R., Pritchard, P.H., Bourguin, A.W., "Dibutyl phthalate degradation in esturine and fresh water sites", *Chemosphere*, **13**, 1283—1294(1984).
9   Saeger, V.W., Tucker, N.V., "Biodegradation of phthalic acid esters in river water and activated sludge", *Appl. Environ. Microbiol.*, **31**, 29—34(1976).
10  Wang, J.L, Liu, P., Qian, Y., "Microbial degradation of di-$n$-butyl phthalate", *Chemosphere*, **31**, 4051—4056(1995).
11  Wang, J.L., Liu, P., Qian, Y., "Biodegradation of phthalic acid esters by immobilized microbial cells", *Environ. Int.*, **23**, 775—782(1997).
12  Wang, J.L., Liu, P., Shi, H.C., Qian, Y., "Kinetics of biodegradation of phthalic acid esters in continuous culture system", *Chemsphere*, **37**, 257—264(1998).
13  Wang, J.L., "Effect of di-$n$-butyl phthalate (DBP) on activated sludge", *Process Biochem.*, **39**, 1831—1836(2004).
14  Wang, J.L., Ye, Y.C., Wu, W.Z., "Comparison of biogradation of di-$n$-methyl phthalate by free and immobilized microbial cells", *Biomed. Environ. Sci.*, **16**, 126—132(2003).
15  O'grady, D.P., Howard, P.H., Werner, A.E., "Activated sludge biodegradation of 12 commercial phthalate esters", *Appl. Environ. Microbiol.*, **49**, 443—445(1985).
16  Sugatt, R H., O'Grady, D.P., Banergee, S., Howard, P.H., Gledhill, W.E., "Shake flask biodegradation of 14 commercial phthalate esters", *Appl. Environ. Microbiol.*, **47**, 601—606(1984).
17  Wang, J.L., Liu, P., Qian, Y., "Biodegradation of phthalic acid esters by acclimated activated sludge", *Environ. Int.*, **22**, 737—741(1997).
18  Wang, J.L., Liu, P., Shi, H.C., Qian, Y., "Kinetics of phthalic acid ester degradation by acclimated activated sludge", *Process Biochem.*, **32**, 567—571(1997).
19  Ziogou, K., Kirk, P.W.W., Lester, N.J., "Behaviour of phthalic acid esters during batch anaerobic digestion of sludge", *Water Res.*, **23**, 743—748(1989).
20  Wang, J.L., Qian, Y., "Microbial metabolism of di-butyl phthalate (DBP) by a denitrifying bacterium", *Process Biochem.*, **34**, 745—749(1999).
21  Wang, J.L., Chen, L.J., Shi, H.C., Qian, Y., "Microbial degradation of phthalic acid esters under anaerobic digestion of sludge", *Chemosphere*, **41**, 1245—1248(2000).
22  Nozawa, T., Maruyama, Y., "Anaerobic metabolism of phthalate and other aromatic compounds by a deitrifying bacterium", *J. Bacteriol.*, **170**, 5778—5784(1988).
23  Huang, X., Zhang, X.J., Fu, Q., "Assessment for biodegradability of organics in municipal wastewater and controlling measure for its refractory organics", *Chin. J. Eiviron. Sci.*, **15**, 15—19(1994).
24  Wang, J.L., Hou, W.H., Qian, Y., "Immobilization of microbial cells using polyvinyl alcohol (PVA)-polyacrylamide gels", *Biotechnol. Techniques*, **10**, 203—208(1995).

25  Wang, J.L., Qian, Y., "Microbial degradation of 4-chlorophenol by microorganisms entrapped in carra-geenan-chitosan gels", *Chemosphere*, **38**, 3109 — 3117(1999).

26  Wang, J.L., Han, L.P., Shi, H.C., Qian, Y., "Biodegrada-tion of quinoline by gel immobilized *Burkholderia* sp.", *Chemosphere*, **44**, 1041—1046(2001).

27  Wang, J.L, Quan, X.C., Han, L.P., Qian, Y., Werner, H., "Microbial degradation of quinoline by immobilized cells of *Burkholderia pickettii*.", *Water Res.*, **36**, 288—296(2002).

28  Shreve, G.S., Vogel, T.M., "Comparison of substrate utilization and growth kinetics between immobilized and suspended *Pseudomonas* cells", *Biotechnol. Bioeng.* **41**, 370—379(1993).

29  Lee, S.T., Rhee, S.K., Lee, G.M., "Biodegradation of pyridine by freely suspended and immobilized *Pimelo-bacter* sp.", *Appl. Microbiol. Biotechnol.*, **41**, 652—657(1994).