BIOMEDICAL AND ENVIRONMENTAL SCIENCES 16, 126-132 (2003)

Comparison of Di-n-methyl Phthalate Biodegradation by Free and Immobilized Microbial Cells[1]

JIAN LONG WANG,[#,*] YU-CAI YE[#] AND WEI-ZHONG WU[+]

[#]*Laboratory of Environmental Technology, Institute of Nuclear Energy Technology, Tsinghua University, Beijing 100085, China;* [+]*School of Environment Science, Peking University, Beijing 100871, China*

Objective To compare the biodegradation of di-n-methyl pathalate by free and immobilized microbial cells. **Methods** The enrichment and isolation technique was used to isolate the microorganism. The PAV-entrapment method was utilized to immobilize the microorganisms. The scanning electron microscophy (SEM) was used to observe the growth and distribution of microbial cells immobilized inside the PVA bead gels. The GC/MS method was used to identify the main intermediates of DMP degradation. **Results** The microbial cells could grow quite well in PVA gel. The metabolic pathway did not change before and after immobilization of the microbial cells. The degradation rate of immobilized cells was higher than that of free cells. **Conclusion** The immobilized microbial cells possess advantages than free cells when applied to the biodegradation of toxic organic pollutants.

Key words: Priority pollutants; Phthalic acid ester; Immobilized microbial cells; Biodegradation

INTRODUCTION

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

PAEs are the most common industrial chemicals widespread in the environment as they have been found in sediments, waters and soils[2,3]. Some of them are suspected to be mutagens[4] and carcinogens[5]. Di-n-butyl phthalate belongs to the family of phthalic acid esters, which is one of the most commonly used plasticizers and is produced in large quantities in China. It has received increasing attention in recent years due to its widespread use and ubiquity in the environment[3].

Metabolic breakdown of PAEs by microorganisms is considered to be one of the major routes of environmental degradation for these widespread pollutants. Numerous studies demonstrated that several PAEs could be biodegraded under aerobic and anaerobic conditions in soil, natural waters and waste water[2,6-17]. These studies focused on the biodegradability of

[1]This work was supported by the National Natural Science Foundation of China (Grant No. 29637010).

*Correspondence should be addressed to JIAN LONG WANG, Laboratory of Environmental Technology, Institute of Nuclear Energy Technology, Tsinghua University, Beijing 100084, China. Tel: 86-10-62784843, Fax: 86-10-62771150, E-mail: wangjl@tsinghua.edu.cn

Biographical note of the first author: Jian-Long WANG, male, born in 1964, Ph.D. degree, majoring in the field of water pollution control and environmental biotechnology.

0895-3988/2003
CN 11-2816

different PAEs and their pathway of degradation. Several types of microorganisms were found to degrade PAEs, including aerobic[9], anaerobic[2] and facultative[18] species.

Immobilization of biocatalysts (enzymes and cells) has received increasing interest in recent years[19]. It offers a promising potential for the improvement of the efficiency of bioprocess. Compared with free cells, immobilized cells have the following several advantages: (1) They can increase the biodegradation rate through a higher cell loading. (2) The bioprocess can be controlled more easily. (3) The continuous process can take place at a high dilution rate without washout. (4) The catalytic stability of biocatalysts as well as its tolerance against toxic compounds can be improved. Biodegradation using immobilized cells has been widely investigated for numerous toxic compounds such as 4-chlorophenol[20, 21], quinoline[22, 23], phthalic acid esters[9,11], pyridine[24], benzene derivatives and chlorobenzoates[25], 2, 4-dichlorophenoxy acetic acid (2, 4-D)[26].

However, to our knowledge, biodegradation of phthalate esters using immobilized cells has not been reported except that by our research group. The object of this study was to compare the biodegradation of di-n-methyl phthalate by free and immobilized microorganisms and to explain the reasons why immobilized cells have some advantages over free cells during the biodegradation of refractory organic pollutants.

MATERIALS AND METHODS

Microorganisms

Several strains of DMP-degrading microorganisms were isolated from the treated wastewater sludge in a coke plant by enrichment and acclimation shaking culture at 25℃. It was acclimated to 500 mg/L DMP as the sole carbon source. The microorganisms were purified by successive streak transfers on agar-plate medium. The strain was tentatively identified as a *Bacillus* sp. on the basis of its characteristics according to Bergey's Mannual[27].

Medium

The basic medium used in this investigation is given in Table 1.

TABLE 1

Composition of Basic Medium

Component	Concentration (g/L)
DMP	0.05-0.5
KH_2PO_4	1.0
KNO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.1
$CaCl_2$	0.1
$FeCl_3$	0.01
NaCl	1.0

Immobilization of Microbial Cells

Ten grams of polyvinyl alcohol (PVA, nominal degree of polymerization=1 750, approx. molecular weight 75 000-80 000) were dissolved in 50 mL of distilled water, cooled to 40℃,

then mixed thoroughly with 50 mL of cell suspension with concentration of ca. 4.0×107 cells/mL. The resulting mixture was dropped into saturated boric acid solution for 1 h to form spherical beads. The formed gel beads were then soaded in 0.5 mol/L sodium orthophosphate solution for 1 h. The particles were washed with physiological saline[28].

Analytical Method

The biomass and liquid phase were separated by centrifugaton, and the supernatant was extracted twice with 5 mL of dichloromethane each time. The two aliquots of dichloromethane were combined and used for DMP analysis. DMP concentration of all samples in this work was analyzed by gas chromatography (Hewlett-Packard model 5890A with a flame ionization detector). The column temperature was 280℃ and the nitrogen gas flow rate was 30 mL/min. The volume of the injected samples was 2 μL, and the detection limit was 1 ng. The mass spectra were recorded using a model 5972 mass spectrometer.

Cell growth was monitored by determination of the optical density at 660 nm and an experimentally derived correlation was used to obtain the concentration.

Degradation of DMP by Free Cells

Free cell inoculum of 1.0 mL was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of sterile medium and shaken at 25℃ and 120 rev/min.

Degradation of DMP by Immobilized Cells

The experiments were carried out in 250 mL Erlenmeyer flasks containing 5 g beads of immobilized cells in 50 mL of sterile medium, and the other conditions were the same as above.

RESULTS AND DISCUSSION

Characteristics of DMP Degradation and the Growth of Microbial Cells

The time course of DMP degradation and the growth of microbial cells were studied using batch culture experiments. The results showed that 100 mg/L DMP could be completely degraded within 38 h and the biomass concentration increased with DMP degradation, indicating that the microorganisms isolated in this study were capable of utilizing DMP as the sole sources of carbon. In addition, microorganisms were found to continue their growth for a certain time after DMP was consumed, which showed that intermediates accumulated during the primary degradation of DMP could be subsequently used for microbial growth.

DMP Degradation by Free Cells

In order to determine the effect of initial DMP concentration rate of free cells, the strain was inoculated and cultured at various DMP concentrations. The results are shown in Fig.1

Fig.1 demonstrates that the lag phase duration of DMP degradation was prolonged with increase of initial DMP concentration, that is to say, the microorganism exhibited extended lag time at higher initial DMP concentration. Although 400 mg/L DMP could also be degraded completely, the time needed for degradation was about 96 h. The lag time lasted

for about 40 h. However, once the degradation started, its degradation rate was not inhibited.

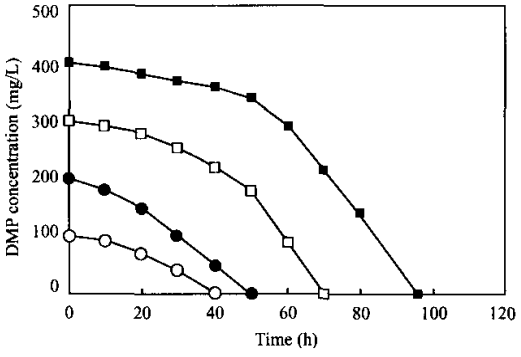

FIG. 1. Biodegradation of DMP by free cells.
Symbols:(○) (●) (■)(□) represent the initial concentration of DMP which is 100, 200, 300, and 400 mg/L, respectively. Data are means of triplicate experiments. Standard deviations are less than 10%.

DMP Degradation by Immobilized Cells

To determine the effect of immobilization on DMP-degrading activity and tolerance of the cells against DMP, the immobilized cells were prepared and used to degrade DBP at various initial concentrations. The results of DMP degradation by immobilized cells at different initial concentrations of DMP are shown in Fig. 2.

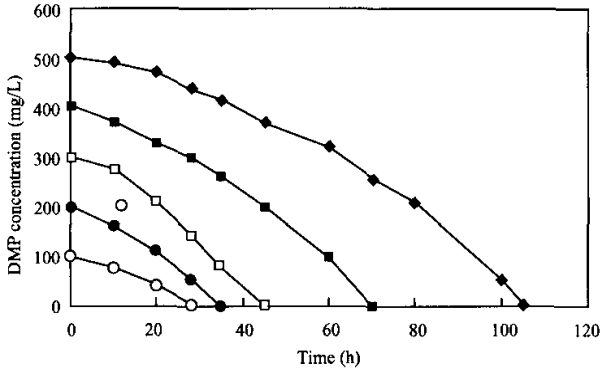

FIG. 2. Biodegradation of DMP by immobilized cells.
Symbols:(○) (●) (■)(□)(◆) represent the initial concentration of DMP which is 100, 200, 300, 400, and 500 mg/L, respectively. Data are means of triplicate experiments. Standard deviations are less than 10%.

The growth and distribution of microbial cells inside the PVA gel beads were observed by scanning electron microscopy, and the SEM graphs are shown in Figs. 3 and 4.

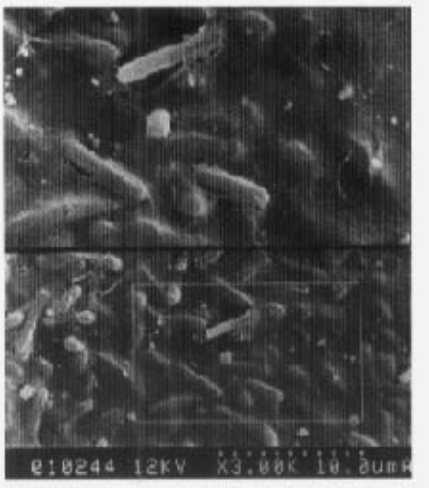

FIG. 3. Immobilized microbial cells on the surface of PVA gel.

FIG. 4. Immobilized microbial cells inside the PVA gel.

It could be seen that the microbial cells grew quite well inside the PVA gel beads.

Comparison of DMP Degradation by Free and Immobilized Cells

As shown in Figs. 1 and 2, the increasing concentration of DMP was better tolerated and more quickly degraded by immobilized cells than by free cells. The time needed for completely degrading various initial concentrations of DMP is depicted in Fig. 5.

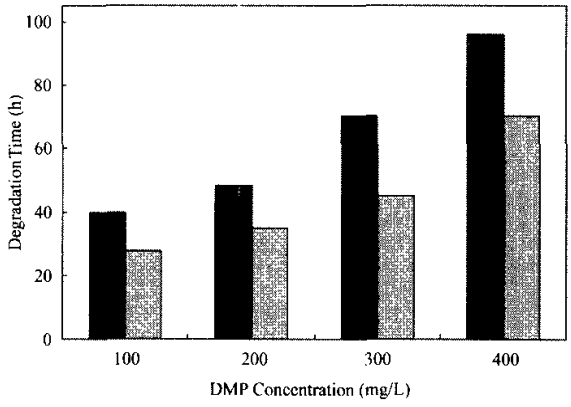

FIG.5. Comparison of DMP degraded by free and immobilized cells .
Symbols: (□) (■)represent the time needed for DMP degradation by free and immobilized cells, respectively. Data are means of triplicate experiments.

As shown in Fig.5, the time needed for complete degradation of DMP at the initial concentration of 100 mg/L was 40 h by free cells and 28 h by immobilized cells, respectively, when the initial concentration of DMP was in the range of 100-400 mg/L, while the time needed for DMP degradation by immobilized cells was shorter than that by free cells, which indicated that DMP could be degraded more quickly by immobilized cells than by free cells. The reasons may be as follows: (1) Immobilized cells can provide a high cell concentration, which results in a high degradation rate; (2) Immobilization of cells can improve the catalytic stability as well as tolerance against toxic compounds, which was also observed when immobilized cells were used to degrade phenol and chlorophenol[29].

Identification of Metabolites

The metabolites of DMP degradation by immobilized cells were extracted using dichloromethane at different time intervals and identified by GC/MS method. Some of the intermediates were isolated and identified.

The results of GC/MS analysis indicated that the main compounds isolated during DMP degradation either by immobilized cells or free cells were mono-methyl phthalate, phthalic acid and protocatechuic acid etc. Therefore, the metabolic pathway of DMP degradation by immobilized cells and free cells was identical. The degradation pathway of DMP by immobilized cells can be tentatively proposed as follows:

di-n-methyl phthalate→mono-methyl phthalate→phthalic acid→

protocatechuic acid→ring cleavage and mineralization

And such proposed pathway is consistent with the conclusion of other studies using free cells. This indicates that immobilization of microbial cells does change their original metabolic pathway.

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(*Received October* 12, 2002 *Accepted January* 20, 2003)