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BIODEGRADATION OF PHTHALIC ACID ESTERS BY IMMOBILIZED MICROBIAL CELLS

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Microorganisms capable of degrading di-n-butyl phthalate (DBP) were immobilized by polyvinyl alcohol (PVA) and used for DBP degradation. The characteristics of DBP degradation by immobilized and free cells were investigated. The experimental results showed that the degradation rate of immobilized cells was higher than that of free cells. The optimal pH and temperature for DBP degradation were determined to be 7.0 and 25°C, respectively. The semi-continuous degradation test demonstrated that high-frequency feeding of low concentration (50 mg/L) was more favourable than low-frequency feeding of high concentration (150 mg/L) for DBP degradation. The main intermediates of DBP degradation by immobilized cells were identified using a gas chromatography/mass spectrometry method. The results revealed the presence of monobutyl phthalate, phthalic acid, and protocatechuate. A tentative pathway for DBP degradation was proposed, which demonstrated that the metabolic mechanism of immobilized cells remained the same as that of the free cells. ©1997 Elsevier Science Ltd

INTRODUCTION

Phthalic acid esters (PAEs) are a class of refractory organic compounds widely used as plasticizers. They are among the most common industrial chemicals and have become widespread in the environment as they have been found in sediments, waters, and soils (Shelton et al. 1984; Fatoki and Ogunfowokan 1993; Huang et al. 1994). Some of them are suspected to be mutagens (Kozumbo et al. 1982) and carcinogens (Huff and Kluwe 1984). Three of the PAEs, namely, 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 (Wang et al. 1996) and the U.S. Environmental Protection Agency (USEPA 1982).

DBP belongs to the family of PAEs, which is a commonly used plasticizer and produced in large quantities in China. It has received increasing attention in recent years due to its widespread use and ubiquity in the environment (Wang et al. 1995b).

The metabolic breakdown of PAEs by microorganisms is considered one of the major routes of environmental degradation for these widespresad pollutants. Numerous studies have demonstrated the biodegradation of several PAEs under aerobic conditions in soil, natural waters, and wastewater (Ribbons et al. 1984). Sugatt et al. (1984) studied the biodegradation of 14 commercial phthalate esters that are commonly used as plasticizers by an acclimated shake flask CO_2 evolution. Nozawa and Maruyama (1988) investigated the anaerobic metabolism of phthalate and other aromatic compounds by the denitrifying bacterium *Pseudonomas sp.* strain P136. Shelton et al. (1984) studied the biodegradation of dimethyl, diethyl, di-n-butyl, and butyl benzyl phthalate esters in anaerobic digester sludge. Inman et al. (1984) conducted incubation experiments to investigate the factors affecting the decomposition of carboxyl-labelled (^{14}C) phthalic acid, monobutyl phthalate, and dibutyl phthalate in

soil under laboratory conditions. Johnson and Lulves (1975) studied the biodegradation of DBP and di-2-ethylhexyl phthalate in freshwater hydrosoil. Saeger and Tucker (1976) studied the primary and ultimate biodegradation of phthalic acid, monobutyl phthalate, and five structurally diverse PAE plasticizers in river water and activated sludge samples using ultraviolet spectrophotometry, gas chromatography, and CO_2 evoltion. Walker et al. (1984) studied the degradation of DBP in estuarine and freshwater sites. Wang et al. (1996) investigated the microbial degradation of three phthalates, i.e., DMP, DBP, and DOP by acclimated activated sludge. These studies focused on the biodegradability of the different PAEs and the pathway of degradation. Several types of microorganisms were found to degrade PAEs, including aerobic (Wang et al. 1995b), anaerobic (Shelton et al. 1984), and facultative (Zhang and Reardon 1990) species.

However, Huang et al. (1994) found that DBP was one of the main refractory organic compounds in municipal wastewater which usually remain in the effluent of the conventional activated sludge plants. One response to this problem is to utilize specific microbial cultures that are highly active in degrading these recalcitrant wastes. The problem then is proper control of culture conditions in an appropriate reactor over a prolonged period.

Immobilization of biocatalysts (enzymes and cells) has received increasing interest in recent years (Wang et al. 1995a). It offers a promising potential for the improvement of the efficiency of bioprocess. Compared with free cell, immobilized cell has several advantages as follows: 1) It 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; and 4) The catalytic stability of biocatalysts as well as the tolerance against toxic compounds can be improved. Biodegradation using immobilized cells has been widely investigated for numerous toxic compounds such as pentachlorophenol (O'Reilly and Crawford 1989), 4-chlorophenol (Balfanz and Rehm 1991), pyridine (Lee and Rhee 1994), benzene derivatives and chlorobenzoates (Sahasrabudhe et al. 1988), and 2,4-dichlorophenoxy acetic acid (2,4-D) (Shreve and Vogel 1993). However, biodegradation of phthalate esters using immobilized cells has not been reported.

The object of this study is to investigate the biodegradation of DBP by immobilized microorganism.

Table 1. Composition of basic medium.

Component	Concentration (g/L)
DBP	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

MATERIALS AND METHODS

Microorganisms

Several strains of DBP-degrading microorganisms were isolated from coke-plant wastewater treatment plant sludge by enrichment and acclimation shaking culture at 25 °C. It was acclimated to 500 mg/L DBP as the sole carbon source. The microorganisms were purified by successive streak transfers on agar-plate medium (Wang et al. 1995b). The strain was identified as a *Pseudomonas sp.* on the basis of its characteristics according to Bergey's Manual (Buchanna and Gibbons 1984).

Medium

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

Immobilization of microbial cells

Ten g of polyvinyl alcohol (PVA, nominal degree of polymerization = 1750, approx. molecular weight 75 000 ~ 80 000) was dissolved in 50 mL of distilled water, cooled to 40 °C, then mixed thoroughly with 50 mL of cell suspension with concentration of about 4.0×10^7 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 soaked in 0.5 M sodium orthophosphate solution for 1 h. The particles were washed with physiological saline (Chen and Lin 1994).

Analytical method

The biomass and the liquid phase were separated by centrifugation and the supernatant was extracted twice with 5 mL of dichloromethane each time. The two aliquots of dichloromethane were combined and used for

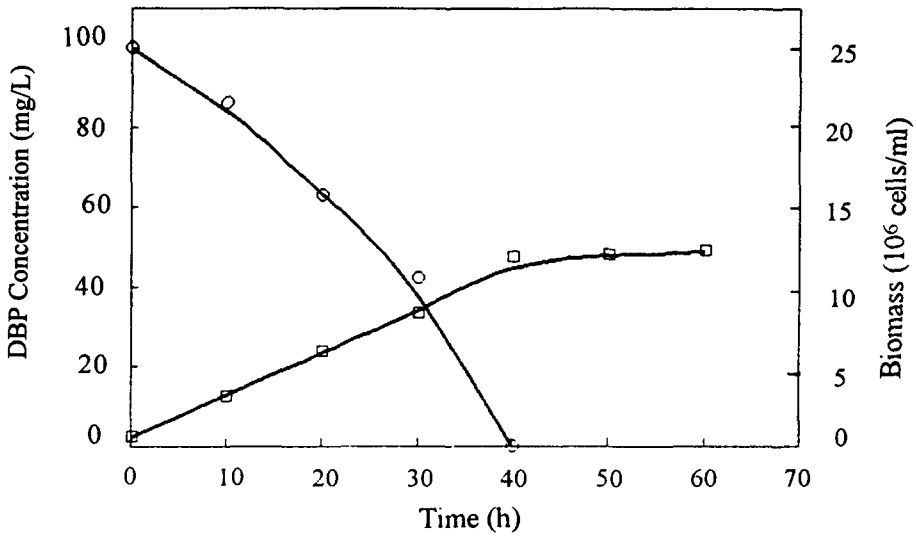

Fig. 1. Characteristics of DBP degradation and microbial growth.

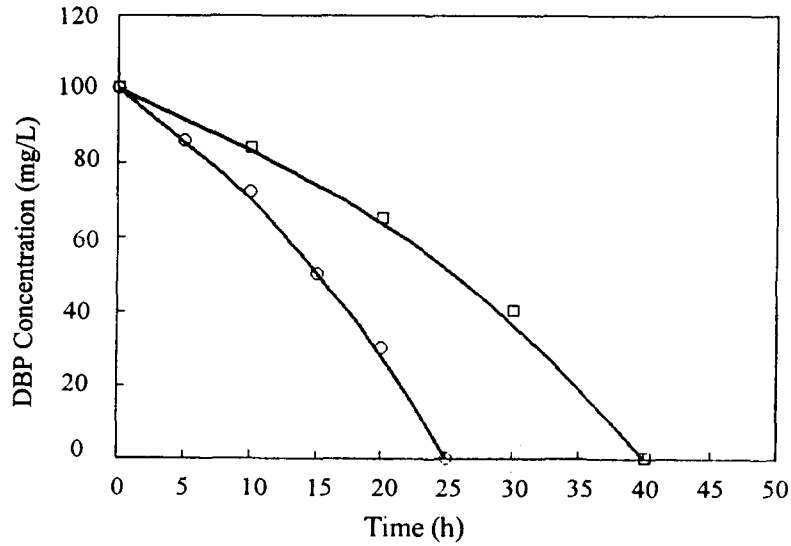

Fig. 2. DBP degradation by free and immobilized cells. (□) free cells; (○) immobilized cells.

for DBP analysis. DBP concentrations of all samples in this work were analyzed by gas chromatography (Hewlett-Packard model 5890A with a flame ionization detector). The column temperature was 280°C 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 using an experimentally derived correlation to obtain the concentration.

Degradation of DBP by free cells

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

Degradation of DBP by immobilized cells

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

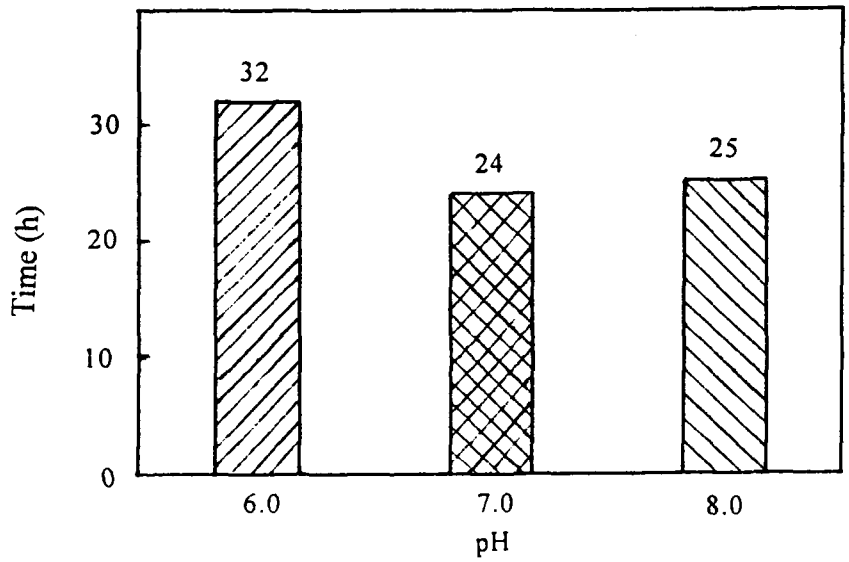

Fig. 3. Effect of pH on DBP degradation.

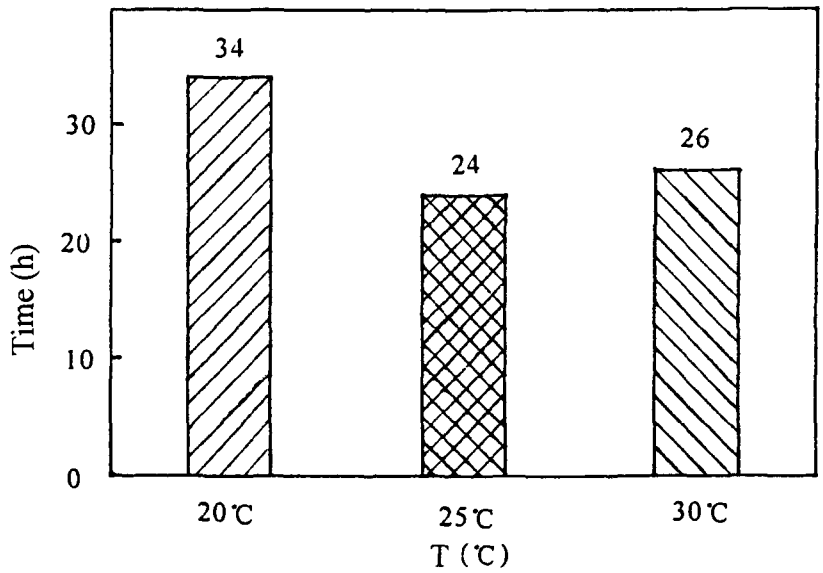

Fig. 4. Effect of temperature on DBP degradation.

RESULTS AND DISCUSSION

Characteristics of DBP degradation and the growth of microbial cells

The time course of DBP degradation and the growth of microbial cells are shown in Fig. 1. It can be seen from Fig. 1 that 100 mg/L DBP can completely be degraded within about 40 h. Fig. 1 also demonstrates that the biomass concentration increased with DBP degradation, indicating that the microorganisms isolated in this study were capable of utilizing DBP as the sole sources of carbon. In addition, it was found that microorganisms continued to grow for a certain time after DBP was consumed, which showed that intermediates accumulating during the primary degradation of DBP could subsequently be used for microbial growth.

Comparison of DBP degradation by free and immobilized cells

The microbial cells were entrapped by PVA. The immobilized cells were used to degrade DBP. The biodegradation of DBP by immobilized cells is shown in Fig. 2. The DBP degradation by free cells is also demonstrated in Fig. 2. As can be seen in Fig. 2, DBP was more quickly degraded by immobilized cells than by free cells.

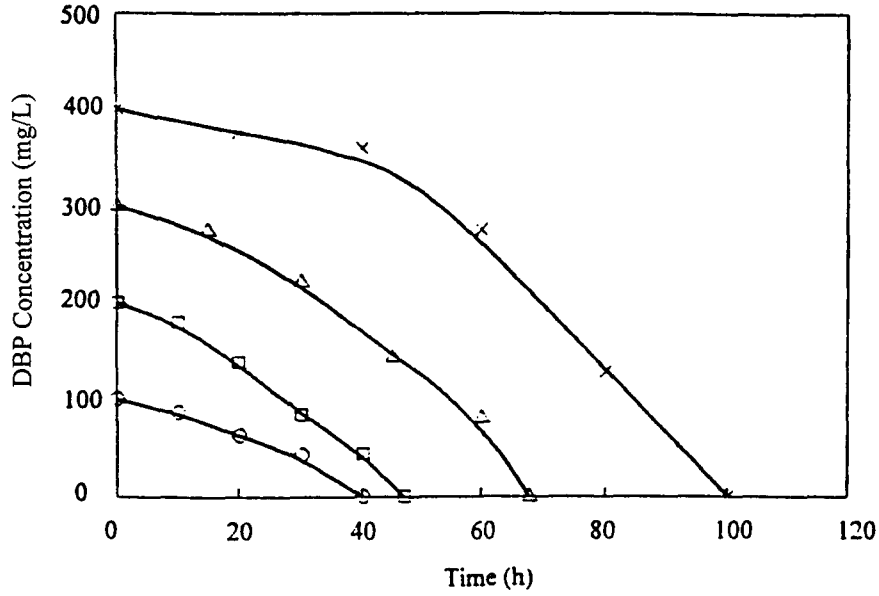

Fig. 5. DBP degradation by free cells.

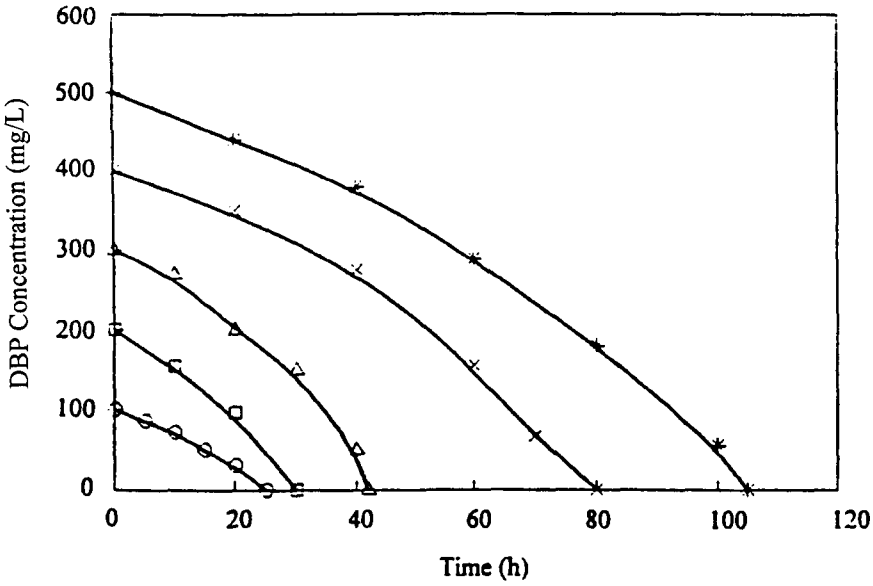

Fig. 6. DBP degradation by immobilized cells.

Effect of pH on DBP degradatiion

The pH value is a key factor in microbial metabolic processes, because it influences the redox-potential and the enzymatic activity. Experiments examining the pH optimum for DBP degradatioin by immobilized cell were performed. The initial pH of the medium was adjusted by 6M NaOH and 6M HCl. The results are shown in Fig. 3. It can be seen that the optimal pH for DBP degradation was 7.0. At pH 7.0, the time of total degradation could be decreased from 32 h to 24 h.

Effect of temperature on DBP degradation

DBP degradation by immobilized cells was carried out at different temperatures. The experimental results are demonstrated in Fig. 4. It is obvious that the optimal temperature was 25 °C.

Effect of initial DBP concentration on degradation rate

To determine the effect of immobilization on DBP-degrading activity and tolerance of the cells against DBP, the immobilized cells were prepared and used to

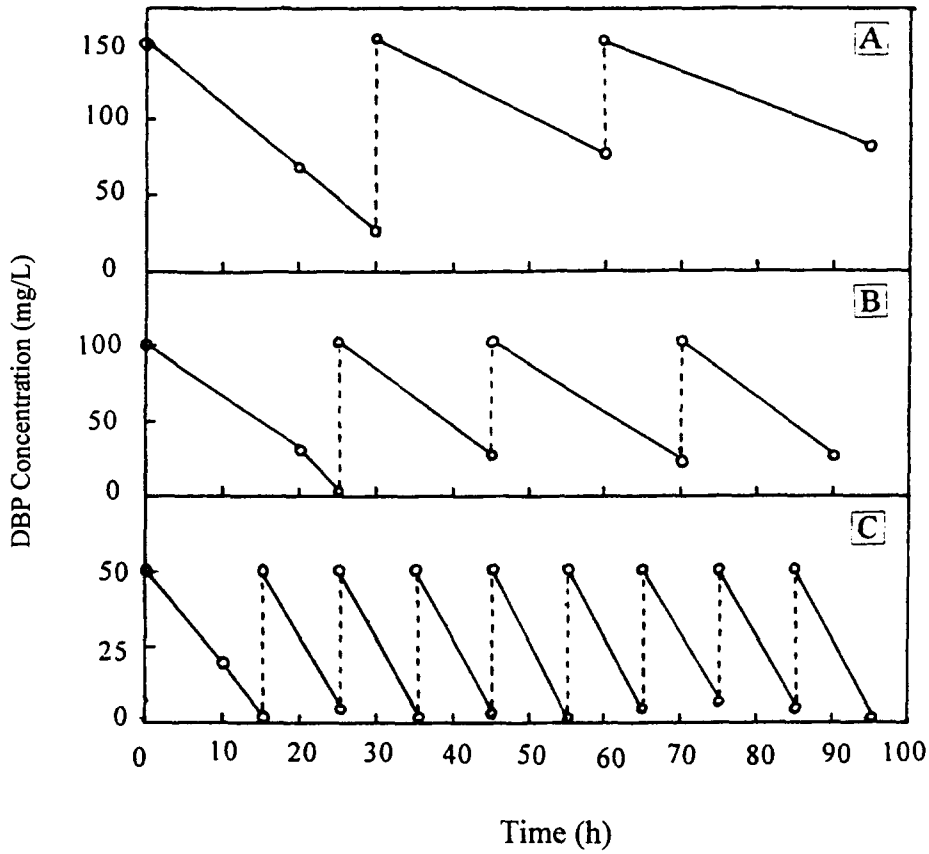

Fig. 7. Semi-continuous degradation of DBP by immobilized cells.

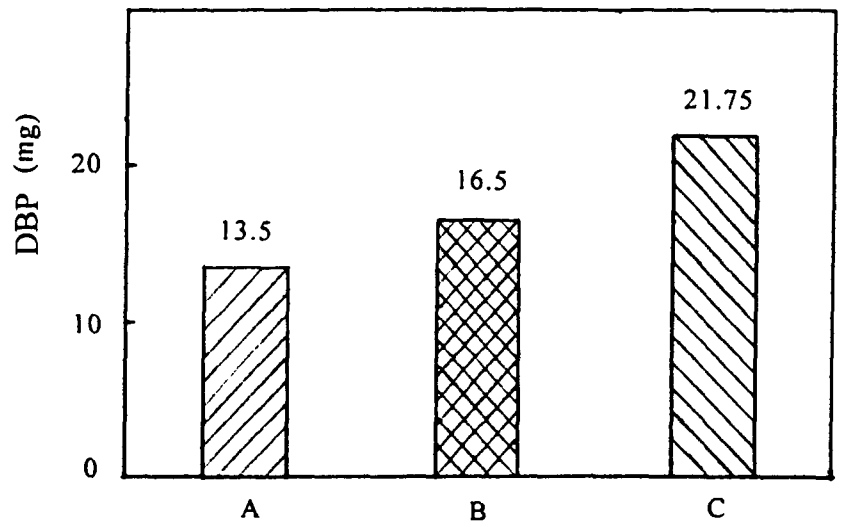

Fig. 8. Effect of feeding dosage on total DBP degraded within 100 h.

degrade DBP at various initial concentrations. The results of DBP degradation by immobilized cells with different initial concentrations of DBP were compared with that of free cells. The biodegradation of DBP by free and immobilized cells is shown in Figs. 5 and 6, respectively.

Figure 5 demonstrates that the lag-phase duration of DBP degradation prolonged with an increase in the initial DBP concentration, that is to say, the microorganism exhibited extended lag time at higher initial DBP concentration. Although 400 mg/L DBP can also be degraded completely, the time needed for degrada-

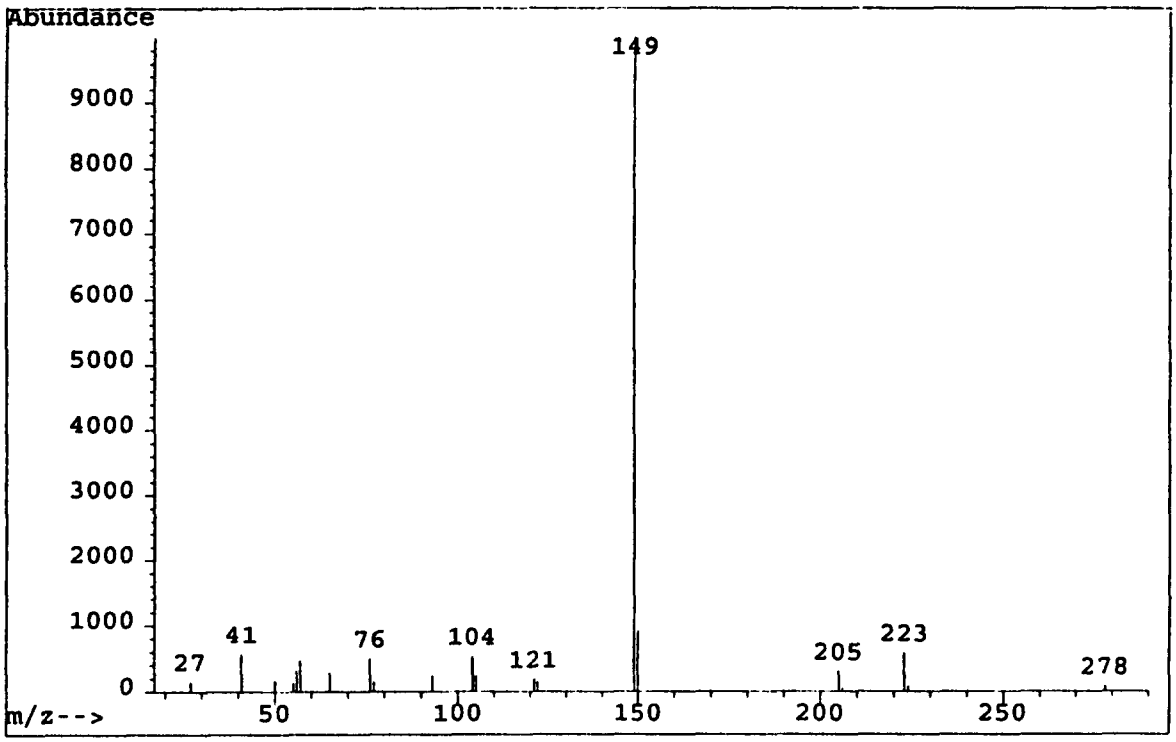

Fig. 9. Mass spectrum of isolated DBP.

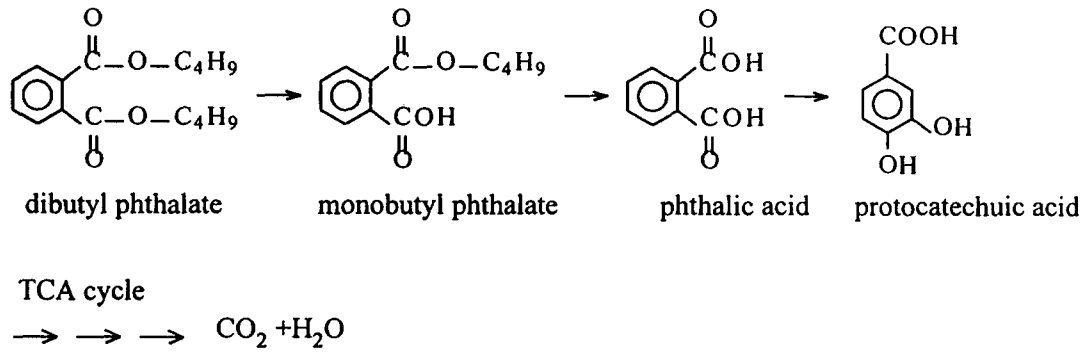

Fig. 10. Proposed pathway for the DBP degradation by immobilized cells.

tion was about 100 h. The lag time lasted about 40 h. However, once the degradation started, its degradation rate was not inhibited.

As can be seen in Figs. 5 and 6, the increasing concentration of DBP was better tolerated and more quickly degraded by immobilized cells than by free cells. The reasons may be that: 1) Immobilized cells can provide a high cell concentration, resulting in a high degradation rate; and 2) Immobilization of cells can improve the catalytic stability as well as tolerance against toxic compounds, which was also observed in the application of immobilized cells to degradation of phenol and chlorophenol (Weistmeier and Rehm 1989.

Semi-continuous degradation of DBP

Semi-continuous tests for DBP degradation by immobilized cells were performed with different concentrations of DBP. When each dose of DBP had been substantially degraded, a further dose was fed. The results are shown in Fig. 7. Figure 8 shows the total amounts of DBP degraded within 100 h with different feeding concentrations.

As can be seen in Figs. 7 and 8, the high-frequency feeding of low concentration (50 mg/L) was more favorable than low-frequency feeding of high concentration (150 mg/L) for DBP degradation.

Identification of metabolites

The metabolites of DBP degradation by immobilized cells were extracted using dichloromethane at different time intervals and identified by the GC/MS method. Some of the intermediates were isolated and identified. The mass spectrum of compound I is shown in Fig. 9.

The mass spectral analysis of compound I showed the parent ion peak at m/z 278 (Fig. 9), which is in good agreement with empirical formula $C_{16}H_{22}O_4$. The fragmentation pattern showed ion peaks at m/z 223 base peak (M-OC_4H_9), and 149 (M-OH).

The other main compounds isolated during DBP degradation were also identified. They are: monobutyl phthalate, phthalic acid, and protocatechuic acid. Therefore, the pathway of DBP degradation by immobilized cells can be tentatively proposed (Fig. 10).

This is consistent with the conclusion reported by former researchers using free cells. This indicated that the immobilization of microbial cells did not change their original metabolic pathway.

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REFERENCES

Balfanz, J.; Rehm, H.J. Biodegradation of 4-chlorophenol by adsorptive immobilized *Alcaligenes sp.* A 7-2 in soil. Appl. Microbiol. Biotechnol. 35: 662-668; 1991.

Buchanna, R.E.; Gibbons, N.E. Bergey's manual of determinative bacteriology. 8th ed. Baltimore: Williams and Wilkins; 1984.

Chen, K.C.; Lin, Y.F. Immobilization of microorganisms with phosphorylated polyvinyl alcohol (PVA) gel. Enzyme. Microb. Technol. 16: 79-83; 1994.

Fatoki, O.S.; Ogunfowokan, A.O. Determination of phthalate ester plasticizers in the aquatic environment of southwestern Nigeria. Environ. Int. 19: 619-623; 1993.

Huang, X.; Zhang, X.J.; Fu, Q. Assessment for biodegradability of organics in municipal wastewater and controlling measure for its refractory organics. Chin. J. Environ. Sci. 15: 5-19; 1994.

Huff, J.E.; Kluwe, W.M. Phthalate esters carcinogenecity in F 3444/N rats and B6C3F mice. Prog. Clin. Biol. Res. 141: 137-145; 1984.

Inman, I.C.; Strachan, S.D.; Sommer, L.E.; Nelson, D.W. The decomposition of phthalate esters in soil. J. Environ. Sci. Health Ser. B. 19: 245-257; 1984.

Johnson, G.; Lulves, W.J. Biodegradation of di-n-butyl phthalate and di-2-ethyl hexyl phthalate in freshwater hydrosoil. J. Fish. Res. Board Can. 32: 333-339; 1975.

Kozumbo, W.J.; Kroll, R.; Rubin, R.J. Assessment of mutagenicity of phthalate esters. Environ. Health Perspect. 45: 103-110; 1982.

Lee, S.T.; Rhee, S.K.; Lee, G.M. Biodegradation of pyridine by freely suspended and immobilized Pimelobacter sp. Appl. Microbio. Biotechnol. 41: 652-657; 1994.

Nozawa, T.; Maruyama, Y. Anaerobic metabolism of phthalate and other aromatic compounds by a denitrifying bacterium. J. Bacteriol. 170: 5778-5784; 1988.

O'Reilly, K.T.; Crawford, R.L. Degradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium* cells. Appl. Environ. Microbiol. 55: 213-218; 1989.

Ribbons, D.W.; Keyser, P.; Kunz, D.A.; Taylor, B.F. Microbial degradation of phthalates. In: Gibson, D.T., ed. Microbial degradation of organic compounds. New York: Dekker; 1984.

Sahasrabudhe, S.R.; Modi, A.J.; Modi, V.V. Dehalogenation of 3-chlorobenzoate by immobilized *Pseudomonas sp* B13 cells. Biotechnol. Bioeng. 31: 89-893; 1988.

Seager, V.W.; Tucker, N.V. Biodegradation of phthalic acid esters in river water and activated sludge. Appl. Environ. Microbiol. 31: 29-34; 1976.

Shelton, D.R.; Boyd, S.A.; Tiedije, J.M. Anaerobic biodegradation of phthalic acid esters in sludge. Environ. Sci. Technol. 18: 93-97; 1984.

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.

Sugatt, R.H.; O'Grady, D.P.; Banerjee, S.; Howard, P.H.; Gledhill, W.E. Shake flask biodegradation of 14 commerical phthalate esters. Appl. Environ. Microbiol. 47: 601-606; 1984.

USEPA (U.S. Environmental Protection Agency). Fate of priority pollutants in publicly owned treatment works. Final report. EPA 440/1-82/303. Cincinnati, OH: USEPA; 1982.

Walker, W.W.; Cripe, C.R.; Corcoran, E.F. Potential for biodegradation of phthalic acid esters in marine regions. Appl. Environ. Microbiol 13: 1283-1294; 1984.

Wang, J.; Hou, W.; Qian, Y. Immobilization of microbial cells using polyvinyl alcohol (PVA)-polyacrylamide gels. Biotechnol. Techniques 9: 203-208; 1995a.

Wang, J.; Liu, P.; Qian, Y. Microbial degradation of di-n-butyl phthalate. Chemosphere 31: 4051-4056; 1995b.

Wang, J.; Liu, P.; Qian, Y. Biodegradation of phthalic acid esters by acclimated activated sludge. Environ. Int. 22: 737-741; 1996.

Weistmeier, F.; Rehm, H.J. Biodegradation of 4-chlorophenol by entrapped *Alcaligenes sp.* A 7-2. Appl. Microbiol. Biotechnol. 22: 301-305; 1985.

Zhang, G.; Reardon, K.F. Parameter study of diethyl phthalate biodegradation. Biotechnol. Lett. 21: 699-704; 1990.