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# Microbial degradation of di-n-butyl phthalate by *Micrococcus* sp. immobilized with polyvinyl alcohol

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ABSTRACT

Di-n-butyl phthalate (DBP) is an endocrine-disrupting chemical and priority pollutant which is difficult to remove by conventional activated sludge process. In this study, a very efficient DBP-degrading strain was isolated and identified as *Micrococcus* sp. The microbial degradation of DBP by immobilized *Micrococcus* sp. was investigated. Polyvinyl alcohol (PVA) gel was used as an immobilizing material for entrapment of microbial cells. The degradation of DBP by cells of *Micrococcus* sp. immobilized by different PVA gel beads was investigated, including PVA-boric acid beads, PVA-sodium nitrate beads, and PVA-orthophosphate beads. The experimental results showed that the PVA-orthophosphate immobilized cells was most efficient for DBP degradation, in comparison with freely suspended cells and the cells immobilized in PVA-boric acid and PVA-sodium nitrate. The PVA-orthophosphate immobilized cells could be reused for more than 12 cycles without losing its degradation capacity; they were more tolerant to pH and temperature changes than freely suspended cells. The kinetics of DBP degradation by immobilized *Micrococcus* sp. conformed to the first-order kinetic model. The metabolic mechanism of immobilized cells remained unchanged.

*Keywords:* Immobilization; Biodegradation; Phthalate; Plasticizer; *Micrococcus* sp.

## 1. Introduction

Phthalic acid esters (PAEs) were commonly referred to as phthalates, they have attracted global attention because of their high production volume, widespread use in consumer products, and deleterious health effects [1]. PAEs have been found in sediments, water bodies and soils, some of them are suspected to be mutagents and carcinogents. Di-n-butyl phthalate is a commonly used plasticizer 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. DBP is a reproductive toxicant which had effects on animals exposed pre- and post-natal. As a result, DBP has been listed as priority pollutant by several regulatory institutions, such as the US Environmental Protection Agency (US EPA, 1991), the European Union, and China National Environmental Monitoring Center [2].

Metabolic breakdown by micro-organisms is one of the major routes for environmental degradation of DBP due to its low rate of the hydrolysis and the photolysis [3,4]. Microbial degradation of DBP was researched in recent years, for example, Patil et al. [5] investigated the degradation of DBP by *Delftia* sp. TBKNP-05. Xu et al. [6] studied the biodegradation of

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an endocrine-disrupting chemical di-n-butyl phthalate ester by *Pseudomonas fluorescens* B-1. Walker et al. [7] reported the dibutyl phthalate degradation in esturine and fresh water sites. Roslev et al. [8] investigated the degradation of phthalate esters in an activated sludge wastewater treatment plant. Luo et al. [9] studied the degradability of the three dimethyl phthalate isomer esters by a *Fusarium* species isolated from mangrove sediment. Pradeep et al. [10] described how *Aspergillus japonicus*, *Penicillium brocae*, and *Purpureocillium lilacinum*, three novel isolates from heavily plastic-contaminated soil completely utilized the plasticizer di(2-ethylhexyl)phthalate bound to PVC blood storage bags in simple basal salt medium by static submerged growth. Biodegradation of different PAEs have been investigated in recent years in China and worldwide [11–17].

However, to our knowledge, there is a little research on the application of immobilized microbial cells for biodegradation of PAEs. The use of free cells for the degradation of various toxic pollutants for industrial applications has a number of disadvantages due to their low mechanical strength, low density of cells, and difficulty in biomass effluent separation. Immobilization techniques can overcome these problems. Immobilization of microbial cells has received increasing attention in the field of wastewater treatment [18]. It offers a promising potential for the improvement of the efficiency of bioprocess. Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems since high densities of specialized micro-organisms are used. They have been widely applied for degradation of numerous toxic pollutants, such as chlorophenol [13], quinoline [19,20], pyridine [21,22], PAEs [23,24], benzene derivatives, and chlorobenzoates [25]. Entrapment of microbial cells in polymeric matrixes is widely used for cell immobilization, and many natural and synthetic polymers have been used.

Polyvinyl alcohol (PVA) is a synthetic polymer, which can be used for microbial immobilization. However, there are some problems when using the PVA-boric acid method for immobilizing microbial cell, such as, the agglomeration of PVA beads, the toxicity of boric acid, and the swelling performance [26].

The objective of this research was to investigate DBP degradation by immobilized *Micrococcus* sp. using PVA as carrier. Three different gelating reagents, that is, boric acid, nitrate, and orthophosphate, were used to improve the PVA immobilization method to develop a suitable and practical microbial immobilization method.

## 2. Materials and methods

### 2.1. Chemicals

Di-n-butyl phthalate used in this study was of commercial grade (Beijing Chemical Plant) and had a purity of at least 99.5%. Hexane used in this study was of HPLC grade (Thermo). All other chemicals and solvents were of analytical grade. All chemicals were used as received without any further purification.

### 2.2. Micro-organisms and medium

The strain used in this study was selected from the activated sludge collected from coking wastewater treatment plant. The micro-organisms were purified by successive streak transfers on agar-plate medium. The strain was identified as *Micrococcus* sp. using biology system.

### 2.3. Immobilization methods

*Micrococcus* sp. was grown on mineral salts medium containing 50 mg/L of DBP. The cells were harvested during the mid-logarithmic growth phase by centrifugation at 10,000 rpm for 10 min at 4°C and then washed twice with 50 mM phosphate buffer (pH 7.0). The cells were immobilized using different PVA matrices.

Ten grams of PVA, (nominal degree of polymerization = 1,750, 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 $4.0 \times 10^7$ cells/mL. The resulting mixture contained 10% (w/v) PVA, 1.0% (w/v) sodium alginate, and about 20 g/L of micro-organisms. The following three different gelating solutions were used to form gel beads [26]:

(1) The mixture was dropped into saturated boric acid and $CaCl_2$ (1% w/v) solution for 1 h to form spherical beads (group B).
(2) The mixture was dropped into saturated boric acid and $CaCl_2$ (1% w/v) solution for 1 h to form gel beads, then transferring to 0.5 M sodium orthophosphate solution and immersing for 1 h (group P).
(3) The mixture was extruded as drops into a solution of sodium nitrate (50% w/v) and $CaCl_2$ (1% w/v), and then immersed for 1 h to form PVA-sodium nitrate beads (group N).

The formed particles were washed with physiological saline solution for 1 h and then stored in de-ionized water at 4°C for further use.

### 2.4. Biodegradation of DBP

All flasks were incubated in a thermostats rotary shaker operated at 30℃ and 150 rpm. Experiments on DBP degradation were conducted in 50-mL Erlenmeyer flasks containing 5 g of immobilized cells and 30 mL medium, with different concentrations of DBP (10–400 mg/L). All tests were conducted in triplicate. Control tests were performed without inoculating micro-organisms.

### 2.5. Analytical method

The degradation products of DBP were determined by gas chromatography (HP4890) and mass spectrometry. The conditions for GC were as follows: helium as the carrier gas, flow rate of 1 mL/min, injector temperature 260℃, detector temperature 285℃, oven temperature initially 80℃ for 5 min then increased to 260℃ by 6℃/min for 5 min, increased to 280℃ by 6℃/min and held for 5 min, increased again to 300℃ by 10℃/min and held for 3 min. The effluent from the GC column was connected to MS, and the spectra were obtained by EI mode, 70 eV ionization energy, and 50–400 amu scan for 2 s. The degradation products of DBP were identified through comparison with the mass spectra library stored in the MS system.

## 3. Results and discussion

### 3.1. Comparison of immobilization methods

In order to investigate the possible degradation of DBP by chemical reactions, such as hydrolysis and oxidation, a sterile control experiment was carried out by autoclaving DBP in mixed medium for 25 min at 121℃. The results suggested that abiotic loss of DBP can be neglected during this study. The adsorption of DBP by immobilizing carries was also neglectable.

Three different kinds of PVA immobilized microbial cells were prepared and designated to be group B, group N, and group P. The biodegradation of DBP by different immobilized cells was carried out, and the experimental results were depicted in Fig. 1.

From Fig. 1 we can see that the degradation efficiency of DBP was 82, 92, and 60% for group N, group P, and group B, respectively, that is to say, the bioactivity of the phosphorylated PVA beads (group P) was highest for DBP degradation, indicating that among three different PVA immobilization methods, PAV-orthophosphate method could maintain the high bioactivity of microbial cells, perhaps due to the low toxicity of gelation solution.

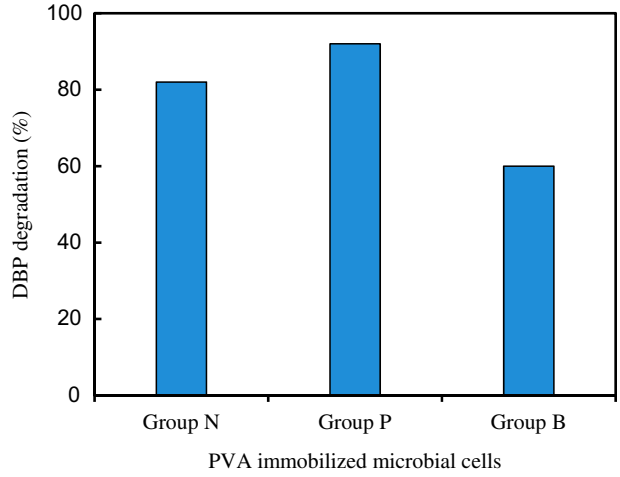


Fig. 1. Comparison of DBP degradation by different immobilized cells.

### 3.2. Degradation of DBP by immobilized cells

The experimental results of DBP degradation by immobilized cells was shown in Fig. 2. The DBP degradation by free cells was also demonstrated in Fig. 2. As it is seen in Fig. 2, DBP was more quickly degraded by immobilized cells than by free cells. The reasons may include: (1) immobilized cells can provide a higher cell concentration, resulting in a higher 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 [13].

The high degrading capacity of PVA-orthophosphate immobilized cells compared to other matrices may be due to its high porosity, mechanical strength,

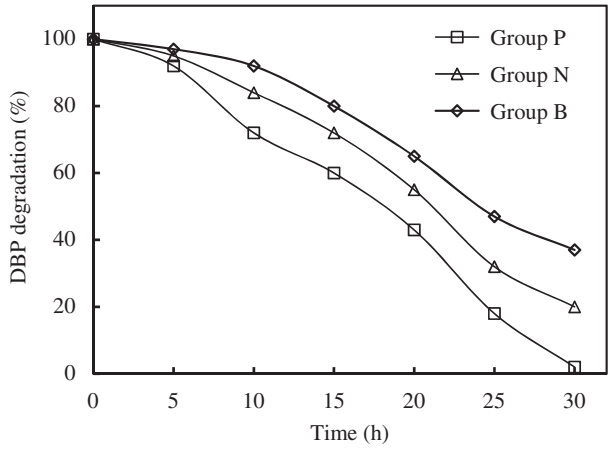


Fig. 2. Degradation of DBP by free and immobilized cells.

stability, and adsorbing capacity. Therefore, PVA-orthophosphate is a suitable carrier for the growth of cells in the medium containing toxic substrate than other matrices.

### 3.3. Effect of pH on DBP degradation

The effect of pH on DBP degradation was studied at pH range of 5.0–9.0. The initial pH of the medium was adjusted by 2 M NaOH and 2 M HC1. The results showed that variation of initial pH between 6.0 and 8.0 had no effect on the degradation of DBP by immobilized cells (Fig. 3), whereas, freely suspended cells were able to degrade at the narrow pH range. The experimental results suggested that the immobilized cells were more stable at wider range of initial pH value. The pH value is a key factor in microbial metabolic processes, because it influences the redox potential and the enzymatic activity. The results indicated that immobilized cells were more suitable for potential actual application in wastewater treatment because of their wider pH range.

### 3.4. 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.

The effect of temperature on DBP degradation showed that immobilized cells had high activity at temperature between 20℃ and 35℃, whereas the freely suspended cells showed high activity at temperature between 25℃ and 35℃. However, the optimum temperature were found to be at 30℃ both for freely suspended cells and immobilized cells in culture medium.

### 3.5. Fed-batch degradation by immobilized cells

The DBP degradation in fed-batch mode by immobilized *Micrococcus* sp. was carried out for every 24 h. The results are shown in Fig. 5. The immobilized cells by PVA-orthophosphate beads could be reused for 12 cycles without losing their degradation capacity. In contrast, the immobilized cells by PVA-boric acid beads could remain to about 50% degradation capacity after repeated use for 12 cycles.

### 3.6. Storage stability of immobilized cells

The stability of freely suspended cells and immobilized cells were tested after storage for 0–60 d at 4℃. The degradation capacity of free cells and immobilized cells was determined after different storage time, the results are shown in Fig. 6.

It can be seen that the immobilized cells can be stored for 60 d at 4℃ without obvious loss of their degradation capacity. But, the freely suspended cells lost their degrading capacity after 60 d and only about 20% of their original degradation capacity was remained, indicating that immobilized cells had much higher stability for long time storage.

### 3.7. Kinetics of DBP degradation

The kinetics of DBP degradation was studied by changing initial DBP concentration at 100, 200, 300, and 400 mg/L, respectively. The DBP degradation at different initial concentrations is shown in Fig. 7.

Numerous kinetic models have been used to describe the biodegradation kinetics of organic pollutants, and the first-order kinetic equation has been

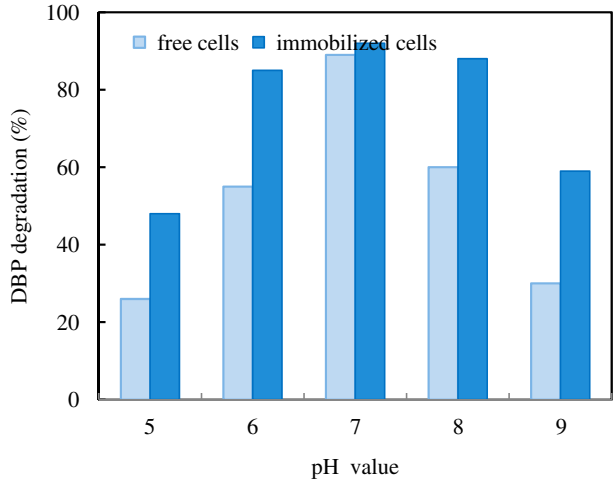


Fig. 3. Effect of pH on DBP degradation.

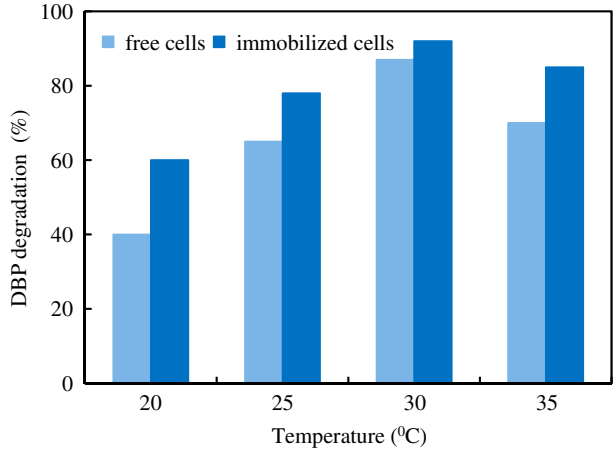


Fig. 4. Effect of temperature on DBP degradation.

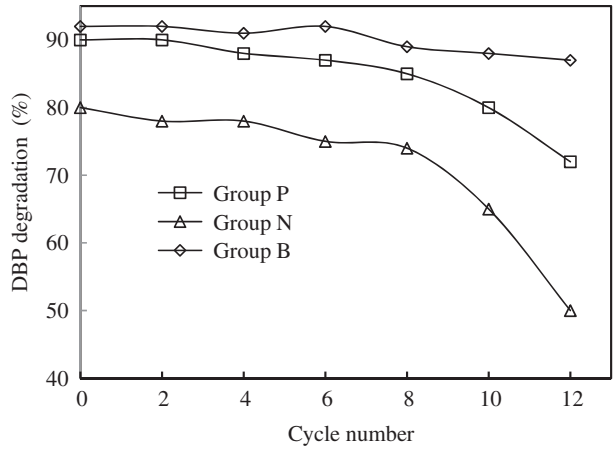


Fig. 5. Fed-batch degradation of DBP by immobilized cells.

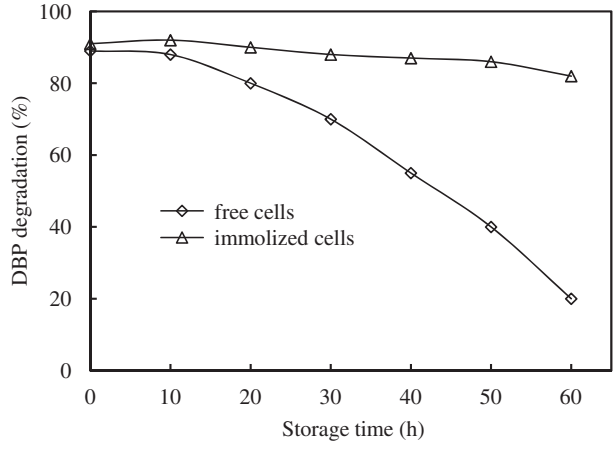


Fig. 6. Storage stability of freely suspended cells and immobilized cells.

used frequently to describe the biodegradation progress at low substrate concentrations [27].

The linear regression was applied to the experimental date shown in Fig. 7 according to the equation:

$$\ln C = a + k_1 t \quad (1)$$

where $C$ is DBP concentration; $t$ is degradation time; and $k_1$ is the first-order rate constant.

The calculated results are depicted in Fig. 8, indicating that the first-order model was suitable to describe the DBP degradation by immobilized *Micrococcus* sp.

The biodegradation half-life of first-order reaction can be calculated according to following equation:

$$t_{1/2} = \frac{0.693}{k_1} \quad (2)$$

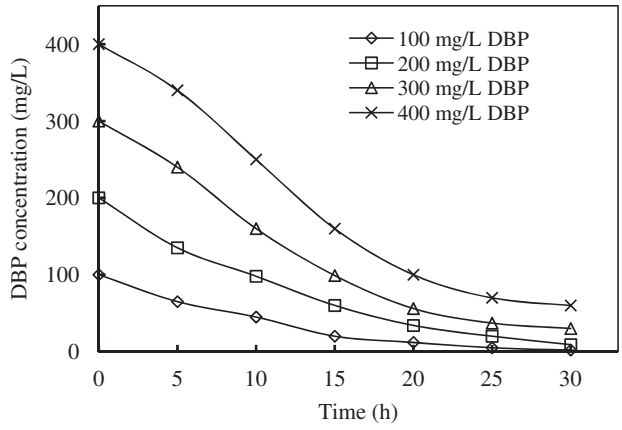


Fig. 7. The kinetics of DBP degradation by immobilized cells.

The kinetic equations of DBP degradation by immobilized *Micrococcus* sp. are summarized in Table 1.

### 3.8. Identification of DBP degradation metabolites

Phthalate esters have the basic structure of an esterified benzene-dicarboxylic acid with two alkyl chains. Usually, the primary biodegradation involves the sequential hydrolysis of the ester linkage between each alkyl chain and the aromatic ring, forming phthalic acid (PA). In this study, mono-butyl phthalate and PA were found in GC analysis as the metabolites or intermediate products, indicating that the metabolic mechanism of immobilized microbial cells remained the same as that of the free cells (Fig. 9).

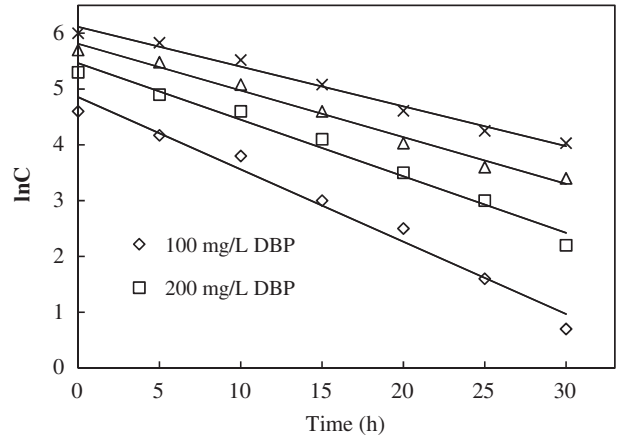


Fig. 8. Relationship between ln $C$ and $t$.

Table 1
The kinetic equations of DBP degradation by immobilized cells

| DBP concentration (mg/L) | Kinetic equations | Rate constant ($h^{-1}$) | Half-life (h) | $R^2$ |
|---|---|---|---|---|
| 100 | $\ln C = 4.854 - 0.1296\ t$ | 0.1296 | 5.35 | 0.9783 |
| 200 | $\ln C = 5.464 - 0.1014\ t$ | 0.1014 | 6.84 | 0.9815 |
| 300 | $\ln C = 5.701 - 0.0797\ t$ | 0.0797 | 8.69 | 0.9920 |
| 400 | $\ln C = 5.977 - 0.0683\ t$ | 0.0683 | 10.15 | 0.9916 |

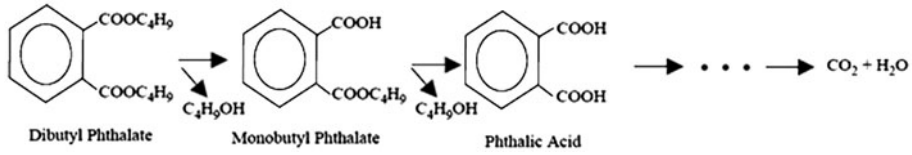


Fig. 9. DBP degradation pathway by immobilized cells.

## 4. Conclusions

This study indicated that the more effective degradation of DBP could be achieved by immobilized cells of *Micrococcus* sp. strain as compared to that of freely suspended cells. The PVA-orthophosphate immobilized cells had a higher degradation rate than PVA-boric acid and PVA-sodium nitrate immobilized cells. The immobilized cells showed more tolerance to pH and temperature changes than freely suspended cells. The immobilized cells could be stored for longer period without losing their degrading capacity. Furthermore, longevity of cells immobilized in PVA-orthophosphate beads and their operational stability was better than other matrices. The kinetics of DBP degradation by immobilized *Micrococcus* sp. conformed to the first-order model. The metabolic mechanism of immobilized microbial cells remained the same as that of the free cells. Thus, the immobilized cells could potentially be used in the treatment of industrial wastewater.

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