**Enhanced biodegradation of di-*n*-butyl phthalate by *Acinetobacter* species
strain LMB-5 coated with magnetic nanoparticles**

Jing Wang^b, Li-Hua Jiang^b, Ying Zhou^{a,b*}, Bang-Ce Ye^{a*}

Abstract

Phthalate acid esters (PAEs) are widely present in a variety of consumer products and they pose a public health concern due to the high mobility and hazards. In this study, we designed an immobilized method to eliminate phthalates by using $\text{Fe}_3\text{O}_4@\text{NH}_2$ nanoparticles (NPs). The surface-modified NPs showed a good dispersion property, and the particle size was approximately 230 nm. The results showed that *Acinetobacter* sp. strain LMB-5 could be coated by the prepared magnetic particles allowing easy separation from solution by applying an external magnetic field. The coated cells possessed adaptability to a wide range of pH and high tolerance towards toxic substances. More importantly, when the temperature was elevated to 60 °C, the coated cells also exhibited remarkable degradability. There was no change in the main metabolites during the biodegradation between the free cells and immobilized ones through GC/MS analysis to detect the degradation intermediates. These results show that immobilization method using magnetic nanoparticles is a promising way for degradation of phthalates in application.

Keywords: phthalate degradation; *Acinetobacter* species; reusability; magnetic separation; nanoparticles

1. Introduction

Phthalates or phthalic acid esters (PAEs) are a group of refractory and hazardous compounds blended in plastics to enhance their plasticity and versatility (Fan et al., 2004; Wang et al., 2004; Xu et al., 2005, Li et al., 2005a, b; Li and Gu, 2006a, b; Xu et al., 2006; Luo et al., 2009; 2011; 2012; Benjamin et al., 2015), which are widely distributed in wastewater effluent, sediment and nature river (Li et al., 2006). Large quantities of PAEs are produced globally to cater the needs of plastic products, but concerns are arising over the fate of them in the environment and potential risk to human health. Phthalate such as DBP is a relative stable compound in the natural environment, and its toxicity could not be ignored completely due to its tendency to accumulate in organisms (Stales et al., 1997).

Many treatment approaches have been attempted to minimize the concentration of phthalates over the past years, including photolysis (Kaneco et al., 2006; Xu et al., 2009), hydrolysis (Lake et al., 1977) and biodegradation (Li et al., 2005a, b; Li and Gu, 2006a, b; Wang and Gu, 2006a, b; Xu et al., 2006; Cheung et al., 2007; Li and Gu, 2007; Xu et al., 2007; Wang et al., 2008; Luo et al., 2016). Among these candidate processes, microbial degradation mainly by bacteria, fungi and enzymes is the main effective process and the mechanism involved has been elucidated (Engelhardt et al., 1977; Kim et al., 2002; Wang et al., 2004; Xu et al., 2005; Li and Gu, 2006a, b; Wang and Gu, 2006a, b; Wu et al., 2010; Luo et al., 2011; 2012). However, when free cells were used to reduce hazardous pollutants directly, loss of activity and separation from the applied conditions are the major obstacles preventing effective and efficient treatment (Kathiravan et al., 2010). Cell immobilization can resolve these problems, providing advantages such as enhanced tolerance against toxic compounds, improved catalytic efficiency and recycle the catalytic cells in further application (Wang et al., 1997).

Cell immobilization and separation by magnetic nanoparticles, which differs from traditional immobilization methodologies, is an innovative and promising technology (Wang et al., 2004; Xu et al., 2011). This immobilization method displays lower diffusional limitations and steric hindrance (Haukanes and Kvam, 1993; Shan et al., 2005). Additionally,

biocompatibility of Fe_3O_4 nanoparticles was also confirmed by in vitro cytotoxicity assay (Ankamwar et al., 2010; Khin et al., 2012). Since high-efficiency degradation bacteria obtained by genetic modification and engineering have potential risks to the ecosystem, recovery of those organisms from application by a magnetic separation method offers major advantage. Cells coated with small superparamagnetic nanoparticles (dozens of nanometers) have been reported for biological desulphurization process (Li et al., 2009; Shan et al., 2005; Xu et al., 2015), phenol degradation (Le et al., 2012) and hexavalent chromium reduction (Xu et al., 2011). Despite these successes, however, it remains a challenge to prepare magnetic nanoparticles with uniform appearance and favorable dispersibility for application use. This largely stems from the aggregation effect between different magnetic dipoles in solution environment (Lyles et al., 2004). To achieve dispersion, encapsulation by silica or stabilization with a surfactant has been widely adopted. However, tedious craft and expensive costs are major obstacles to overcome (Jang and Lim, 2010). Therefore, the need to simplify the preparation process of magnetic vectors and ensure the immobilization effect is a driving force in the field of materials and biotechnology.

In this study, *Acinetobacter* sp. strain LMB-5, a previously isolated bacterium by our laboratory (Supplementary information), was successfully immobilized with $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs (about 230 nm). We also examined many important factors including the effects of pH, temperature and heavy metal contaminants, as well as the effect of particle size on the biodegradation rate of DBP by free and immobilized cells. These results will improve our understanding of magnetic immobilization methods.

2. Materials and methods

2.1. Materials

Unless specified, all chemicals in this study were analytical grade and used without further purification. Purified water from a Milli-Q system was used throughout the experiment. Di-n-butyl phthalate (DBP), dimethyl phthalate (DMP) and phthalic acid (1,2-benzenedicarboxylic acid, PA) used as reference standards were obtained from Sigma-Aldrich (Germany) with purity >99.5%. LC-grade methanol was purchased from CNW Technologies GmbH (Düsseldorf, Germany). 3-aminopropyltriethoxysilane (3-APTES) used

was from TCI (Tokyo, Japan). Small Fe_3O_4 nanoparticles (20 nm, type A) were obtained directly from Aladdin with purity >99.5%, while large Fe_3O_4 nanoparticles were synthesized in the lab (230 nm, type B). Solid phase extraction (SPE) C18 cartridges (0.5 g, 3 ml, polypropylene) used in the experiment were purchased from Supelco Corporation.

2.2. Synthesis of Fe_3O_4 NPs and NH_2 -functionalized Fe_3O_4

Fe_3O_4 NPs were prepared according to a protocol proposed by Yang (2015). To synthesize the $\text{Fe}_3\text{O}_4@\text{NH}_2$ nanoparticles, 0.5 g dried Fe_3O_4 NPs was dispersed in 100 ml alcohol solution and 1 ml ultrapure water. After 30 minutes of ultrasonic dispersion, 0.25 ml concentrated ammonia solution was added as a catalyst. A volume of 0.5 ml of APTES was added dropwise for 1 min, and the reaction proceeded for 24 h under vigorous stirring at the ambient temperature. After dried under vacuum, the obtained nanoparticles were stored in a vacuum dryer. The concentration of magnetic nanoparticles was expressed in terms of dry weight per unit volume of suspension medium (g l^{-1}).

2.3. Bacterial strain cultivation and immobilization

Acinetobacter sp. strain LMB-5 cells were grown in 5 ml TSB medium overnight at 37 °C. Then 1 ml of the seed culture was transferred to 50 ml TSB medium, and incubation was continued for 12 h to the late exponential phase. The dry weight of cells was approximate 1.96 g l^{-1} (Fig. A.1). Then the cells were separated from the medium by centrifugation at 4000 rpm for 10 min, and washed 2 times with phosphate buffer saline (PBS, pH 7.4) under sterile conditions, then re-suspended in the MSM medium (Supplement information) to an OD_{600} of 0.2 before adding several volumes of NH_2 -functionalized Fe_3O_4 NPs solution (10 mg ml^{-1}). The cells were coated by absorbing the magnetic NPs.

The growth rate of the cells was monitored by determining the optical density value at 600 nm (OD_{600}) using an automatic microplate reader (Bio-Tek, America). The optimum ratio for subsequent experiments was NPs: DCW=2:1 (w/w, Fig. A.2), and at this point 93.9% immobilization efficiency was achieved, which was calculated from the decreased OD value relative to a reference without magnetic fluids.

16S rRNA gene sequence of the strain LMB-5 was submitted to the GenBank (Accession No. KM981462).

2.4. Characteristics of naked Fe_3O_4 and $\text{Fe}_3\text{O}_4 @\text{NH}_2$ nanoparticles

The morphologies of Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs were examined by JEOL SM-6360LV scanning electron microscopy (SEM). Size analysis of particles in SEM was carried out using the NanoMeasurer 1.2 software. X-ray diffraction (XRD) measurement was performed on a D/Max 2550 VB X-ray diffractometer (Rigaku Co., Japan) at room temperature. The number of NH_2 incorporated into magnetite nanoparticles was determined by thermogravimetric analysis (Thermax 700, America), which was carried out in N_2 atmosphere at a heating rate of $10\text{ }^\circ\text{C}/\text{min}$ from 30 to $900\text{ }^\circ\text{C}$. A 7407 vibrating sample magnetometer (VSM, Lake Shore, USA) was used to evaluate the magnetic properties of the prepared NPs.

2.5. The optimal conditions of DBP degradation

Comparison the effect of different sizes of nanoparticles coated on the strains, we found that type B exhibited a better biodegradability on DBP than type A (Fig. A.3). Therefore, following experiments are concentrated on type B. After coated with the magnetite NPs, the cells were washed two times with sterilized PBS buffer to remove all non-coated cells and suspended in the same MSM medium. A strong magnet was used to separate the coated cells from the washing solution. 1 ml of those cells was inoculated into 20 ml MSM medium containing 100 mg l^{-1} DBP. The blank experiment was conducted to measure the DBP reduction efficiency of $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs by adding 1 ml NPs (10 mg ml^{-1}) without any cells and incubated under the same conditions.

To assess the effect of temperature and pH on the degradation ability of coated cells, all the other parameters were remain unchanged, while temperature varied from 20 to $60\text{ }^\circ\text{C}$ and pH from 5.0 to 8.0 . Then aliquots of solution were withdrawn at designed intervals, and DBP was extracted from the culture by SPE procedure before suffering a high-performance liquid chromatography (HPLC) analysis. All experimental results reported in this study were based on averaging results of three repeated experimental runs.

2.6. Effect of metal ions on DBP degradation

PAEs are ubiquitous pollutants in several industrial wastewater streams. However, it is noteworthy that accumulation of heavy metals (HMs) within wastewater always occurs simultaneously, which is caused by fertilizer industries, solid-waste disposal and pesticide applications (Fu and Wang, 2011). Heavy metals existed in the ecosystem may have detrimental effects on the activity of the bacteria and therefore may hinder biodegradation

ability of corresponding organisms. The DBP concentration in the culture was determined by incubation with $K_2Cr_2O_7$, $PbCl_2$, $CuCl_2$, $ZnCl_2$ and $NiCl_2$, respectively, with the concentration of 10 mg l^{-1} (Bystrzejewski et al., 2009). Each flask was incubated for 48 h aerobically at $37\text{ }^{\circ}\text{C}$. The control group was conducted by adding the same volume of deionized water instead of HMs.

2.8. Analysis of DBP-derived degradation products by immobilized cells

To study the influence of magnetic nanoparticles on metabolic pathway of *Acinetobacter* sp. strain LMB-5, the samples containing nanoparticles were extracted and dissolved in $300\text{ }\mu\text{l}$ ethyl acetate as above. After filtration with a $0.22\text{ }\mu\text{m}$ membrane (Millipore, USA), $0.4\text{ }\mu\text{l}$ filtrate was injected into GC/MS system. The following detection conditions were employed: an HP-5 MS column ($30\text{ m} \times 250\text{ }\mu\text{m} \times 0.25\text{ }\mu\text{m}$, United States) with helium as a carrier gas at a flow rate of 1.0 ml min^{-1} . The injector temperature was set at $250\text{ }^{\circ}\text{C}$. The interface temperature and ion source temperature were both set to $280\text{ }^{\circ}\text{C}$. The column temperature was increased from 100°C for 1 min, raised at $10\text{ }^{\circ}\text{C min}^{-1}$ to $200\text{ }^{\circ}\text{C}$, then at 6°C/min to $280\text{ }^{\circ}\text{C}$ (3.00 min hold). The MS analysis was operated in the positive electron ionization (EI) mode with an electron ionization energy of 70 eV. The metabolites identified by mass spectrometry analysis were matched with authentic standard compounds from the National Institute of Standards and Technology (NIST, USA) library database.

2.9. Reusability of immobilized cells

To examine the reusability of LMB-5 coated with NH_2 -functionalized Fe_3O_4 , the coated cells were repeatedly tested to degrade DBP (100 mg l^{-1}) for 60 h as a cycle period, which time point was selected because we believe that their behavior change can be reflected better near the terminal point. At the end of each batch, the coated cells were retrieved from the reaction mixture by applying of an external magnetic field from the outside of the conical flask, then the old medium was discarded, and the coated cells were washed three times with PBS buffer. Then a new medium was added for a new degradation batch cycle. The degradation efficiency of the coated cells at the first cycle was defined as the control and attributed with the relative activity of 100%.

3.0. Analytical methods

3.0.1. SPE procedures

SPE experiments were performed on a Supelclean solid phase extraction apparatus with the function of inhibiting cross-contamination. For reversed-phase SPE of DBP from aqueous solutions, the cartridges were preconditioned with 2 ml MeOH and 2 ml water. The culture medium (20 ml) was centrifuged and the supernatant was adjusted to pH 6.0 with 1 M of hydrochloric acid solution. Then, samples was passed through the cartridge as slowly as possible (approximately 1 ml min^{-1}). Finally, DBP was eluted with methanol ($2 \text{ ml} \times 2$) and purged to dryness with pure nitrogen gas. The residue was re-dissolved in 1 ml of methanol. Before analyzing the eluate by HPLC, the analytes were passed through a $0.22 \mu\text{m}$ membrane filter and recoveries of DBP after SPE were 89%.

3.0.2. Determination of DBP

DBP concentration was analyzed by HPLC (Shimadzu Co. Ltd, China) equipped with an SPD-20A prominence UV/Vis detector and a DGU-20A5 prominence degasser and a LC-20AT prominence liquid chromatograph. A GL Science Inc. Inertsil ODS-4 column ($250 \times 4.0 \text{ mm}$, particle size $5.0 \mu\text{m}$) was used as a separation column. The mobile phase for detecting DBP was a mixture of methanol and water (90:10, v/v), and 1 ml/min was selected as the flow rate of the mobile phase. The UV wavelength was 254 nm , and $20 \mu\text{l}$ samples were injected into the system for each analysis. Quantification calculation was based on the peak area using external standards method.

3. Results and discussion

3.1. Characteristics of magnetic nanoparticles

The Fe_3O_4 nanoparticles had an average diameter of about 229 nm (Fig. A.4c) and the surface was rough (Fig. A.4a), which was due to the fact that each Fe_3O_4 microsphere was composed of many smaller particles (Shen et al., 2012), while the dimension of the particles did not change greatly after modification (Fig. A.4d). In addition, the surface of $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs became smoother than before modification (Fig. A.4b). X-ray diffraction (XRD) experiments were performed to confirm the chemical composition of the particles obtained. The pattern of experimentally obtained particles was identical to that of the standard Fe_3O_4 . XRD patterns of Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{NH}_2$ are depicted in Fig. 1a. The specific peaks at 2θ values of 30° , 35.4° , 43.3° , 53.4° , 57.1° and 62.7° corresponds to the (220), (311), (400),

(422), (511) and (440) lattice planes of cubic Fe_3O_4 for the synthesized nanoparticles. These data indicated that the NPs were pure Fe_3O_4 with an inverse cubic spinel structure.

As shown in Fig. 1b, NH_2 -modified NPs display a mass loss of about 16.70% after heating to 900 °C. In contrast, this value for the bare Fe_3O_4 NPs was only about 13.10%. Based on this observation, the content of NH_2 in $\text{Fe}_3\text{O}_4 @\text{NH}_2$ was speculated to contribute to the 3.60% relative to the whole weight. The saturation magnetization (M_s) values of bare Fe_3O_4 and $\text{Fe}_3\text{O}_4 @\text{NH}_2$ were 52.546 emu/g and 43.783 emu g^{-1} , respectively (Fig. 1c). Although the saturation magnetization value decreased over the modification process, the prepared materials were mainly superparamagnetic, meaning that its coercivity and remanence can be neglected. This result indicated that the $\text{Fe}_3\text{O}_4 @\text{NH}_2$ nanoparticles could be separated by applying a magnetic field in application (Fig. A.5). TEM image of the coated cell is presented in Fig. 1d. The size of LMB-5 was about 1 μm in length and 500-600 nm in width, which indicated that only a few nanoparticles could be coated on the cell surface. According to some researches, most of small size nanoparticles are likely to be internalized into cells (Zhang et al., 2002), and excessively dense coverage may constitute obstacles in the mass transfer process. From this perspective, magnetic materials with relatively larger sizes are advantageous to serve as adsorptive support for recovery purpose.

3.2. DBP degradation and determination of optimal degradation conditions

The performance of free and immobilized cells on DBP degradation was compared (Fig. 2). The immobilized cells exhibited similar activities to planktonic free cells, although the degradation performance of the coated cells was a little lagging behind. Both of them could completely degraded 100 mg l^{-1} DBP after 72 h of incubation, suggesting that mass transfer hindrance did not happen in this system.

The optimal temperature and pH for free LMB-5 was 37 °C (Fig. 3a) and 6.0 (Fig. 3b), respectively. Notably, when the cultivation temperature was increased to 60 °C, the activity of free cells showed a drastic decline after 48 hours of incubation, with the degradation efficiency of 34%, while this value remained as high as 65% for the coated cells. These data showed that the immobilized cells had the ability to adjust the temperature variety, especially at the higher temperature. There was no significant difference between free cells and immobilized cells in DBP degradation when initial pH was 6.0. However, the coated cells had

a stronger tolerance to pH than the free cells under the same conditions. Consequently, this immobilized technique by magnetic nanoparticles is feasible to tolerate acid and alkali in a realistic wastewater.

3.3. Effect of different heavy metals on DBP degradation

Investigation on the tolerance of degrading bacteria to HMs is critical for actual application. As presented in Fig. 4, Cr^{2+} , Pb^{2+} , Cu^{2+} and Ni^{2+} had different inhibitory effects (10%-50% suppression) on the activities of free cells, with the DBP degradation efficiency of 31%, 50%, 35% and 48%, respectively. Fortunately, the inhibitory effect was alleviated after coated by magnetic NPs and almost recovered to the control levels. However, it was observed that the DBP reduction in both cases was enhanced (about 10%) in all Zn^{2+} -containing samples relative to the control groups. One possible reason is that the activities of related enzymes were promoted in the presence of Zn^{2+} . Additionally, the DBP reduction capability for coated cells in the addition of Cr^{2+} , Cu^{2+} and Ni^{2+} increased 1.8, 1.9 and 1.4-fold than before treatment. Thus, these results indicated that the activity of LMB-5 was disproportionately influenced by the type of HMs and protected by coating with magnetic NPs.

3.4. Analysis of DBP-derived degradation products by immobilized cells

The DBP degradation intermediates in the culture of the immobilized samples were analyzed by GC/MS. As shown in Figure 6, four main compounds were identified as DBP, BMP, DMP and PA. Coincidentally, the same metabolic products were also detected in the free cells at the same time (data not shown). Therefore, these results suggested that the degrading pathway of DBP did not show any change after the strain LMB-5 was immobilized by nanoparticles. The predicted chemical structures, retention times (RT), and characterized ions of the mass spectra (m/z) are summarized in Table 1. Furthermore, the existence of DMP and BMP suggests that biodegradation of DBP by *Acinetobacter* sp. strain LMB-5 might be through reduction of the carbon chain rather than hydrolysis of ester group, which differs from the initial hydrolysis of ester bonds reported before (Xu et al., 2005a, b; Li and Gu., 2005a, b; 2006a, b; Wang and Gu, 2006a, b; Li and Gu, 2007; Luo et al., 2009, 2011, 2012).

3.5. Recycling stability

The magnetically immobilized cells could be well separated from the culture medium, and used at least four consecutive cycles in the reaction mixture under the same conditions (Fig. 5). It was observed that the excellent activities of coated cells (>90%) were still obtained after four cycles compared with the original. However, with increasing reaction cycles, the reduction rate of DBP turned unusually disappointing (data not shown), which could be attributed to a gradual loss of the coated cells after each cycle when performing the separation action.

4. Conclusions

A new cell immobilization technology for phthalates degradation has been developed, by using large size $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs via electrostatic attraction, which integrated the advantages of magnetic nanomaterials and biodegradation by microorganisms. *Acinetobacter* sp. strain LMB-5 showed enhanced biodegradation capability after coated with magnetic nanoparticles with improved tolerance of high temperature, extensive pH range and toxic contaminants in the process of DBP degradation. The involvement of nanoparticles did not change the transformation pathway. The results suggested that the immobilization technology using large magnetic nanoparticles is feasible and advantageous to enhance biodegradation of phthalates in wastewater or soils.

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Figure legends

Fig. 1 Wide-angle XRD patterns of Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs (a); TGA curves of Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs (b); Magnetization curves of Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs (c); and TEM images of LMB-5 coated by $\text{Fe}_3\text{O}_4@\text{NH}_2$ NPs (d).

Fig. 2 Time courses of DBP reduction by free and Fe_3O_4 NPs-coated cells. The initial DBP concentration was 100 mg l^{-1} .

Fig. 3 DBP degradation under different temperature (a) and pH (b).

Fig. 4 Degradation of DBP in the presence of heavy metals (pH 6.0, 37°C).

Fig. 5 Reuse and stability of coated LMB-5 for DBP reduction (pH 6.0, 37°C).

Fig. 6 GC chromatograms of DBP biodegradation products of immobilized cells in MSM (a); a close up (b); and MS spectra of the intermediates detected from DBP transformation (c).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1 Characterization of degradation products of DBP determined by GC/MS analysis.

Name	Chemical formula	Molecular weight	Retention Time(min)	Major peaks (m/z)
PA	C ₈ H ₆ O ₄	166.13	7.918	50,76,104,148
DMP	C ₁₀ H ₁₀ O ₄	194.19	9.556	50,77,104,133,163
BMP	C ₁₀ H ₂₂ O ₄	206.28	12.702	55,77,105,133,163
DBP	C ₁₀ H ₂₂ O ₄	278.35	15.753	41,75,104,149,223,278