

Biochemical pathways and enhanced degradation of dioctyl phthalate (DEHP) by sodium alginate immobilization in MBR system

Ke Zhang, Xiangling Wu, Hongbing Luo, Wei Wang, Siqiao Yang, Jian Chen, Wei Chen, Jia Chen, You Mo and Lin Li

ABSTRACT

As one of the most representative endocrine disrupting compounds, dioctyl phthalate (DEHP) is difficult to remove due to its bio-refractory characteristic. In this study, an immobilization technology was applied in an MBR system to improve the degradation of DEHP. The degradation efficiency of DEHP was significantly improved and the number of degradation genes increased by 1/3. A bacterial strain that could effectively degrade DEHP was isolated from activated sludge and identified as *Bacillus* sp. The degradation pathway of DEHP was analyzed by GC-MS. DEHP was decomposed into phthalates (DBP) and Diuretic sylycol (DEP), then further to Phthalic acid (PA). PA was oxidized, dehydrogenated, and decarboxylated into protocatechins, further entered the TCA cycle through orthotopic ring opening. The DEHP degrading strain was immobilized by sodium alginate and calcium chloride under the optimized immobilization conditions, and added to MBR systems. The removal rate of DEHP (5 mg/L) (91.9%) and the number of 3, 4-dioxygenase gene copies was significantly improved by adding immobilized bacteria. *Micromonospora*, *Rhodococcus*, *Bacteroides* and *Pseudomonas* were the dominant genres, and the results of bacterial community structure analysis show that immobilization technology is beneficial to system stability. The results showed the potential applications of the immobilized technique in DEHP wastewater treatment in MBR.

Key words | 3, 4-dioxygenase genes, bacterial community dynamics, biodegradation pathway, enhanced biodegradation, immobilized DEHP degrading bacteria, MBR system

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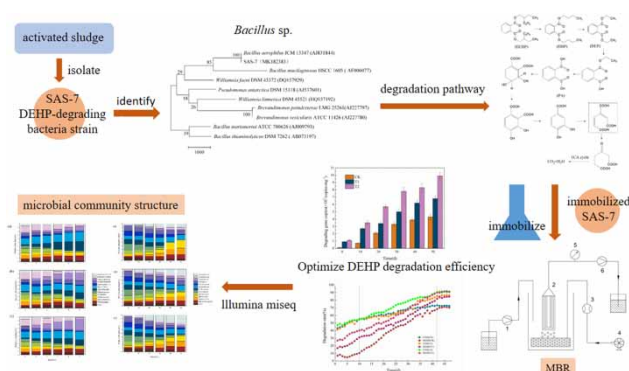
HIGHLIGHTS

- The bacterial strain (SAS-7) isolated from activated sludge, is capable of completely degrading DEHP under aerobic conditions.
- The immobilization technique used in this paper helped increase the number of DEHP degrading genes.
- The immobilized cells removed 91.9% of DEHP and 90.7% of COD.
- The composition of bacteria that were responsible for DEHP biodegradation in the MBR system was examined.

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GRAPHICAL ABSTRACT



INTRODUCTION

Phthalate (PAEs) are a series of endocrine disruptors (Bergman *et al.* 2013), widely used as plasticizers in industry (Lyche *et al.* 2009). The annual production and consumption exceed 2.5 million t, accounting for 80% of the total production of plasticizers (Yin *et al.* 2003). The binding of PAEs to plastic molecules is mainly through hydrogen bonds and van der Waals forces. There is no direct connection of chemical bond: they enter the environment easily during the production, storage, use and processing of industrial products (Per Axel *et al.* 2007; Liu *et al.* 2013). Many studies have shown that industrial and agricultural areas in China are suffering from PAEs pollution to varying degrees (Xia *et al.* 2011; Ma *et al.* 2012). PAEs from waste plastic recycling stations in salt beaches range from 200 to 1200 ng·m⁻³ (Huang *et al.* 2013). The concentrations of DEHP, DMP, DBP and DOP in the water samples of Anshan city were 18.1863 mg/L (DOP)–0.0446 mg/L (DBP) (Yao *et al.* 2011). PAEs in the soils of many greenhouse vegetable bases in China exceed the standard (Xi *et al.* 2002; Xu *et al.* 2008). DEHP is toxic to wheat root tissue (Gao *et al.* 2018). DnBP in soil can change the abundance, structure and composition of bacteria in the rhizosphere of cucumber seedlings, and ultimately lead to a decrease of the protein content in roots (Zhang *et al.* 2015). As the most widely used PAEs, Di-(2-ethylhexyl) phthalate (DEHP) has been detected around the world in recent years (Kong *et al.* 2012; Yan *et al.* 2018). DEHP has stable chemical properties and is difficult to degrade in the environment. The hydrolysis half-life of DEHP can reach several decades (Muchangos *et al.* 2019). Many

studies have revealed that DEHP has strong toxicity, including carcinogenicity, teratogenicity, reproductive toxicity, hepatotoxicity, mutagenicity and endocrine disruption toxicity (Zhang *et al.* 2018; Wu *et al.* 2019). It is necessary and urgent to conduct DEHP remediation research.

Due to the extremely slow photodegradation of DEHP under natural conditions, abiotic degradation is rarely used to remove DEHP (Gledhill *et al.* 1980; Staples *et al.* 1997). Biodegradation has been widely used because of its fast growth cycle, high removal rate and low cost (Ahuactzin-Pã *et al.* 2016). The MBR is an effective and reliable biological wastewater treatment technology. Compared with the traditional biological process, MBRs have the advantages of small footprint size of the treatment unit, less sludge production, complete retention of solids, and flexibility in operation (Boonnorat *et al.* 2016). Previous studies have shown that MBRs can be used to remove pollutants with complex structures, such as drug compounds, pesticides and EDCs (Boonnorat *et al.* 2016; Fudala-Ksiazek *et al.* 2018).

Many bacteria capable of degrading PAEs have been isolated from biofilter reactors (Meng *et al.* 2015), and activated sludge (He *et al.* 2013), most of which belong to the genera *Rhodococcus*, *Gordonia*, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Sphingobacterium*, *Chryseomicrobium*, *Rhizobium*, *Mycobacterium*, *Pseudoxanthomonas* (Meng *et al.* 2015), *Achromobacter* (He *et al.* 2013) and *Ensifer* (Song *et al.* 2019). Most of these bacteria use dimethyl phthalate or DBP as their only carbon source, and the few isolated bacteria with DEHP as carbon source mainly belong to the genera *Rhizobium* (Fang *et al.* 2017) and *Ensifer* (Jin *et al.*

2010), *Bacillus* (Ren *et al.* 2018), *Pseudoxanthomonas* (Meng *et al.* 2015). In previous study, 3, 4-dioxygenase genes were detected to reflect DEHP degradation ability (Zhao *et al.* 2018). In most cases, these degrading bacteria do not perform as expected in complex communities. It is speculated that the degrading bacteria are not active biodegradable bacteria in their natural habitat and the environment of the degradation system is not suitable for bacterial growth, which limits the degradation efficiency of microorganisms. New techniques are urgently needed to explore these microorganisms to be cultivated. The existing research on DEHP degradation mainly focused on anaerobic conditions, with the disadvantages of incomplete degradation and long cycle. Compared with anaerobic degradation, aerobic degradation has the advantages of complete and rapid degradation (Tang 2016), but there is still a lack of relevant research. This study was carried out under aerobic conditions and the biochemical degradation pathways were investigated.

Microbial immobilization technology confines free cells to specific space areas by chemical or physical methods in order to maintain cell viability (Cho & Knorr 1993). In recent years, it has mainly appeared in research into sewage denitrification and ammonia removal. The commonly used embedding methods include interception, adsorption, encapsulation and synthesis of polymers. According to a previous study, when the molar ratio of COD to $\text{NO}_3\text{-N}$ was 2.0, the filling rate of carrier volume was 20%, and the hydraulic retention time was 38 h, the TN removal rate of wastewater in the reactor was 98% (Dong *et al.* 2017). In general, previous studies have shown that immobilization technology can effectively improve the efficiency of cell degradation of organic compounds (Jiang *et al.* 2018). However, there are few reports on the immobilization of DEHP-degrading bacteria. As a refractory organics, DEHP has a low degradation rate. Immobilization technology may promote DEHP. The effects of immobilized technique on DEHP degradation and the associated microbial mechanism are still not clear. In this study, we measured the degradation rate of DEHP and the number of degradation genes, and found that immobilization technology had a significant effect on improving the degradation rate of DEHP by bacteria.

In this study, aerobic degradation pathways of DEHP and orthogonal experiments of immobilization conditions were conducted, and the feasibility of its application in bioreactors and associated microbial processes were further discussed. The results from this study are expected to provide valuable information for DEHP wastewater treatment.

MATERIALS AND METHODS

Chemicals and medium

The purity of DEHP used in this experiment is 99% (Sigma-Aldrich Company, St. Louis, MO, USA). The solvents (methanol, ethyl acetate) are of high performance liquid chromatography (HPLC), and the other chemical reagents are analytically pure. Minimal Salt Medium [MSM]: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05 g, K_2HPO_4 1.5 g, KH_2PO_4 1.52 g, $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ 0.04 g, NaNO_3 0.5 g, $(\text{NH}_4)_2\text{SO}_4$ 1.00 g, FeCl_3 0.14 g, H_2O 1,000 mL. LB liquid medium: yeast extract 5 g, peptone 10 g, NaCl 5 g, H_2O 1,000 mL, pH 7. The solid medium was prepared by adding 20 g agar into the above two liquid media, and the DEHP concentration was added into MSM according to actual needs.

Isolation and identification of degrading bacteria

SAS-7 is a DEHP-degrading bacteria strain with high efficiency, domesticated from the activated sludge, which were collected from the secondary sedimentation tank of Xinjin Municipal Sewage Treatment Plant in Sichuan Province ($30^\circ 24' 49''$ N $103^\circ 49' 11''$ E). Sludge samples were concentrated according to literature (Fang *et al.* 2010). The pH value of culture medium was adjusted to 7.0 by HCl and NaOH. After enrichment, the concentration of DEHP was gradually increased to 5 mg/L by gradient pressure domestication. The DEHP concentration in the culture medium decreased with the increase of bacteria, and then the bacteria were confirmed to be DEHP-degrading bacteria. The bacteria were further purified by plate method. Finally, the isolate was re-inoculated into MSM agar plate containing 5 mg/L DEHP to confirm its degradation ability. The strain SAS-7 with the best degradation effect of DEHP was selected and stored in a slant medium at 4°C .

The bacterial genomic DNA was extracted by the extraction kit (TianGen Biotechnology Co., Ltd, Beijing) according to the instructions. The bacterial primers used in this study were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-GGCTACCTTGTTACGACTT-3'). PCR (Bio-Rad, USA) was carried out under the following conditions: preheating at 95°C for 2 min, denaturing at 94°C for 1 min, annealing at 56°C for 1 min, expanding at 72°C for 3 min, cycling 30 times and expanding at 72°C for 8 min. The PCR product was purified from the universal DNA Gel Extraction Kit (Tiangen Bio Polytron Technologies Inc, Beijing), cloned into the pGM-T vector of

Escherichia coli, and plasmids were extracted from positive clones by plasmid Mini Kit, and DNA sequencing was performed by Sangon company (Shanghai). Then, a BLAST X homology search was performed on the obtained sequences, and phylogenetic tree was constructed by using neighborhood connection method in MEGA5 (version 6.0, USA) software package.

Analysis of metabolites and degradation pathways

DEHP concentration was determined by high performance liquid chromatography (Agilent 1200) with ultraviolet detector and Water Sunfire C18 column (20 mm × 4.6 mm × 5 μm). The chromatographic conditions were as follows: the volume ratio of mobile ethanol to water (90:10) and the flow rate (1 mL/min). The extraction and purification methods were as follows: ethyl acetate was added to each sample, shaken and mixed for 5 minutes, and the water and organic phase were separated at a speed of 8,000 rpm, and rotated for 5 minutes. The aqueous phase was extracted twice under the same volume of ethyl acetate, which was dried and evaporated on anhydrous sodium sulfate and then dissolved in 10 mL methanol. Then 1 mL methanol mixed with DEHP was passed through a filter membrane with a pore diameter of 0.22 μm, and finally 20 μL filtrate was injected into the HPLC system.

We established the QA/QC (quality assurance and quality control) process for DEHP analysis. This process mainly includes regular measurement of standard samples to help master the status of the instrument, the analysis and calibration of actual samples. DEHP biodegradation were analyzed by GC-MS, using a Agilent 6890 N gas chromatograph coupled to a Agilent 5972 mass selective detector equipped with a capillary Agilent column HP-5MS. Helium with purity of 99.99% was used as the carrier gas at 1.0 mL min⁻¹, 2 μL of sample was injected in splitless mode at a port temperature of 250 °C. The detection work was completed by Sichuan Keyuan Engineering Technology co., LTD, China.

Immobilization of SAS-7 strain

The immobilization method was mainly according to previous studies with some modifications (Massalha et al. 2015). Sodium alginate and calcium chloride were used as embedding and crosslinking materials. SAS-7 cell resuspension (OD₆₀₀ = 1.0, 5 mL) was mixed with MSM (20 mL) in a conical flask (200 mL), and then the conical flask was sterilized. Subsequently, the MSM containing SAS-7 strain was combined

with sterile SA, and the mixture was dripped into the gel solution (CaCl₂) with the help of a syringe at 4 °C. Thus, spherical immobilized bacterial beads with a diameter of 4–5 mm were obtained. Blank control beads without SAS-7 cells were prepared by the same method. In order to obtain the optimal immobilization conditions, the mass fraction of sodium alginate, the mass fraction of CaCl₂, the volume ratio of bacteria to sodium alginate, and the crosslinking time were taken as factors to design the four-factor and three-level orthogonal experiment L₉ (3)⁴ (Table 1). Immobilized beads were washed three times with sterile distilled water and then stored at 4 °C for subsequent experiments.

Reactor start-up and operation

In this study, three experimental groups (T1, T2 and CK) were set up (T1, T2 and CK refers to the experimental group with added free SAS-7 bacteria, immobilized SAS-7 bacteria and no SAS-7 bacteria, respectively). Each MBR reactor was inoculated with 0.6 L activated sludge. Through pair-wise comparison between the systems, the degradation effect of SAS-7 bacteria on DEHP and the application of embedding technology can be obtained respectively. The reactor was initially fed with a synthetic glucose wastewater at room temperature. After the domestication of glucose wastewater, a synthetic DEHP wastewater containing different concentration of DEHP was fed. NH₄SO₄ was the nitrogen source, KH₂PO₄ was the phosphorus source, KCl 5,000 mg/L was added, trace elements FeSO₄·7H₂O 5,000 mg/L, ZnCl₂ 1,000 mg/L, MnCl₂ 1,000 mg/L, CoCl₂·6H₂O 200 mg/L, CuSO₄·5H₂O 100 mg/L were added, and the pH was adjusted to 7.0. According to the relevant experiments on the amount of bacteria inoculated and the relative results of aerobic degradation (Tahhan et al. 2011), the inoculation amount of DEHP degradation bacteria in this experiment was 7% of the volume of sludge inoculation. Added strain SAS-7 with the same biomass to both systems (the immobilized biomass amount is the same of suspended). The MBR reactor system was controlled by relay. The process flow diagram is shown in

Table 1 | Factors and levels of orthogonal experiment design

	Factor A SA	Factor B CaCl ₂	Factor C Ratio	Factor D
Levels	concentration (%)	concentration (%)	of bacteria to SA (%)	Cross-linking time (h)
1	4	3	1:1	5
2	5	4	1:2	6
3	6	5	2:1	7

Figure 1. Continuous influent was adopted, dissolved oxygen was 4–5 mg/L, operating temperature was 24 °C, hydraulic retention time was 48 h. The degradation rate of DEHP in the system was monitored daily for 50 consecutive days.

Detection of 3,4-dioxygenase gene in MBR by q-PCR

According to previous study, 3, 4-dioxygenase gene is the DEHP degrading gene, which drives bacteria to degrade DEHP (Zhu *et al.* 2020). Total number of DEHP degrading bacteria 3, 4-dioxygenase genes in MBR were detected by q-PCR using ABI 7500 Real-time Polymerase Chain Reactor (Applied Biosystems, USA). The product of 3,4-Dioxygenase 16S rRNA PCR was purified and ligated into pMD18-T vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5a (TaKaRa, Japan). Plasmid DNA was extracted by Plasmid Extraction Kit (Transgen, Beijing). DNA concentration and quality were measured by NanoDrop ND-1000 (American Thermal Fisheries Science Corporation). The 20 µL reaction mixture contains 10 µL SYBR Premix Ex Taq II (Ex Taq II enzyme pre-mixed with SYBR dye for fluorescence quantification) (Takara, Japan). Each primer extracts 0.6 µL, 1 µL genomic DNA, and the remainder is filled by double-distilled hydrogen peroxide. The program is set as follows: preheating for 5 minutes at 95 °C, denaturation for 1 minute at 94 °C, annealing for 1 minute at 56 °C, expansion for 3 minutes and 30 cycles at 72 °C. The plasmid containing 16S rRNA gene sequence of bacteria was diluted to a series of concentrations, each concentration differed by 10 times. The standard curve of plasmid was drawn. The R^2 of the standard curve was >0.99. All standards and samples are in triplicate.

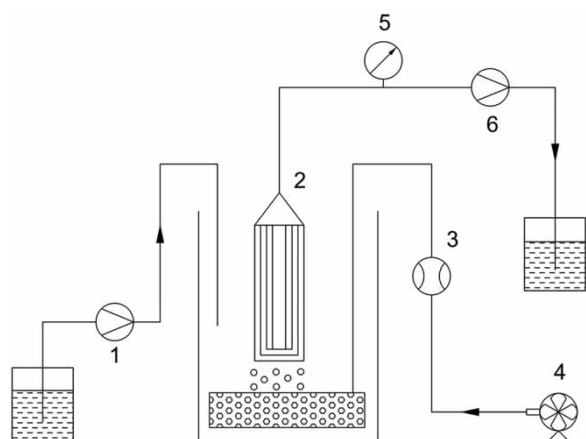


Figure 1 | MBR flow chart and reactor 1. Water creeping pump; 2. Membrane module; 3. Flow meter; 4. Aeration device; 5. Pressure gauge; 6. Outlet peristaltic pump; 7. Water bath circulating device.

Microbial community analysis

In order to explore the bacterial community structure dynamics after the free and immobilized degradation bacteria SAS-7 were introduced into the MBRs, the sewage samples in the MBR were collected every 10 days for Illumina MiSeq sequencing. Three samples were collected at a time. After the immobilized beads were crushed, total DNA was extracted through Power Soil DNA Isolation Kit (MOBIO Laboratories Inc., USA). For gene amplification of 16s rDNA V3-V4 area, choose 341F (CCCTACACGACGCTCTTCCGATCTGCCTACGGGNGGCWGCAG) and 80 R (GACTGGAGTTCCTTGGCACCCGAGAATTCCAGACTACHVGGGTATCTAATCC) as a primer. Both forward and reverse primers were tagged with adapter, pad and linker sequences. Each bar code sequence was added to the reverse primers, and multiple samples were collected in one MiSeq sequence (Jing *et al.* 2015). The amplified products were loaded onto 1.2% agarose gel and purified by E.Z.N.A.TM Gel Extraction Kit (Omega BioTek Inc., Norcross, Georgia) (Gao 2015). FTC-3000 real-time PCR method was used to determine DNA concentration and quality. The amplicon was then used for MiSeq sequencing. After sequencing, the data were analyzed by different procedures combined with the quantitative analysis of standard pipes in QIIME Pipeline (Fadrosh *et al.* 2014). Eliminate low-quality reads, adapters, barcodes and primers. Using the UCLUST algorithm, the remaining 16S rRNA sequences were clustered into operational classified nitrogen sources (OTUs) with a distance limit of 0.03. The Shannon's diversity index of each sample was calculated in MOTHUR by clustering file to estimate the diversity of the community.

Statistical analysis

All data were analyzed by SPSS statistical software package (Version 21.0). Two-way ANOVA was performed to determine significant difference between means ($n=3$, $P<0.05$). Graphical work was carried out using the software Origin 9.0 and Chemdraw.

RESULTS AND ANALYSIS

Identification of strains

A Gram-positive bacterium named SAS-7 was obtained, which can effectively utilize DEHP (5 mg/L) as a carbon source and energy source. Strain SAS-7 showed light

yellow, wet surface of the colony, with a round shape and neat edge. Electron microscopy showed that cells were rod-shaped with spores.

The 16S rDNA sequence of strain SAS-7 was stored in NCBI with the Accession number MK182383. A phylogenetic tree based on the 16S rRNA gene sequences of strain SAS-7 and its close relatives was constructed (Figure 2). According to Figure 2, the similarity between SAS-7 and *Bacillus aerophilus* JCM 13347 (AJ831844) was 100%. Based on the morphological characteristics of the strain and 16S rRNA gene sequence analysis, the strain was identified as *Bacillus* sp.

Degradation pathways of DEHP

To ensure that DEHP will not cause secondary pollution to the environment in the process of bioremediation, the metabolites of DEHP under the degradation of SAS-7 were detected by GC-MS. These intermediates include 2-ethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), diethyl phthalate (DEP), phthalic acid (PA) and protocatechol, etc.

Based on the results of GC-MS, the degradation pathways of DEHP by SAS-7 were analyzed. The degradation pathways were inferred as shown in Figure 3. 2-ethylhexyl phthalate (DEHP) was first decomposed into dibutyl phthalate (DBP), then hydrolyzed to diethyl phthalate (DEP), which was converted into phthalic acid (PA) through continuous deesterification. Phthalic acid (PA) is oxidized, dehydrogenated and decarboxylated to produce protocatechol. Protocatechol enters the TCA cycle through ortho-ring opening, and finally completes the mineralization of DEHP.

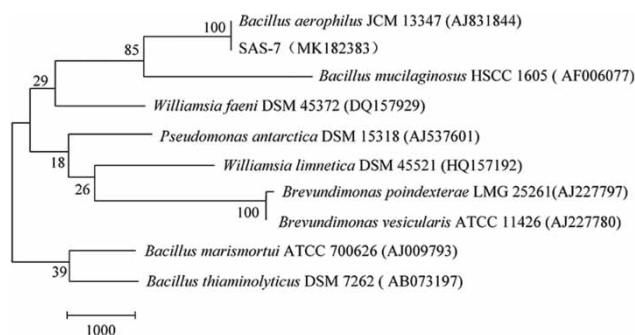


Figure 2 | Phylogenetic tree of SAS-7 and its related species based on 16S rRNA gene sequence (the gene library number of each bacterial isolate is written in parentheses, and the number on each branch node is based on the guiding value of 1,000 resampling).

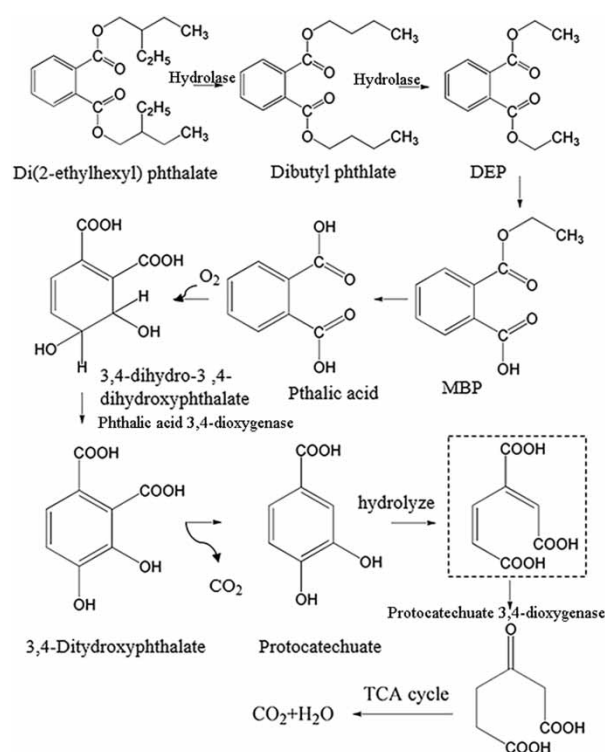


Figure 3 | Proposed pathways of DEHP degradation by strain SAS-7 (substrates marked by dotted lines are undetected in GC-MS).

Optimization of immobilization conditions

To optimize the immobilization conditions, L9 (3^4) orthogonal experiment (Table 1) was designed. Sodium alginate mass fraction (A), CaCl_2 mass fraction (B), ratio of bacteria to SA (C) and crosslinking time (D) were taken as factors. According to the R value shown in Table 2, the order of the influence of each factor on DEHP removal rate is: $B > D > A > C$. Different CaCl_2 mass fraction has the greatest influence on DEHP removal rate, followed by crosslinking time. The ratio of bacteria to SA has the smallest influence on DEHP removal rate. According to the K value, the optimum immobilization conditions were $A_1B_3C_1D_2$; that is, the SA mass fraction was 4%, CaCl_2 mass fraction was 5%, bacteria to SA ratio was 1:1, and cross-linking time was 6 hours.

DEHP removal rate in MBR reactor

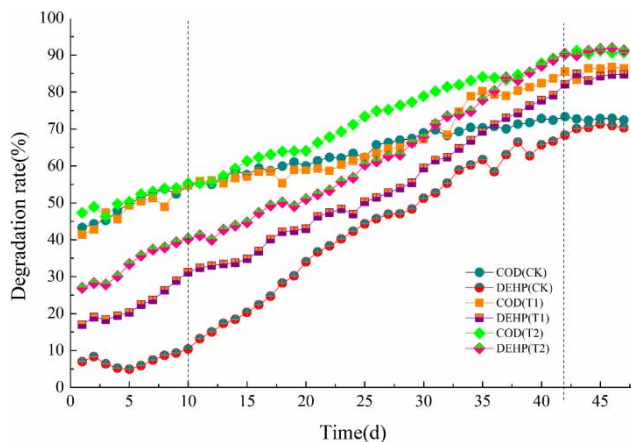
To evaluate the performance of the bioreactor, the effluent samples of the MBR reactor were collected daily and the removal rates of COD and DEHP were measured. The synthetic glucose wastewater containing the glucose and 5 mg/L DEHP were pumped into the three reactors. As shown in Figure 4, there was no significant difference in

Table 2 | Orthogonal experimental results of immobilized DEHP degrading bacteria

Trial No.	Factors				Degradation rate (%)
	A	B	C	D	
1	1	1	1	1	63.4
2	1	2	2	2	57.8
3	1	3	3	3	61.3
4	2	1	2	3	54.8
5	2	2	3	1	49.7
6	2	3	1	2	66.3
7	3	1	3	2	56.9
8	3	2	1	3	50.3
9	3	3	2	1	61.5
k_1^a	60.833	58.367	60.000	58.200	
k_2	56.933	52.600	58.033	60.333	
k_3	56.233	63.033	55.967	55.467	
R^b	4.600	10.433	4.033	4.866	

R^b indicates the range of DOP degradation under different factors.

k_1^a , k_2 and k_3 represent the average degradation rates of DEHP at 1, 2 and 3 levels under different factors, respectively. Factors A, B, C and D represent SA concentration, CaCl_2 concentration, ratio of bacteria to SA, and cross-linking time, respectively.

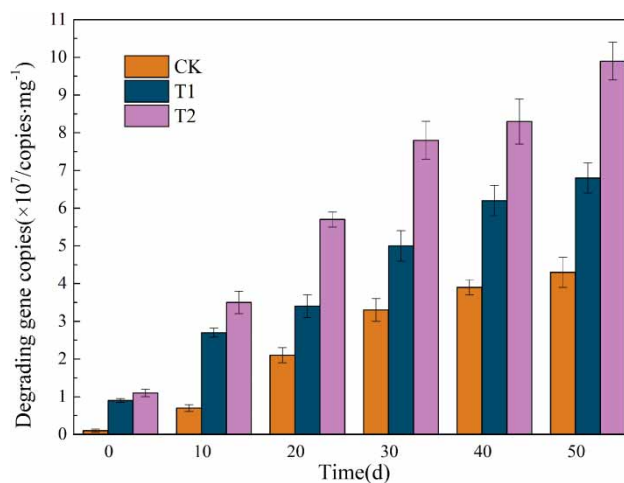
**Figure 4** | Removal effect of free bacteria (T1) and immobilized bacteria (T2) in MBR reactor on DEHP.

the removal rate of organic pollutants in the first 10 days under different conditions. On the 12th day, the removal rates of DEHP in CK, T1 and T2 were 15, 33 and 40%, respectively, and the removal rates of COD were between 55 and 56%. On the 28th day, the removal rate of DEHP in sewage treated with immobilized bacteria reached 63%, which was 9% higher than that of free bacteria. At 37 days, the remaining amount of DEHP between T1 and T2 sewage showed the greatest difference. The removal rate of

DEHP in T1 was 84%, the removal rate of T2 was 73%, and the difference was 11%. From the 28th day to the 37th day, the degradation rate of the T2 system increased faster than that of the T1 system, indicating that immobilization technology played a role in significantly improving the degradation rate. When the DEHP removal rate of MBRs stopped increasing, the system reaction was considered to be over. At the end of the experiment (day 46), the removal rates of DEHP and COD in T2 were 91.9% and 90.7%, respectively, while the removal rates of DEHP and COD in T1 were 84.7% and 86.7%, respectively. The results showed that the immobilized bacteria not only accelerated the degradation rate but also improved the degradation effect. In general, the removal effect of immobilized bacteria on DEHP and COD in sewage was better than that of free bacteria.

q-PCR analysis of degrading gene 3,4-dioxygenase

To investigate the effect of immobilization technology on the quantitative characteristics of DEHP degrading genes in bio-reactors, samples from MBRs were collected every 10 days, and the degrading genes in the MBR system were detected by q-PCR. As shown in Figure 5, within 50 days after the introduction of bacteria, the number of copies of degrading genes in the immobilized bacteria system was constantly greater than that in the free bacteria system. In the first 10 days, DEHP degrading genes in CK system increased by 0.6×10^7 copies·mg⁻¹, while T1 and T2 increased by 1.8×10^7 copies·mg⁻¹ and 2.4×10^7 copies·mg⁻¹, respectively. This result proved that immobilization can shorten

**Figure 5** | Number of degrading gene (3,4-dioxygenase) copies in sludge samples after introducing blank pellets (CK), free bacteria (T1) and immobilized bacteria (T2) by q-PCR extraction.

the start-up time of the reactor. Then, the number of DEHP degrading genes in the immobilized and non-immobilized systems continued to increase, while the growth rate of DEHP degrading genes in the immobilized system was faster than that in the non-immobilized system. On the fiftieth day, the difference of 3, 4-Dioxygenase gene number between T1 and T2 systems was the greatest. The number of 3, 4-Dioxygenase genes in the T1 system was 6.8×10^7 copies·mg⁻¹, which was 3.1×10^7 copies·mg⁻¹ less than that in the T2 system. These results indicated that immobilization technology can promote the increase of the number of the degrading gene, 3, 4-dioxygenase.

Dynamic analysis of bacterial community structure

Activated sludge samples were collected every 10 days and sequencing by Illumina MiSeq. Figure 6 shows the change of bacterial community composition at the phylum level with time in the system, and the inoculation of free bacteria and immobilized bacteria both changed the bacterial community structure. As shown in Figure 6(a), β -proteobacteria, Bacteroidetes, Firmicutes and Deferribacteres were the main phyla in the primary activated sludge, accounting for about 55% of the total. With the process of domestication, the contents of Fibrobacteres, Actinobacteria and γ -Proteobacteria increased. Notably, the relative abundance of γ -Proteobacteria increased significantly after the inoculation of free bacteria, from 2% to 20% (Figure 6(b)). This was related to the addition of SAS-7. Similar to the system with free bacteria (T1), γ -Proteobacteria also significantly increased in the system with immobilized bacteria (T2) added. However, in the T2 system, γ -Proteobacteria increased more significantly, from 2% to 29% (Figure 6(c)). In a word, the trend of bacterial community change was similar in the T1 and T2 systems, but the change was more obvious in T2 systems, and Fibrobacteres, Actinomycetes and γ -Proteobacteria were the predominant phylum for DEHP biodegradation in the activated sludge.

The bacterial community dynamics in MBR system at the genus level is shown in Figure 7. As shown in Figure 7(a), *Nitrospira*, *Clostridium*, *Nitrosomonas*, *Comamonas* and *Micropruina* were the main bacterial groups at the genus level in the primary activated sludge, accounting for about 69% of the total. As the domestication process deepened, all these dominant genera decreased significantly, with *Nitrospira*, *Clostridium* and *Nitrosomonas* dropping to 1 and 2% respectively, and *Micropruina* and *Comamonas* disappeared, suggesting these genera were less tolerant to DEHP toxicity. As shown in Figure 7(b), in the system

inoculated with free bacteria, *Pseudomonas* increased significantly, and *Nitrospira* remained at a relatively stable constant during the whole trial. Similar to the T1 system, *Pseudomonas* also increased significantly in the T2 system, which may be related to the addition of SAS-7 bacteria (Figure 7(c)). It was notable that some genera disappeared in both the CK and T1 system, while no genus disappeared in the T2 system. These results suggested that adopting immobilization technology had a positive effect on maintaining the microbial diversity of the MBR system.

DISCUSSION

In this study, sodium alginate and calcium chloride were used as embedding and cross-linking materials to immobilize the DEHP degradable strain (*Bacillus sp.*, SAS-7) isolated from activated sludge. Orthogonal experimental results showed that the effects of four factors on the immobilization were in the following order: the mass fraction of sodium alginate > CaCl₂ mass fraction > bacteria and sodium alginate volume ratio > cross-linking time. Previous studies have shown that excessively low CaCl₂ mass fraction may reduce the exchange rate between Ca²⁺ and Na⁺, thus reducing the degree of cross-linking between Ca²⁺ and sodium alginate, weakening the coagulation ability, and finally leading to low DEHP removal rate (Demirkan *et al.* 2011; Hedayati *et al.* 2016). The optimal crosslinking time was between 5 and 7 h, possibly because too short a cross-linking time may lead to cell instability and leakage, and too long a crosslinking time may lead to reduced bacterial activity (Zhang *et al.* 2017). Whether a better immobilization condition exists remains to be studied.

To ensure microorganisms will not cause secondary pollution when degrading strains are applied to environmental remediation, it is necessary to detect the metabolites and intermediates of degradation bacteria before putting them into use. The metabolites detected in this paper were similar to those reported in the past (Stingley 2004). DEHP was completely mineralized during the degradation process. The aerobic DEHP degradation involves several enzymes, including oxygenases, hydrolases, decarboxylases, and dehydrogenases (Roslev *et al.* 1998). Acinetobacter had been found to produce esterolytic enzymes (Shabtai & Gutnick 1985), which are widely used for ester-bond hydrolysis and transesterification (Rao *et al.* 2013). According to Liang *et al.* 2008, PA can be further converted to protocatechuate (3, 4-dihydroxy-benzoate) through cis-4, 5-dihydroxy-4, 5-dihydrophthalate and 4, 5-dihydroxyphthalate via the

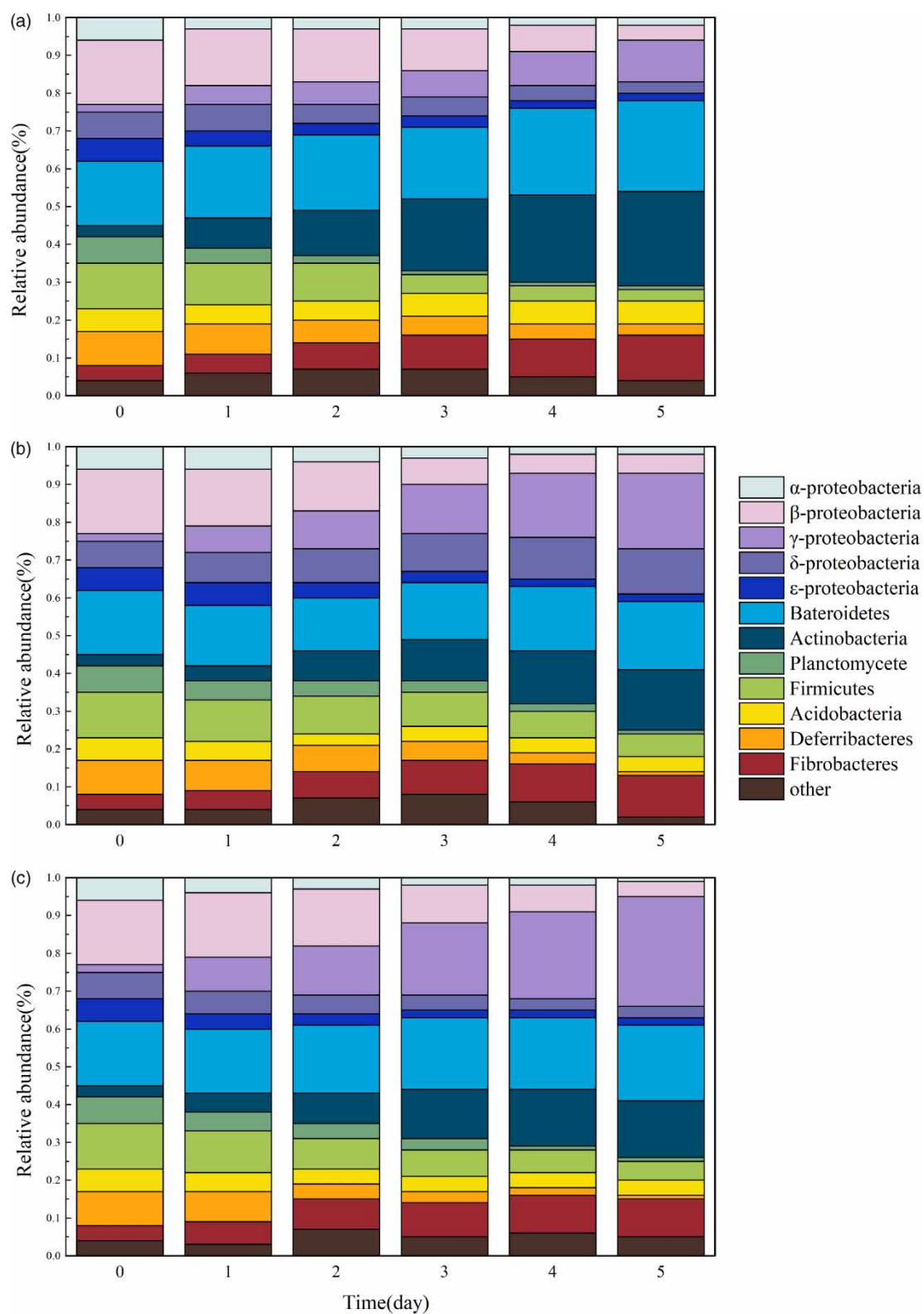


Figure 6 | Bacterial community composition of activated sludge samples at phylum classification level (a) blank control CK; (b) free bacterial system T1; and (c) immobilized bacterial system T2.

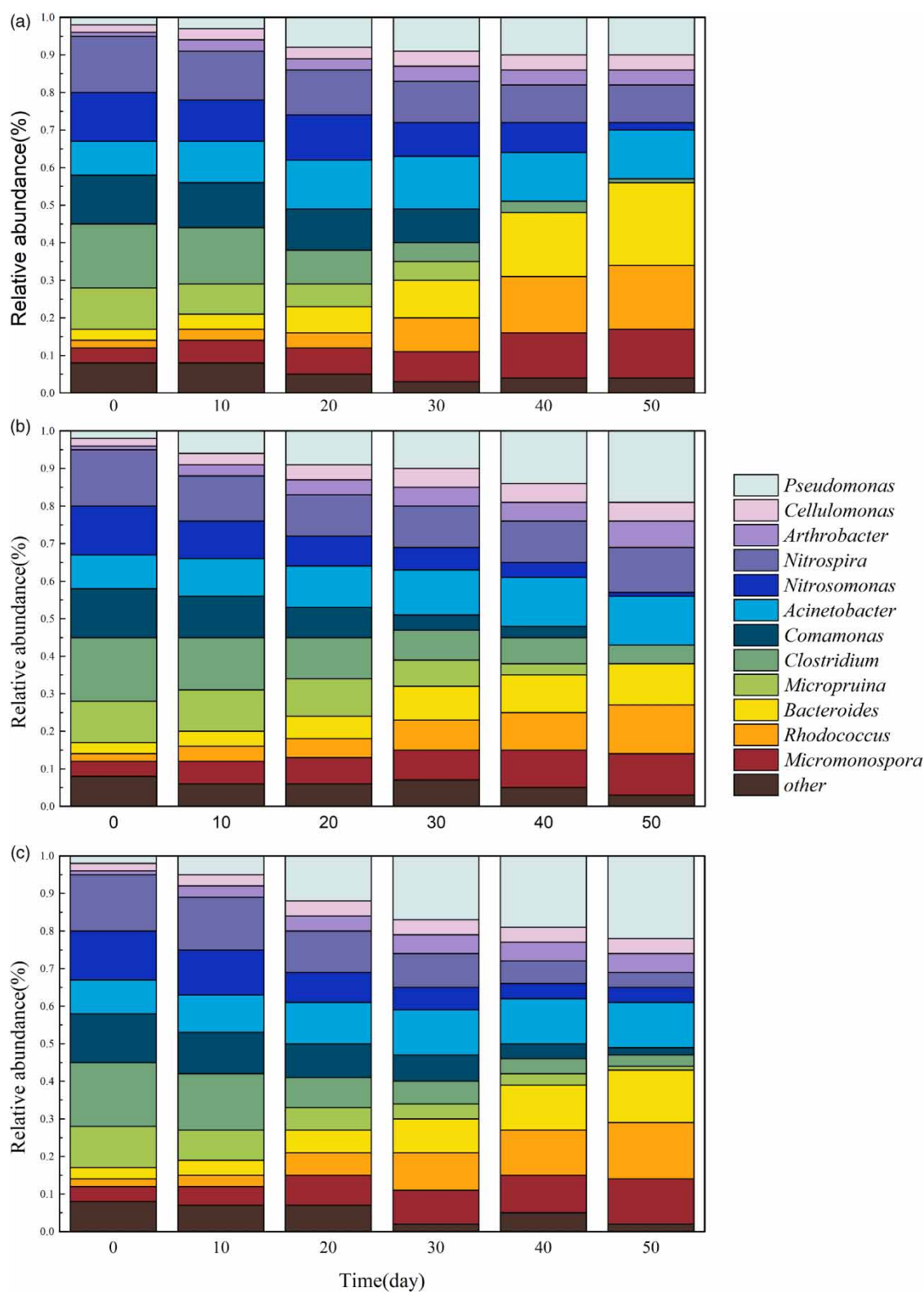


Figure 7 | Bacterial community composition of activated sludge samples at genus classification level (a) blank control CK; (b) free bacterial system T1; and (c) immobilized bacterial system T2.

dioxygenase-catalyzed pathways (Wu *et al.* 2013). We speculate that the degradation pathway of DEHP by SAS-7 is as follows: the 3–4 bit carbon chain of DEHP was shortened and hydrolyzed to DBP, and further to DEP. DEP was gradually hydrolyzed to PA through deesterification. PCA was formed by PA degradation via 3, 4-dihydroxyphthalate pathway by PA 3, 4-dioxygenase. PCA was further hydrolyzed to small molecule organic acids by protocatechuate 3, 4- α and 3, 4- β dioxygenase, and finally converted into CO₂ and H₂O through the tricarboxylic acid (TCA) cycle. The result is similar to previous studies (Tang 2016; Zhao *et al.* 2018). And according to previous papers, it had been proved to be a common and effective pathway in PAEs bioremediation; the gram-positive bacteria oxidize and dehydrogenate at the C-3-4 position of phthalic acid to form 3, 4-dihydroxy phthalic acid, protocatechins and finally enter TCA cycle (Stingley 2004; Xu *et al.* 2017). Different from Predee, we have detected PA in the degradation progress (Pradeep *et al.* 2015): PA used to be regarded as the key to complete the mineralization of DEHP, as it is easier for microorganisms to utilize than DEHP (Roslev *et al.* 1998). What's more, our result is different from previous reports on *Acinetobacter* SN13: these did not detect DEP in the degradation process, but only detected MEHP (Pradeep *et al.* 2015; Xu *et al.* 2017). On the one hand, this could be because the reaction was too fast for detecting these intermediates; on the other hand, it may be due to a better and unknown degradation pathway existing. In short, there are still many problems in the degradation pathway of DEHP by *Bacillus aerophilus* that remain unresolved. Further study is worthwhile.

The DEHP degrading bacteria were immobilized under the optimum conditions. Then, we introduced the immobilized degrading bacteria and the free degrading bacteria into the reactors. The removal rates of COD and DEHP were tested once a day. During the first ten days of the experiment, there was no significant change in the degradation rates, which may be because the microbes were in the adaptive phase. From the 12th day, the degradation rate of DEHP in each reactor increased rapidly, which indicated that bacteria began to multiply in large quantities. The DEHP and COD degradation rate (on the 12th day) of T2 system was significantly higher than that of T1 and CK, indicating that immobilization technology shortened the start-up time of the reactor. The degradation effect of immobilized bacteria was greater than that of free bacteria (from the 28th day to the 37th day), which indicated that the immobilization technology could improve the degradation efficiency of DEHP. What is more, according to the change of COD and

DEHP removal rates, we speculated that the T2 system was stable on the 44th day, while the T1 system was stable on the 45th day: this indicates that adding the immobilized degrading bacteria could shorten the start-up time of the reactor. On the 46th day, the removal rates of DEHP and COD were significantly higher than those of T1 and CK systems. This may be because there were some strains or substances that were competitive or poisonous with DEHP degrading bacteria in the wastewater. And the immobilized technique can be used to isolate and protect bacteria to a certain extent, creating a better living environment for degrading bacteria (Ahmad *et al.* 2020). The adsorption of DEHP by sodium alginate in the immobilized bacteria may also helped the MBR system (T2) to achieve a better DEHP removal rate (Fu *et al.* 2020). The result indicates that the introduction of bio-immobilization technology could not only shorten the start-up time of the reactor, but also improve the degradation rate and effect of DEHP. This result is also confirmed in other studies (Alessandrello *et al.* 2017; Zhang *et al.* 2017). In short, adopting the immobilized technique improved the DEHP removal efficiency of the MBR system: it is confirmed to be available to the MBR system, which deserves further study.

The 3, 4-Dioxygenase gene in the reactor was analyzed by q-PCR. It was found that the number of DEHP degrading genes in the T2 system was significantly more than that in the T1 system. It is speculated that compared to free cells, the immobilization technology can isolate and protect bacteria to a certain extent, and the growth of degraded bacteria was better, which led to a larger number of degrading genes. Combining with the analysis of Figures 6 and 7, the ratio of DEHP degrading genes and the removal rate of DEHP was proportional, which indicated that the high copy number of DEHP degrading genes was the guarantee of the removal rate of DEHP. The number of 3, 4-Dioxygenase gene copies in the immobilized bacteria system was 1/3 higher than that in the free bacteria system, which indicated that the use of immobilization technology could promote the growth of DEHP degradation gene copies. The quantitative relationship of DEHP degrading genes in free bacteria and immobilized bacteria systems can also explain the difference in the DEHP degradation rate (Figure 5).

In the MBR system, microorganisms are the key to removing organic pollutants. The reason for the higher efficiency of immobilized reactor treatment is that the relative abundance of *Bacteroides* sp. (SAS-7) added to the system has been maintained at a high level. It's speculated that immobilization technology can effectively protect target bacteria from invasion of competitors and natural competition

of native microorganisms. What's more, the immobilization technology may improve the removal efficiency of pollutants through surface adsorption, internal adsorption and biodegradation of pollutants (Fu *et al.* 2020). As the degrading bacteria were introduced, the bacterial population structure in the system changed; this caused a change in the degradation effect. As we were using next generation sequencing (NGS) rather than metagenomic sequencing and analysis, we could only identify microbial community structures to the level of genera. After introducing the immobilized degrading bacteria to the T2 system, *Micromonospora*, *Rhodococcus*, *Bacteroides* and *Pseudomonas*, which have been reported to be able to remove DEHP, increased and finally became the dominant genera in the system (Xue *et al.* 2013; Wen *et al.* 2014). This indicates that the change of microbial community caused by immobilized bacteria is more conducive to DEHP degradation. What's more, no bacteria in the T2 system disappeared during the domestication process, while *Comamonas* and *Micropruina* gradually disappeared in the CK and T1 systems. This result shows that the addition of immobilized bacteria may have a certain equilibrium effect on the bacterial community structure in the reactor. Generally speaking, adopting immobilization technology can not only protect degrading bacteria, but also regulate and balance the bacterial structure in the system.

CONCLUSION

A novel bacterial strain, *Bacillus* sp. SAS-7, capable of utilizing DEHP as growth substrates was isolated and characterized. The intermediates analyzed by GC-MS elucidated the degradation pathway of DEHP. The introduction of the immobilization technique significantly improved the biodegradation efficiency of DEHP in the MBR. Under the optimal immobilization conditions, the MBR system with immobilized strain SAS-7 could efficiently degrade DEHP with high removal rate (91.1%), and the copy number of degrading genes was 1/3 higher than that of the MBR system with free bacteria. The effects of strain SAS-7 and immobilization technology on the diversity and activities of indigenous microflora in the system were investigated through the detection of Illumina MiSeq sequencing. The immobilization method can not only ensure the living environment of the target strain (SAS-7), but also maintain the stability of the microbial structure of the system. Thus, strain SAS-7 has great potential and advantages in DEHP remediation and safe water. It should be noted that the

principle of sodium alginate and DEHP adsorption in immobilized bacteria by analyzing kinetic molecular simulation needs to be further studied as well as the expression levels of key genes associated with DEHP degradation by strain SAS-7.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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