

Article

Biochemical Pathways and Enhanced Degradation of Diethyl Phthalate (DEHP) by Sodium Alginate Immobilization in MBR System

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Abstract: A bacterial strain that could effectively degrade DEHP was isolated from the activated sludge and identified as *Bacillus* sp. by DNA sequencing. The biochemical degradation pathway of DEHP was further analyzed by GC-MS, and the results showed that DEHP was first decomposed into phthalates (DBP). Diuretic glycol (DEP) was then generated, and phthalates (PA) were generated by a continuous de-esterification reaction. Phthalic acid (PA) was oxidized, dehydrogenated, and decarboxylated into protocatechins. Protocatechins enter the TCA cycle through orthotopic ring opening. To enhance DEHP degradation, sodium alginate and calcium chloride were used as embedding and cross-linking materials, and the strain was immobilized. The immobilization conditions were optimized via an orthogonal experiment, and the results showed that the optimal immobilization conditions were SA mass fraction of 4%, CaCl₂ mass fraction of 5%, ratio of bacteria to SA of 1:1, and the crosslinking time of 6 hours. The immobilized bacteria agent was further applied to MBR systems. The results showed that the removal rate of DEHP (5mg/L) in the system by immobilized bacteria was 91.9%, which is significantly higher than that of free bacteria. The 3, 4-dioxygenase gene and microbial community dynamics were analyzed by q-PCR and Illumina Miseq sequencing. The q-PCR results showed that the number of copies of 3, 4-dioxygenase gene in the immobilized system was significantly higher than that of free bacteria. Illumina Miseq sequencing results showed that *Micromonospora*, *Rhodococcus*, *Bacteroides* and *Pseudomonas* were the dominant genera in the MBR system. The analysis of bacterial community structure indicated that immobilization technology had a positive impact on the system stability. The results implied that this immobilized technique had potential applications in DEHP wastewater treatment.

Keywords: DEHP; biochemical pathways; immobilization; MBR; bacterial community dynamics

1. Introduction

Phthalate (PAEs) are a series of endocrine disruptors [1], widely used as plasticizers in industry [2]. Its annual production and consumption exceed 2.5 million t, accounting for 80% of the total production of plasticizers [3]. The binding of PAEs to plastic molecules is mainly through hydrogen bonds and van der Waals. There is no direct connection of chemical bond: it is easy to enter the environment during the production, storage, use and processing of industrial products [4, 5]. Many studies have shown that industrial and agricultural areas in China are suffering from PAEs pollution to varying degrees [6, 7]. PAEs of waste plastic recycling stations in salt beach range from 200 - 1200 ng·m⁻³ [8]. 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) [9]. PAEs in the soil of many greenhouse vegetable bases in China exceed the standard [10, 11]. DEHP is toxic to wheat root tissue [12]. DnBP in soil can change the abundance, structure and composition of bacteria in rhizosphere of cucumber seedlings, and ultimately lead to the decrease of protein content in roots [13]. PAEs are not easy to volatilize and insoluble in water, and they can be stable in the soil, which has serious harm to soil

microorganisms, plants and human health. As the most widely used PAEs, Di-(2-ethylhexyl) phthalate (DEHP) has been detected around the world in recent years [14]. Many studies have proved that DEHP has strong toxicity, including carcinogenicity, teratogenicity, reproductive toxicity, hepatotoxicity, mutagenicity and endocrine disruption toxicity [15, 16]. In Japan, DEHP has caused 13.7 million disability-adjusted life years (DALYs) to humans [17]. DEHP also exist extensively in sewage treatment plants [18]. Therefore, it is necessary and urgent to conduct DEHP remediation research.

Biodegradation has been widely used because of its fast growth cycle, high removal rate and low cost [19]. Abiotic degradation is rarely used to remove DEHP. Due to the extremely slow photodegradation of DEHP under natural conditions, the hydrolysis rate of DEHP under neutral acid and alkaline conditions is almost negligible, with a half-life of about 2000 years [20, 21]. At present, many DEHP-degrading bacteria have been isolated from different environments, such as *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Bacillus*, etc. [22, 23]. Previous studies on the degradation pathways of DEHP have shown that DEHP may be converted to MEHP through esterase hydrolysis, then further hydrolyzed to PA, and finally gradually mineralized [24-26]. It has also been reported that the DEHP degradation pathway is that DEHP first shortens the side chains by β -oxidation to form medium-chain DBP, then hydrolyzes into short-chain DEP and finally converts into PA [27]. In short, the degradation pathways of DEHP are not clear yet. Moreover, 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. This study was mainly carried out under aerobic conditions and the biochemical degradation pathways were investigated. Microbial immobilization technology is to confine free cells to specific space areas by chemical or physical methods in order to maintain cell viability [28]. In recent years, it mainly appears in the research of sewage denitrification and ammonia removal. The commonly used embedding methods include interception, adsorption, encapsulation and synthesis of polymers. DONG et al. [29] found that 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%. ZHANG et al. [30] used corncob-SA to immobilize DOP-degrading bacteria to degrade DOP in soil. The removal rate was 60 and 2.3 times higher than that of corn cob or free bacteria, respectively. In general, past studies have shown that immobilization technology can effectively improve the efficiency of cell degradation of organic compounds [31]. 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. However, the effects of immobilized technique on DEHP degradation and the associated microbial mechanism are still not clear.

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

2. Materials and Methods

2.1. Chemicals and medium

The purity of DEHP used in this experiment is 99% (Aldrich-Sigma Company in St. Louis, USA). The solvents (methanol, ethyl acetate) are of high performance liquid chromatography (HPLC), and the other chemical reagents are analytical 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 1000 mL. LB liquid medium: yeast extract 5 g, peptone 10 g, NaCl 5 g, H_2O 1000 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.

2.2 Identification of degrading bacteria

Activated sludge samples were collected from the secondary sedimentation tank of Xinjin Municipal Sewage Treatment Plant in Sichuan Province (30°24'49" N 103°49'11" E). Sludge samples were concentrated according to literature [32]. 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 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, 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.

2.3 Analysis of metabolites and degradation pathways

DEHP concentration was determined by high performance liquid chromatography (Aglient 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: adding ethyl acetate to each sample, shaking and mixing for 5 minutes, separating water and organic phase at a speed of 8000 rpm, and rotating 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 taken through a filter membrane with a pore diameter of 0.22 µm, and finally 20 µL filtrate was injected into the HPLC system.

DEHP degradation intermediates were detected by gas chromatography-mass spectrometry (GC-MS), and the obtained data were compared with the mass spectrometry library in the MS system to determine the types of metabolites. The detection work was completed by Sichuan Keyuan Engineering Technology co., LTD, China.

2.4. Immobilization of SAS-7 strain

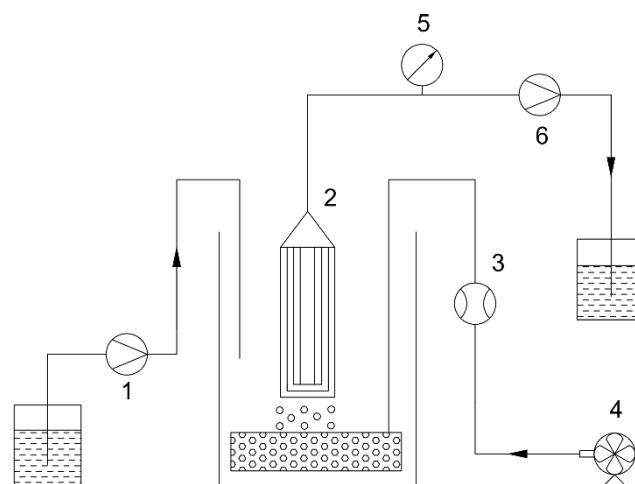
Sodium alginate and calcium chloride were used as embedding and crosslinking materials. SAS-7 cell resuspension ($OD_{600}=1.0$, 5 mL) was mixed with MSM (20 mL) in a conical flask (200 mL), and then the conical flask was sterilized. Subsequently, the MSM containing SAS-7 strain was combined with sterile SA, and the mixture was dripped into the gel solution ($CaCl_2$) 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 $CaCl_2$, 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 $L_9(3)^4$ (Table 1). Immobilized beads were washed three times with sterile distilled water and then stored at 4 °C for subsequent experiments.

Table 1. Factors and levels of orthogonal experiment design.

Levels	Factor A	Factor B	Factor C	Factor D
	SA concentration (%)	CaCl ₂ concentration (%)	Ratio 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

2.5. Reactor start-up and operation

In this study, each MBR reactor was inoculated with 0.6 L activated sludge. Artificial water distribution was used as reactor feed water, in which NH_4SO_4 was the nitrogen source, KH_2PO_4 was the phosphorus source, KCl 5000 mg/L was added, trace elements $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5000 mg/L, ZnCl_2 1000 mg/L, MnCl_2 1000 mg/L, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 200 mg/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 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 [33], the inoculation amount of DEHP degradation bacteria in this experiment was 7% of the volume of sludge inoculation. MBR reactor system was controlled by relay. The process flow diagram is shown in figure 1. Continuous influent was adopted, dissolved oxygen was 4~5 mg/L, operating temperature was 24 °C, hydraulic retention time was 48 h.

**Figure 1.** MBR flow chart and reactor.

2.6. Detection of 3,4-Dioxygenase gene in MBR by q-PCR

The total number of 3,4-Dioxygenase genes of DEHP-degrading bacteria in MBR were detected by q-PCR using ABI 7500 Real-time Polymerase Chain Reactor (Applied Biosystem, 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 MI 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.

2.7. 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 [34]. The amplified products were loaded onto 1.2% agarose gel and purified by E.Z.N.A.™ Gel Extraction Kit (Omega BioTek Inc., Norcross, Georgia) [35]. 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 [36] Eliminate low-quality reads, adapters, barcodes and primers. Using 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.

3. Results

3.1. Isolation and identification of strains

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

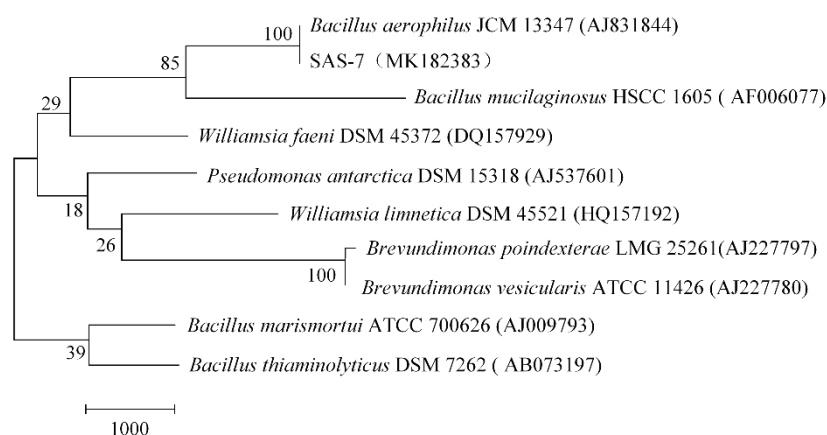


Figure 2. Phylogenetic tree of SAS-7 and its related species based on 16S rRNA gene sequence.

3.2. Degradation pathways of DEHP

To ensure that DEHP will not cause secondary pollution to the environment in the process of bioremediation. In this study, 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 TCA cycle through ortho-ring opening, and finally completes the mineralization of DEHP.

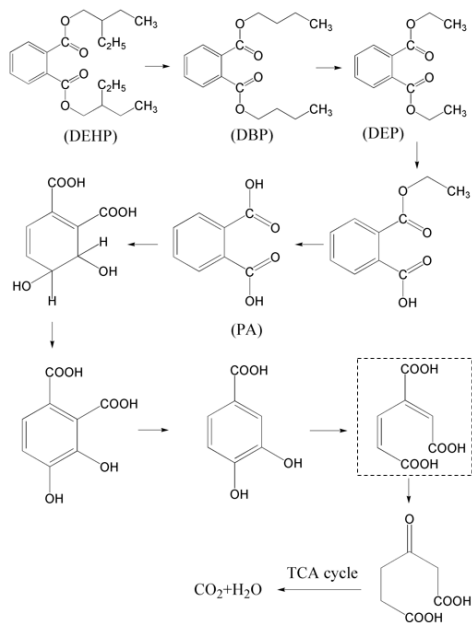


Figure 3. Proposed pathways of DEHP degradation by strain. (Material marked by dotted lines is undetected in GC-MS)

3.3. Optimization of immobilization conditions

To optimize the immobilization conditions, L9 (3⁴) orthogonal experiment (Table 1) was designed. Sodium alginate mass fraction (A), CaCl₂ mass fraction (B), ratio of bacteria to SA (C) and crosslinking time (D) were taken as factors. According to the R value showed in table 1, the order of the influence of each factor on DEHP removal rate is: B > D > A > C. Different CaCl₂ 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 A₁B₃C₁D₂, that is, SA mass fraction was 4%, CaCl₂ mass fraction was 5%, bacteria to SA ratio was 1:1, cross-linking time was 6 hours.

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_{1a}	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_{1a} , 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, cross-linking time, respectively.

3.4. DEHP removal rate in MBR reactor

To evaluate the performance of the bioreactor, the effluent samples of MBR reactor were collected daily and the removal rates of COD and DEHP were measured. Three experimental groups T1, T2 and CK were set up (T1, T2 and CK refer to the experimental group with adding free SAS-7 bacteria, immobilized SAS-7 bacteria and no SAS-7 bacteria, respectively). The wastewater containing 5 mg/L DEHP were pumped into the three reactors. As shown in Fig. 4, the removal rates of organic pollutants in the first 10 days were not significantly different 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%. Obviously, the DEHP and COD degradation rate of T2 system was significantly higher than that of T1 and CK, indicating that immobilization technology shortened the start-up time of the reactor. 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 T2 system increased faster than that of T1 system, indicating that immobilization technology played a role in significantly improving the degradation rate. 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.

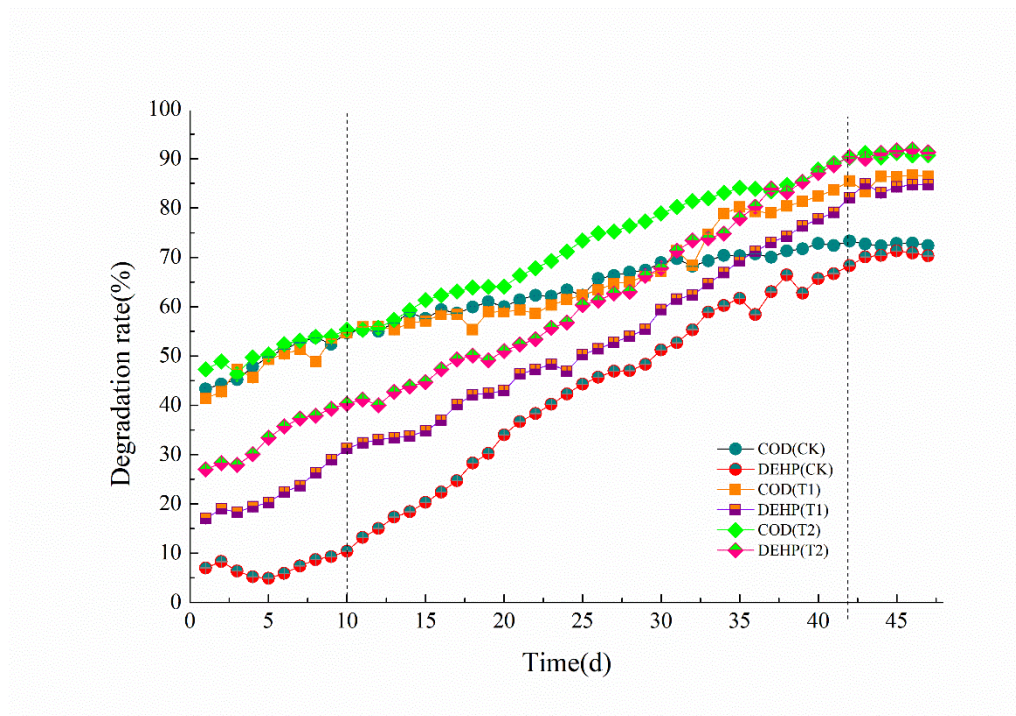


Figure 4. Removal effect of free bacteria (T1) and immobilized bacteria (T2) in MBR reactor on DEHP

3.5. *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 bioreactors, samples from MBRs were collected every 10 days, and the degrading genes in 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 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 T1 system was 6.8×10^7 copies·mg⁻¹, which was 3.1×10^7 copies·mg⁻¹ less than that in T2 system. These results indicated that immobilization technology can promote the increase of the number of the degrading gene, 3,4-dioxygenase.

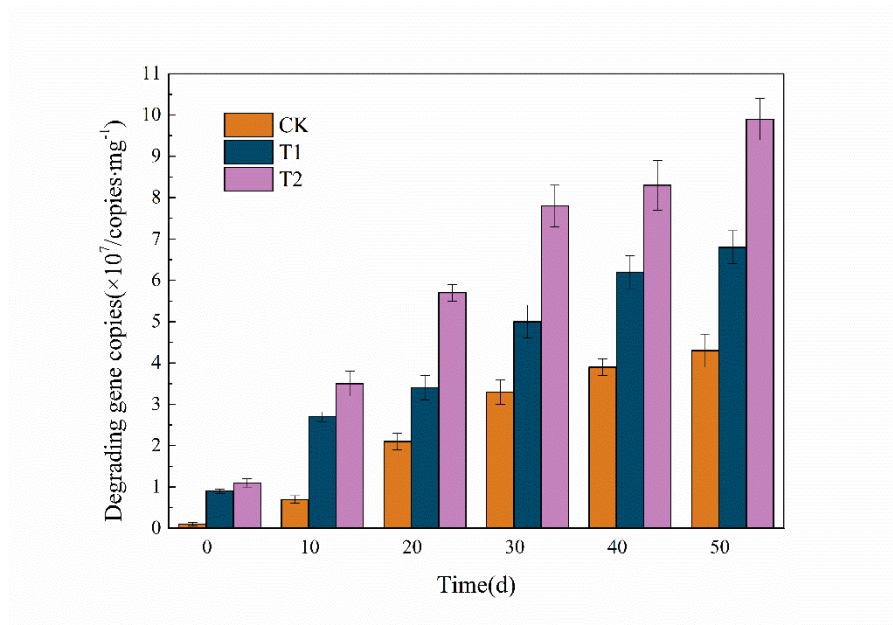


Figure 5. Number of bacterial 16S rDNA gene copies in sludge samples after introducing blank pellets (CK), free bacteria (T1) and immobilized bacteria (T2) by q-PCR extraction.

3.6. 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 phylum 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)). It was related to the addition of SAS-7. Similar to the system with free bacteria (T1), γ -Proteobacteria was also significantly increased in the system with immobilized bacteria (T2) were 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 T1 and T2 system, 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.

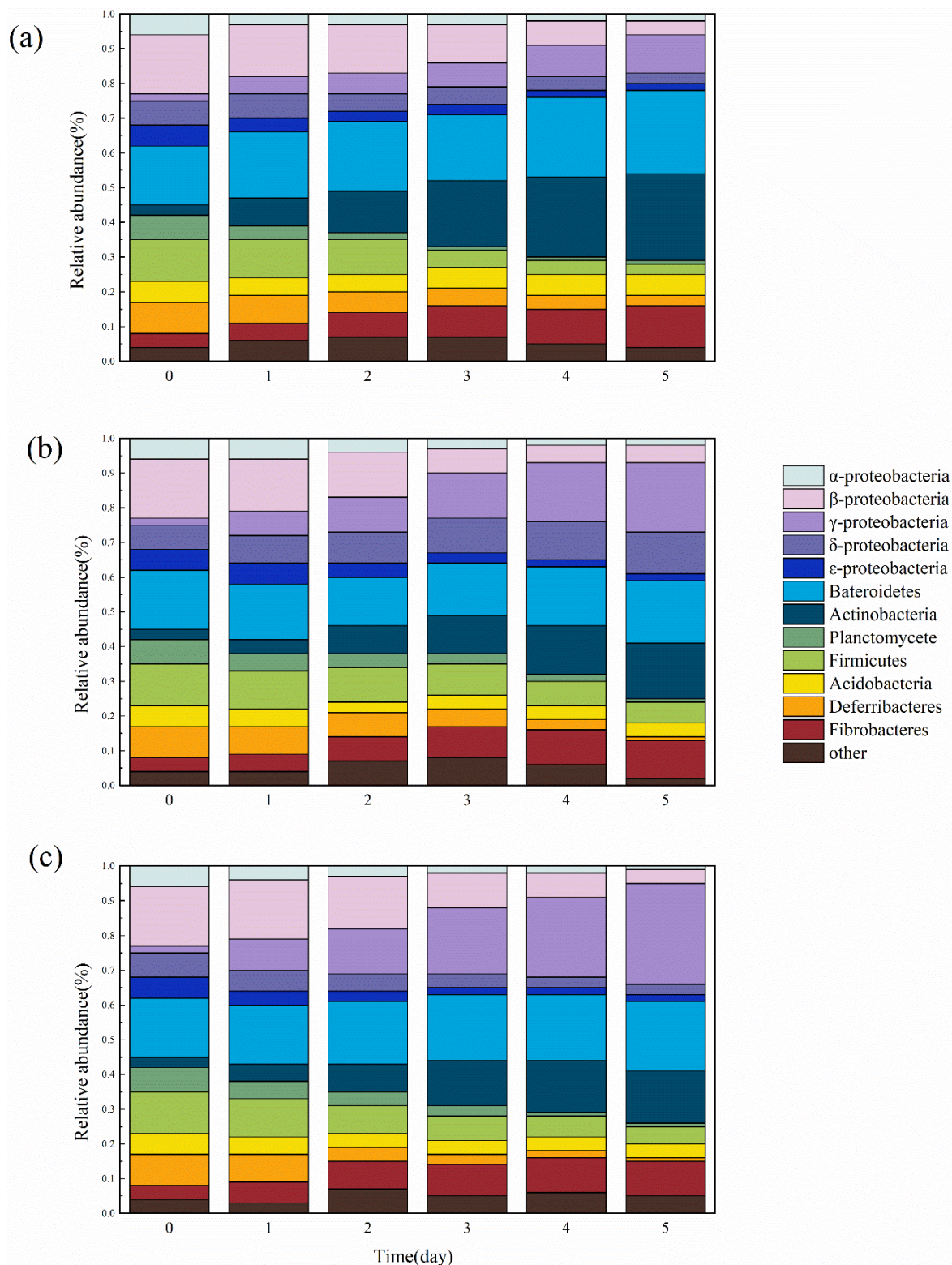


Figure 6. Bacterial community composition of activated sludge samples at phylum classification level (a) blank control CK; (b) free bacterial system T1; (c) immobilized bacterial system T2

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* dropped 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 there were some genera disappeared in both 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.

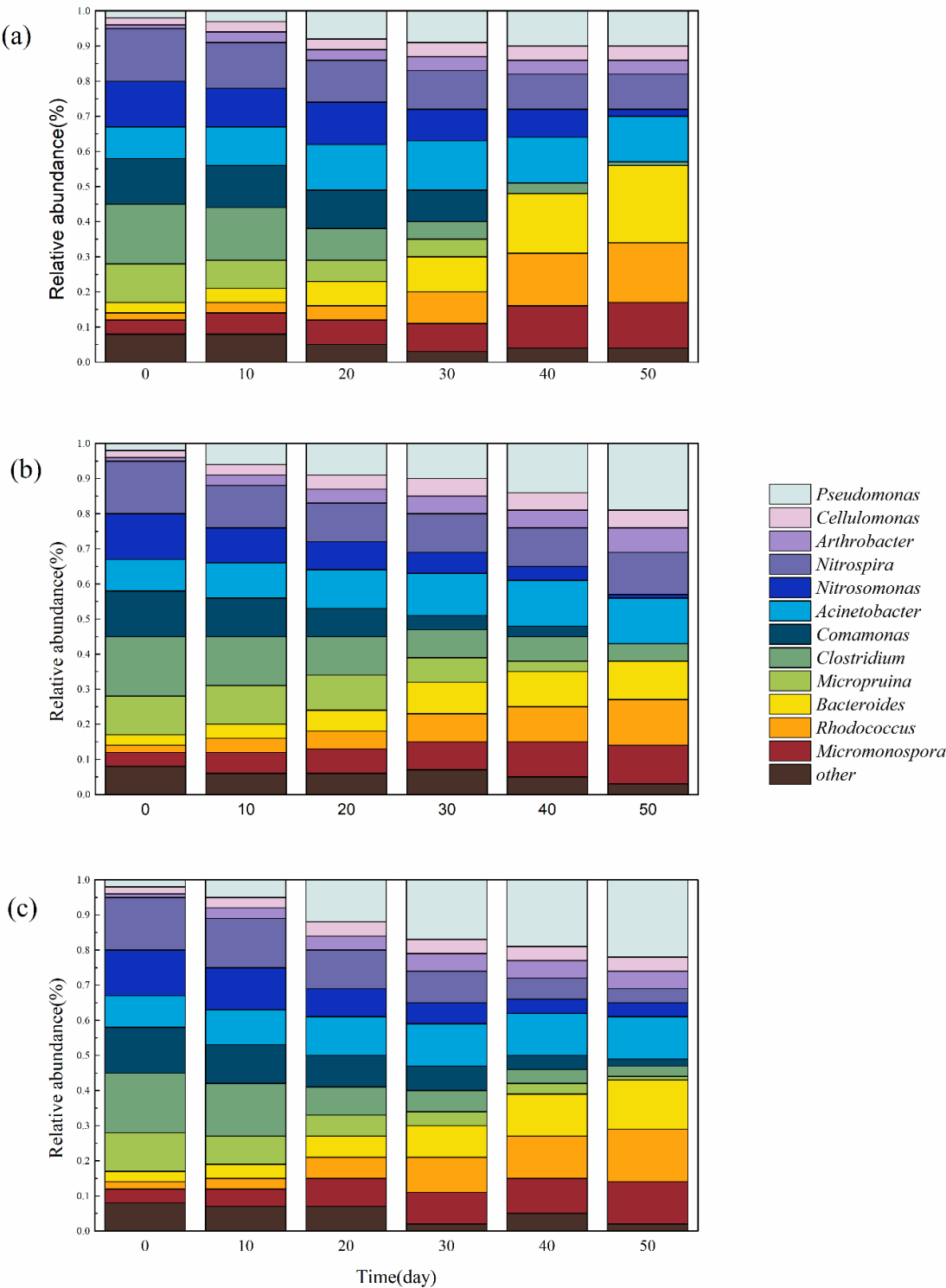


Figure 7. Bacterial community composition of activated sludge samples at genus classification level (a) blank control CK; (b) free bacterial system T1; (c) immobilized bacterial system T2

4. Discussion

In this study, sodium alginate and calcium chloride were used as embedding and cross-linking materials to immobilize a DEHP degradable strain of *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 > CaCl_2 mass fraction > bacteria and sodium alginate volume ratio > cross-linking time. Previous studies have shown that excessively low CaCl_2 mass fraction may reduce the exchange rate between Ca^{2+} and Na^+ , thus reducing the degree of cross-linking between Ca^{2+} and sodium alginate, weakening the coagulation ability, and finally leading to low DEHP removal rate [37, 38]. The optimal crosslinking time was between 5 h and 7 h, possibly because too short a crosslinking time may lead to cell instability and leakage, and too long a crosslinking time may lead to reduced bacterial activity [39]. However, whether a better immobilization condition exists remains to be studied.

To ensure microorganisms will not cause secondary pollution when degrading strains were applied to environment 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 (W, 2001; Stingley, 2004). DEHP was completely mineralized during the degradation process. *Acinetobacter* had been found to produce esterolytic enzymes [40], which are widely used for ester-bond hydrolysis and transesterification [41]. We speculate the degradation pathway of DEHP by SAS-7 was as follows: the 3-4 bit carbon chain of DEHP was shortened and decomposed into DBP to further generate DEP, and then DEP was gradually transformed into PA through deesterification. The result is similar to Tang's (Tang *et al.* 2016). 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 [42, 43]. Different from Predee, we have detected PA in the degradation progress [44]: PA used to be regarded as the key to complete the mineralization of DEHP, as it is easier to be utilized by microorganisms than DEHP [45]. What's more, our result is different from Xu *et al.*'s reports on *Acinetobacter* SN13: They didn't have detected DEP in the degradation process, but only did detected MEHP [44, 46]. On the one hand, this could be because the reaction was too fast for detecting these intermediates, on the other hand, it may due to a better and unknown degradation pathway existed. In short, there are still many problems on the degrading pathway of DEHP by *Bacillus aerophilus* remain unresolved. Therefore 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 degradation effect of immobilized bacteria was greater than that of free bacteria, which indicated that the immobilization technology could improve the degradation efficiency of DEHP. What's more, according to the change of COD and DEHP removal rates, we speculated 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 47th day, the removal rates of DEHP and COD were significantly higher than those of T1 and CK systems. This 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 [39, 47]. It may be because there were some strains or substances which were competitive or poisonous with DEHP degrading bacteria in the wastewater. And immobilized technique can be used to isolate and protect bacteria to a certain extent, creating a better living environment for degrading bacteria. In shorts, adopting the immobilized technique improved the DEHP removal efficiency of the MBR system: It is confirmed to be available to 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 T1 system was significantly less than that in T2 system. Combining with the analysis of figure 6 and figure 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 immobilized bacteria system was 1/3 higher than that in 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 of DEHP degrading rate (figure 5).

In the MBR system, microorganism is the key to remove 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. This result indicates that immobilization technology can effectively protect target bacteria from invasion of competitors and natural competition of native microorganisms. In addition, as the degrading bacteria was introduced, the bacterial population structure in the system changed; It caused a change in the degradation effect. After introduced the immobilized degrading bacteria to 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 [48, 49]. This indicates that the change of microbial community caused by immobilized bacteria is more conducive to DEHP degradation. What's more, there was no bacteria in T2 system that disappeared, during the domestication process, while *Comamonas* and *Micropruina* were gradually disappeared in 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.

5. Conclusions

A strain capable of highly degrading DEHP was isolated from activated sludge, and identified as *Bacillus* sp.. The degradation pathways were deduced as follows: from phthalic acid (2-ethylhexyl) ester (DEHP) to dibutyl phthalate (DBP) and diethyl phthalate (DEP), then further enzymatic hydrolysis to phthalic acid (PA), and finally completed mineralization. Orthogonal experiment indicates that the optimal immobilization condition is SA mass fraction of 4%, CaCl_2 mass fraction of 5%, ratio of bacteria to SA of 1:1, and crosslinking time of 6 hours. The immobilization technique significantly improved the biodegradation efficiency of DEHP in MBR. The removal rate of DEHP in the immobilized system was 91.1%, and the copy number of degraded genes was 1/3 higher than that in the free bacteria system. The 3, 4-dioxygenase gene was positively correlated to the DEHP degradation. Immobilized technique effectively protected the DEHP degrading bacteria in MBR, and *Micromonospora*, *Rhodococcus*, *Bacteroides* and *Pseudomonas* were the dominant genera. Results from this study suggest that immobilization technique has potential application for DEHP removal.

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Figure captions

Fig.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)

Fig.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 1000 resampling.)

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

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

Fig.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 .

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

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

Table list

Table 1. Factors and levels of orthogonal experiment design

Table 2. Orthogonal experimental results of immobilized DEHP degrading bacteria.

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