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Succession of bacterial community in anaerobic–anoxic–aerobic (A^2O) bioreactor using sludge fermentation liquid as carbon source

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ABSTRACT

To analyze the effect of sludge fermentation liquid on succession of bacterial community as carbon source, a three-month-long experiment was conducted in anaerobic–anoxic–aerobic process at room temperature. Experimental results showed that the removal efficiencies of total nitrogen and total phosphorus were improved to 84.7 and 94.3% from 64.5 and 54.2% at flow ratio of fermentation liquid and municipal wastewater, 1:35. Meanwhile, the principal component analysis demonstrated that significant shifts of bacterial community were observed with fermentation liquid addition. In particular, fermentation liquid appeared to be selective for the phosphorus-removal bacteria *Gammaproteobacteria*, denitrifying bacteria *Comamonas* sp., nitrogen-removal bacteria *Betaproteobacteria*, and denitrifying phosphate accumulating organisms *Sphingobacterium*, indicating simultaneous nitrification and denitrification, and denitrifying dephosphatation might be present after fermentation liquid addition.

Keywords: Bacterial community; Waste activated sludge; Nutrient removal; Carbon source; Anaerobic fermentation

1. Introduction

Biological nutrient (nitrogen and phosphorus) removal (BNR), especially phosphorus removal, has become the main objective of wastewater treatment to minimize water eutrophication. Sometimes, the nutrient removal performance is difficult to satisfy the requirement for increasingly strict legislation. Concentrations of readily biodegradable chemical oxygen demand (COD), such as volatile fatty acids (VFAs), in wastewater strongly affect the nutrient removal efficiency [1]. Earlier researches indicated that 3–4 mg COD as acetic acid is demanded to denitrify 1 mg total nitrogen (TN) [2], and that 6–9 mg VFAs is required for biological removal of 1 mg phosphorus [3]. The root cause of low nutrient removal is the competition between polyphosphate accumulating organisms (PAOs) and denitrifying bacteria for the limited carbon source in wastewater [4]. Hence, external carbon sources, such as acetate, are usually utilized to achieve a desirable nutrient removal performance in wastewater treatment plants (WWTPs).

VFAs are the preferred carbon sources for nutrient removal [5], which can be obtained from anaerobic

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fermentation of waste activated sludge (WAS) [6,7]. Simultaneously, many efforts have been made to investigate the effect of sludge fermentation liquid on nutrient removal performances in bench-scale [8] and pilot-scale experiments [9]. And results demonstrated that fermentation liquid addition could meet the demand of PAOs and heterotrophic denitrification bacteria for carbon sources and improve the removal efficiency of TN and phosphate (PO_4^{3-}-P) to 83.2 and 92.9% [10]. However, the effects of fermentation liquid addition on bacterial community structure and its dynamic shifts have not been entirely clear.

It is well known that bacteria are major participants in BNR process. The bacterial community is altered strongly by significant external parameter changes, which may further have important impact on the performance of the overall BNR process [11]. For this reason, it is quite essential to profile the bacterial community alternations caused by fermentation liquid addition for maintaining stable and efficient operations of BNR process. Recently, some molecular biotechnologies have been employed to analyze microbial information regarding community diversity, physiological needs, and complex syntrophic and symbiotic relations. Among them, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technology, coupled with sequencing and phylogenetic analysis, can provide an excellent overview of the microbial composition and diversity in a given system [12].

The objective of this work was to characterize the bacterial community structure in the anaerobic–anoxic–aerobic (A^2O) bioreactor using fermentation liquid as an external carbon source. Simultaneously, the dynamic changes of bacterial community caused by fermentation liquid were analyzed by PCR-DGGE technology, and the main functional species in A^2O bioreactor were identified by cloning analysis.

2. Material and methods

2.1. Experimental material

The WAS was taken from secondary sedimentation tank of a WWTP in Harbin city, China. The sludge was settled for 30 min at 4 ℃ with the supernatant discharged, and then was pretreated by ultrasound (ultrasonic density 0.6 W/mL, ultrasonic time 5 min) and alkali (pH 12) before 5-d anaerobic fermentation. The fermentation liquid, after simultaneous recovery of PO_4^{3-}-P and ammonia nitrogen (NH_4^+-N) in the formation of struvite [13], had the following characteristics: COD 8,120 mg/L, VFAs 5,061 mg/L, total phosphorus (TP) 47.2 mg/L, PO_4^{3-}-P 28.7 mg/L, TN 256.3 mg/L, NH_4^+-N 38.1 mg/L, soluble proteins 279 mg/L, soluble carbohydrates 91 mg/L, and pH 6.9–7.3.

2.2. Experimental device

The schematic diagram of A^2O reactor is shown in Fig. 1. The total working volume of reactor was 324 L, which was divided into an anaerobic tank (17% v/v), an anoxic tank (33% v/v), and an aerobic tank (50% v/v). The hydraulic retention times in anaerobic, anoxic, and aerobic tank were 2, 2, and 6 h, respectively. The recycling rates of sludge, nitrified liquor, and denitrified mixed liquor were 50, 200, and 100% of influent flow, respectively. Mixed liquor suspended solid concentration and sludge retention time were maintained at 4,000 ± 500 mg/L and 15 d, respectively. The dissolved oxygen in aerobic and anoxic tank was controlled at 3.0–3.5 and 0.3–0.6 mg/L, respectively. The reactor was operated at room temperature.

In the first 20 d, only municipal wastewater was treated in aforementioned A^2O process on steady state, and afterwards, the fermentation liquid was supplemented into reactor as external carbon source. The flow ratio of fermentation liquid and municipal wastewater was set to be 1:35. The influent characteristics before and after fermentation liquid addition are summarized in Table 1.

2.3. Chemical analysis

The fermentation liquid was centrifuged at 10,000 rpm for 5 min, and the supernatant was filtered (0.45 μm CAM) prior to analyses. The SCOD, TP, PO_4^{3-}-P, TN, and NH_4^+-N were measured in accordance with standard methods [14]. Proteins content was assayed on the basis of the Lowry–Folin method with bovine serum albumin as the standard [15]. Carbohydrates were determined according to the phenol–sulfuric method with glucose as the standard [16]. To analyze VFAs concentration, the supernatant was placed in a 1.5 mL gas chromatography (GC) vial with 85% formic acid of 100 μL added to decrease the pH to 4.0. The amounts of VFAs, including acetic, propionic, n-butyric, iso-butyric, n-valeric, and iso-valeric acids, were determined by an Aglient 7890A GC equipped with a flame ionization detector (FID) and a capillary column (35 m × 530 μm × 1 μm). Column, injection port, and detector temperatures were 240, 170, and 240 ℃, respectively, with nitrogen gas at 60 mL/min, hydrogen gas at 40 mL/min, and air at 400 mL/min as carrier gases [17].

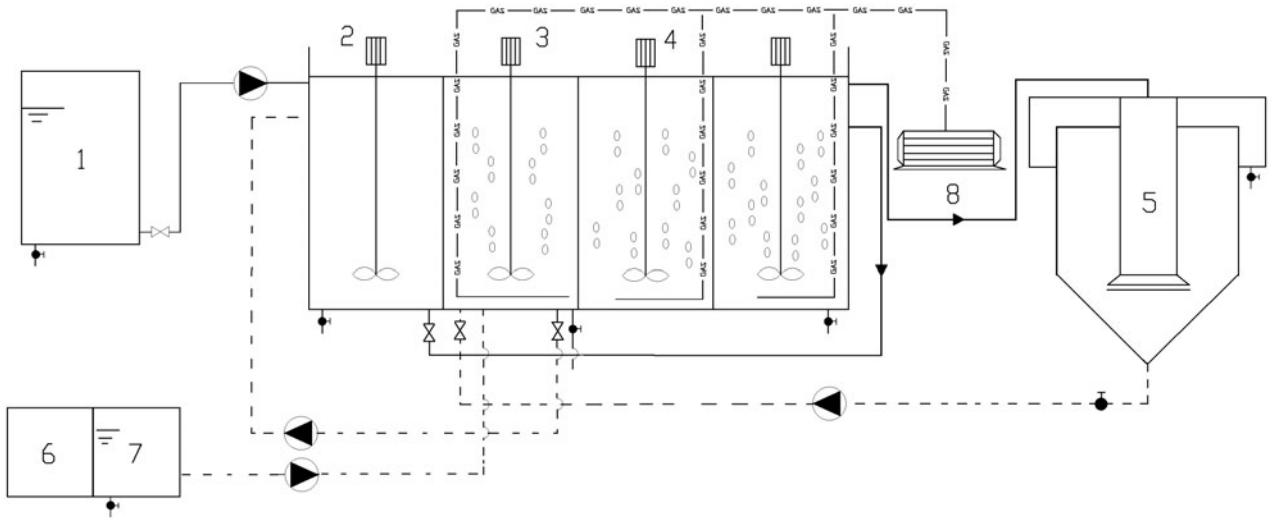

Fig. 1. Schematic diagram of A^2O process: (1) influent tank, (2) anaerobic tank, (3) anoxic tank, (4) aerobic tank, (5) sedimentation tank, (6) sludge treatment tank, (7) fermentation storage tank, and (8) aerators.

Table 1
Influent characteristic before and after sludge fermentation liquid addition

Items	Municipal wastewater	Mixed liquor
COD (mg/L)	183.5	403.9
TN (mg/L)	44.8	50.7
NH_4^+-N (mg/L)	31.9	32.1
TP (mg/L)	8.3	9.4
PO_4^{3-}-P (mg/L)	4.2	4.9
VFAs (mg COD/L)[a]	9.2	149.5

[a]The VFAs include acetic acid 38.2%, propionic acid 30.6%, n-butyric acid 8.7%, iso-butyric acid 9.5%, n-valeric acid 2.9%, and iso-valeric acid 10.1%.

2.4. DNA extraction and PCR amplification

The genomic DNA was extracted from sludge samples using the E.Z.N.A Soil DNA Kit (Omega Bio-Tek. Inc.). The V3 region of 16S rRNA for extracted DNA was amplified by PCR using the forward primer 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer 534 R (5′-ATTACCGCGGCTGCTGG-3′) with a GC clamp. The PCR reaction system accorded with the previous literature [18].

2.5. DGGE

DGGE analysis was performed with the DCode™ Universal Mutation Detection system (Bio-Rad, USA). The PCR samples were applied directly to 7.5% (w/v) polyacrylamide gels in 1 × TAE buffer (pH 8.0) with a denaturing gradient ranging from 40 to 60%. Electrophoresis was carried out at a constant voltage of 70 V for 8 h in 1× TAE buffer of 60℃ [19]. After electrophoresis, the gel was stained with ethidium bromide and then scanned under UV transillumination [20].

2.6. Statistical analysis

Principal component analysis (PCA) is used to plot the maximum variance of the bacterial community band classes in two dimensions. In a PCA ordination plot, samples with similar fingerprints are placed close to one another, and those that are dissimilar are placed far apart [21].

2.7. Cloning, sequencing, and phylogenetic analysis of 16S rRNA gene fragments

The gel slice excised from DGGE bands was crushed and 50 μL sterile water was added to resolve the DNA overnight at 4℃. The eluted DNA was reamplified by PCR with primers 338F and 534R without a GC clamp. The PCR products were purified and ligated with E.Z.N.A Cycle Pure Kit (Omega Bio-Tek. Inc.) and PMD19-T Vector (Takara, Dalian, China). Then the formated plasmid was transformed into *E. coli* Competent Cells DH5α (Takara, Dalian, China) according to the manufacturer's instruction [22]. At last, 16S rRNA gene fragment clones were sequenced, and acquired sequences were compared with the closest reference micro-organisms in the GenBank

database using Basic Local Alignment Search Tool. Neighbor-joining trees were constructed for phylogenetic analysis using MEGA 3.0 software.

3. Results and discussion

3.1. Removal performances of COD, TN, and TP

After the reactor was operated for three months, the effluent COD, TN, and TP became stable, and the removal performances under different influent conditions are presented in Fig. 2. With the addition of fermentation liquid, the influent COD was abruptly increased 2.2-folds, but there was only a slight increase of influent TN and TP. Compared with municipal wastewater, the effluent COD was not deteriorated (only increased from 21.3 to 32.4 mg/L), and the effluent TN and TP were decreased to 5.4 and 0.5 mg/L by fermentation liquid. It can be seen that

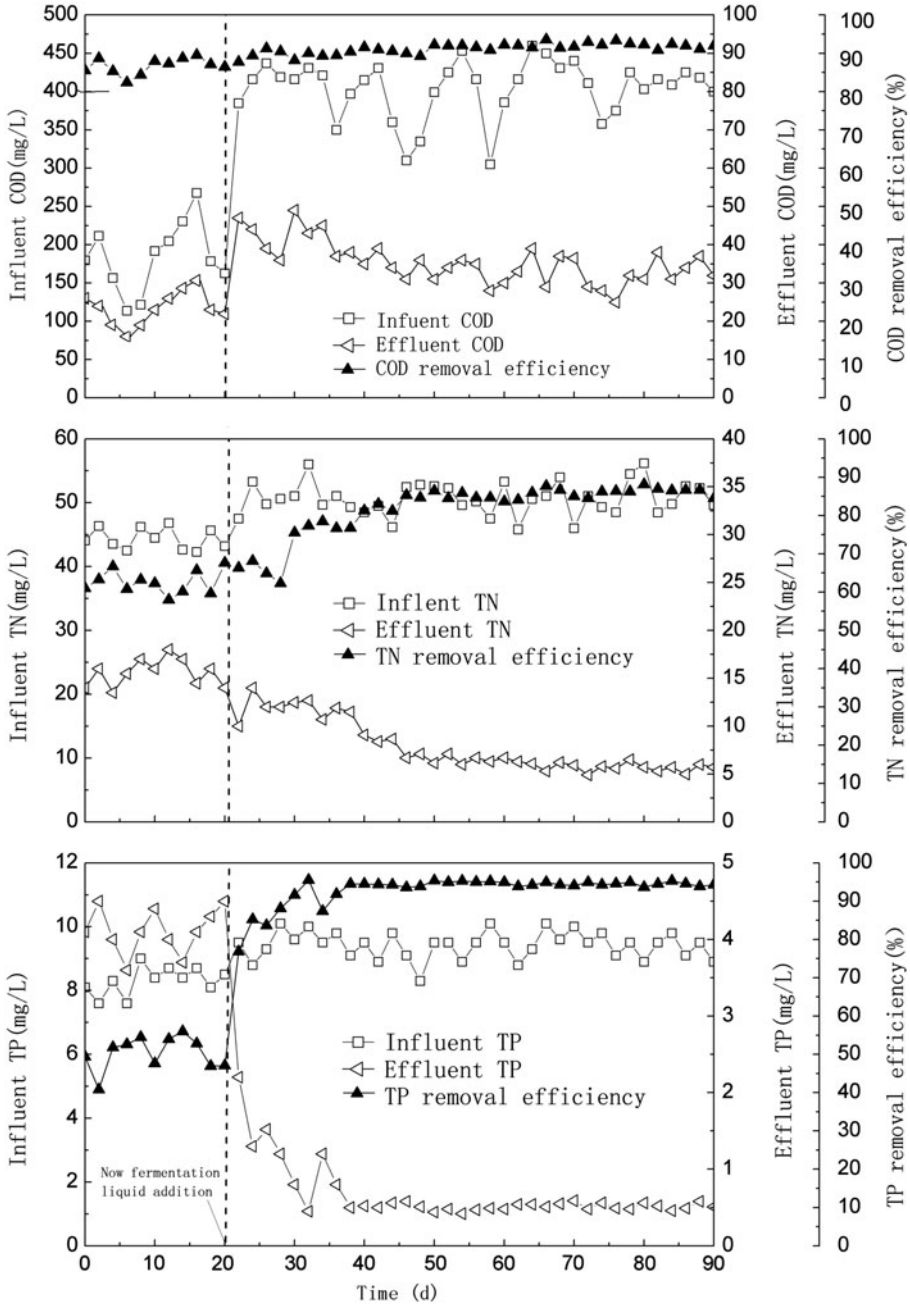

Fig. 2. Variances of COD, TN, and TP before and after fermentation liquid addition.

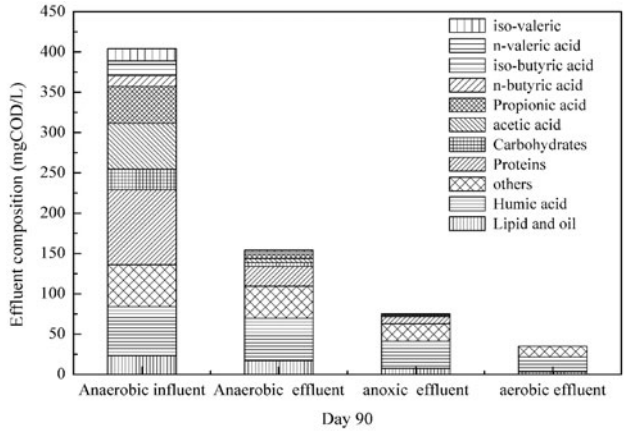

Fig. 3. Variances of effluent composition in anaerobic anoxic and aerobic tanks.

the fermentation liquid addition caused an obvious increase of the removal efficiency of TN (from 64.5 to 84.7%) and TP (from 54.2 to 94.3%). The increase of TP removal efficiency was much greater than that of TN and COD. It seems that flow ratio of fermentation liquid and municipal wastewater, 1:35, was benefit for nutrient removal. Also, in Fig. 2, owing to the application of fermentation liquid, the effluent COD, TN, and TP experienced a 25-d fluctuation period and then restored to steady-state again.

An extensive study on nutrient removal performance on the 90th day was conducted, and the influent and effluent compositions in anaerobic, anoxic, and aerobic tank were analyzed. It is well known that denitrification usually occurred in the anaerobic and/or anoxic period(s), and phosphorus release and uptake happened in the anaerobic stage and anoxic and/or aerobic stage(s), respectively. The presence of adequate carbon source (such as VFAs) is necessary for the denitrification and phosphorus uptake. The variations of effluent COD composition in anaerobic, anoxic, and aerobic phases are shown in Fig. 3. During the anaerobic phase, the COD concentration decreased to 154.3 mg/L, especially, the VFAs concentration decreased to 15.6 mg/L. In the following anoxic tank, soluble proteins and carbohydrates were further utilized and the VFAs were not detectable. Only HA, lipid, and oil were detected in the effluent of aerobic tank, and the increase of effluent COD was mainly attributed to its high HA input along with fermentation liquid.

As seen in Figs. 2 and 3, there was no significant difference in the influent TN and TP before and after fermentation liquid addition. Nevertheless, greater TN and TP removal was received after fermentation liquid addition, which should be explained as follows. Firstly, fermentation liquid enhanced the ratio of carbon and nitrogen (C/N) from 4.1 to 9, making the competition for carbon sources between PAOs and denitrifying heterotrophs weakened [4]. Simultaneously, abundant VFAs in fermentation liquid supplied favorable carbon source for the growth of PAOs and denitrifying bacteria.

3.2. DGGE analysis

In order to investigate the effects of sludge fermentation liquid on bacterial community structure, sludge samples taken from bioreactor on 1, 5, 10, 20, 30, 45, 60, 75, and 90 d were analyzed by PCR-DGGE

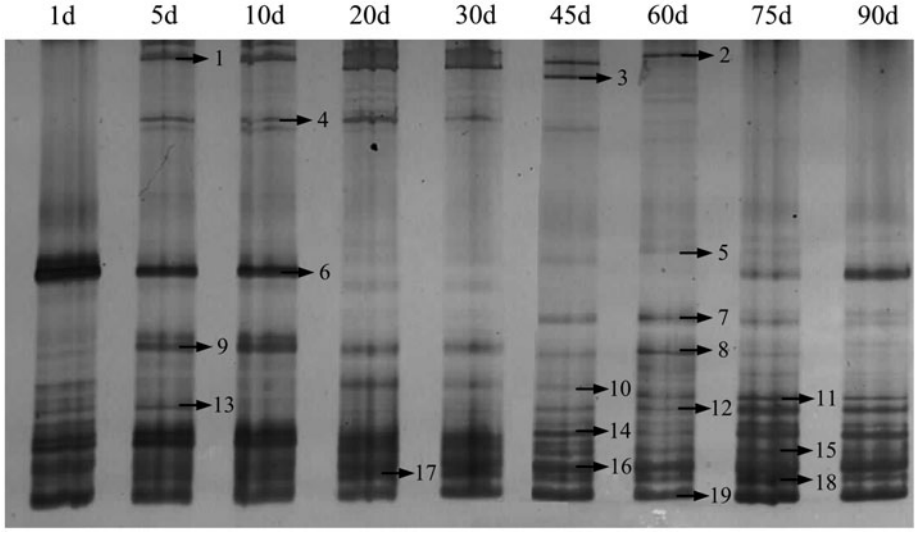

Fig. 4. Denaturing gradient gel electrophoresis (DGGE) profiles of the V3 region of the 16S rRNA gene-amplified products.

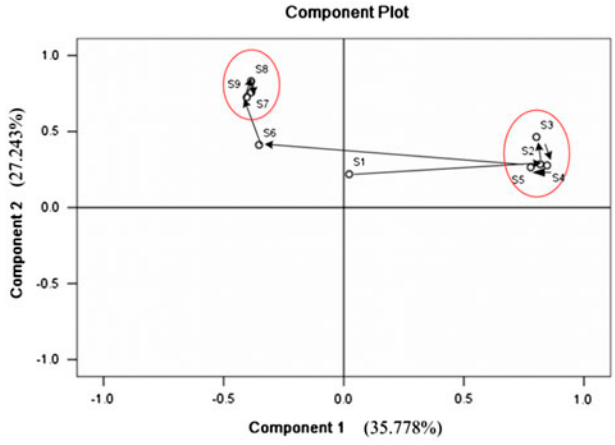

Fig. 5. PCA of DGGE fingerprint obtained from sludge samples of A^{2}O process (the number of samples was 9).

technology. A total of 19 bright bands were detected in DGGE gels, and pronounced shifts of bands numbers and intensities are observed in Fig. 4. The numbers of band reduced in 20–45 d, and then increased in 45–90 d. Bands 1, 4, 6, 9, and 13 were receded with the application of fermentation liquid; bands 2, 3, and 10 only appeared in the period of acclimation; bands 7 and 8 are strongest on the 60 d; other bands were gradually strengthened with fermentation liquid addition. It seems that fermentation liquid affected the nutrient removal performances of bioreactor by influencing the quantity and species of dominant bacteria.

The PCA results of DGGE fingerprint data are revealed in Fig. 5. Three distinct groups were formed, and the data tended to vary more obviously with principal component 1 than component 2. In this study, samples 1–9 (S1-9) of PCA correspond to samples of DGGE (1–90 d). S1, S2-5, and S6-9 belonged to different groups, indicating that the bacterial community structure was remarkably changed by influent conditions. Sludge fermentation liquid was added into A^{2}O reactor from the 20th day, and the bacterial community was changed and shifted to another group on the 45th day. Meanwhile, bacterial community compositions of S2-5 or S6-9 were similar in the dominant species because of relatively stable influent condition. It can be deduced that the fermentation liquid contributed to the establishment of bacterial communities in this experiment.

3.3. Phylogenetic analysis

Nineteen DGGE bands, presumed to represent unique microbial population, were carefully excised

Table 2
The most closely matched species of 16S rRNA sequences in NCBI

Bands	Closest relative (Accession No.)	Phylum	Identity (%)	Putative function
1	*Uncultured Sphaerobacter* sp. (GQ421032.1)	*Chloroflexi*	99	Sludge bulking
2	*Uncultured Actinobacteria bacterium* (CU925307.1)	*Actinobacteria*	99	Organics degradation
3	*Lactococcus* sp. (EU689105.1)	*Firmicutes*	100	Protein degradation
4	*Uncultured Chlorobi bacterium* (CU927353.1)	*Proteobacteria*	100	Sulfur compounds degradation
5	*Uncultured Bacteroidetes bacterium* (CU922346.1)	*Bacteroidetes*	100	Hydrolysis
6	*Uncultured TM7 bacterium* (CU917528.1)	*TM 7*	98	Nutrient removal
7	*Acinetobacter* sp. (AB488778.1)	*Proteobacteria*	100	Denitrifying phosphorus removal
8	*Uncultured Trichococcus* sp. (HQ183758.1)	*Firmicutes*	100	Hydrolysis
9	*Uncultured Nitrosospra* sp. (EF042985)	*Proteobacteria*	100	Ammonium oxidation
10	*Chloroflexi bacterium ET9* (EU875530)	*Chloroflexi*	100	Sludge bulking
11	*Comamonas denitrificans strain 2B7* (EU337122)	*Proteobacteria*	98	Denitrification
12	*Uncultured Sphingobacteriaceae bacterium* (GU257878.1)	*Bacteroidetes*	100	Denitrifying dephosphatation
13	*Uncultured Actinobacteria bacterium* (CU926640.1)	*Actinobacteria*	100	Protein degradation
14	*Uncultured Sphingobacteriaceae bacterium* (GU257891.1)	*Bacteroidetes*	97	Denitrifying dephosphatation
15	*Uncultured Betaproteobacteria bacterium* (CU927437.1)	*Bacteroidetes*	100	Denitrification
16	*Lactococcus lactis* (JN792511.1)	*Firmicutes*	100	Protein and hydrocarbon degradation
17	*Uncultured Actinobacteria bacterium* (CU922264.1)	*Actinobacteria*	100	Organics degradation
18	*Uncultured gamma proteobacterium* (DQ640661.1)	*Proteobacteria*	99	Phosphorus removal
19	*Uncultured Alphaproteobacteria bacterium* (CU926948.1)	*Proteobacteria*	100	Nutrient removal

for sequencing and further analysis. By comparing with GenBank database, all nucleotide sequences were found to be 97–100% homologous with previously identified 16S rRNA gene sequences. The sequences were determined and their closest phylogenetic affiliations are summarized in Table 2. And the phylogenetic relation based on the sequence results is illustrated in Fig. 6.

Bands 11 and 15 similar to *Comamonas* sp. and *Betaproteobacteria* have been identified to be dominant denitrifying bacteria and nitrogen-removal bacteria in nutrient removal reactor [23,24]. In this experiment, the change regulation of these two bacteria was consistent with TN removal efficiency, indicating that they played a very important role in nitrogen removal using fermentation liquid as carbon source. It also can be concluded that simultaneous nitrification and denitrification (SND) occurred after fermentation liquid addition, because C/N ratio of 9 created good survival conditions for SND. Band 19 had 100% homology

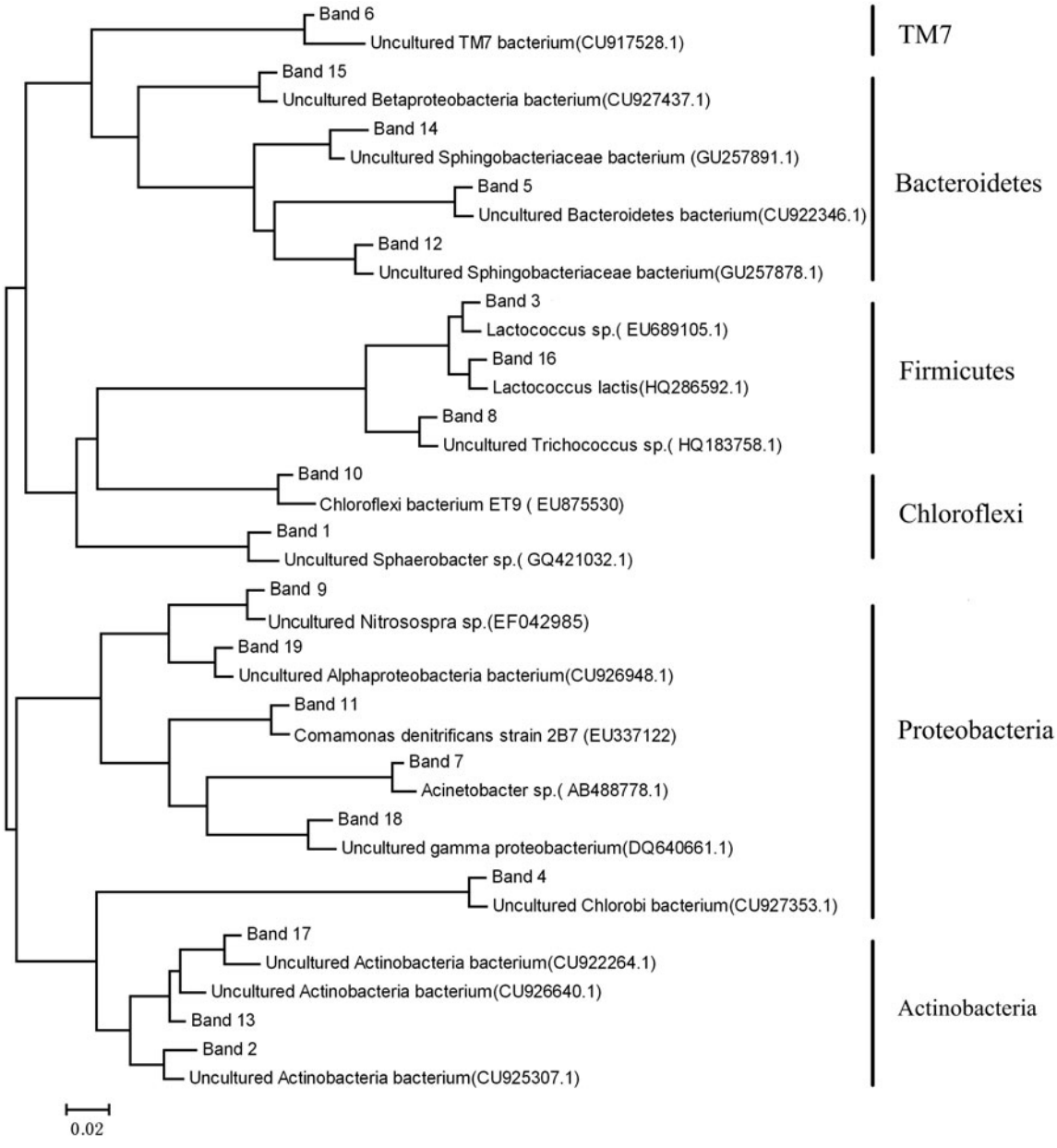

Fig. 6. Phylogenetic tree of 16S rRNA sequences from DGGE profiles represented the relationship of the sludge population. The scale bar represents two substitutions per 100 nucleotide positions.

similarity with *Gammaproteobacteria*, and the good agreement between its intensity and the TP removal efficiency stated that it was responsible for phosphorus removal. Band 14 most closely related to *Sphingobacteriaceae* has been confirmed to be denitrifying phosphate accumulating organisms (DPAOs) [25]. Its variation, increasing gradually and reaching maximum on the 90th day, should be ascribed to the moderate fermentation liquid addition. Appropriate VFAs concentration supplemented into reactor not only weakened the competition between DPAOs and denitrifying bacteria for carbon source, but also prevents the accumulation of glycogen accumulating organisms available. Bacterial analysis and nutrient removal performances demonstrated that fermentation liquid had certain selectivity for functional microorganisms.

4. Conclusions

Sludge fermentation liquid was supplemented into municipal wastewater to test its contribution to nutrient removal and bacterial community in A^2O reactor. With fermentation liquid addition, significant shifts of bacterial communities were observed, microbes in 5–30 and 45–90 d belonged to different groups. Simultaneously, some functional bacteria such as phosphorus-removal bacteria *Gammaproteobacteria*, nitrogen-removal bacteria *Betaproteobacteria*, and denitrifying bacteria *Comamonas* sp. were selected by fermentation liquid, which resulted in TN and TP removal efficiencies reached to 84.7 and 94.3% from 64.5 and 54.2%. Notably, DPAOs *Sphingobacterium* were detected in this experiment, indicating that denitrifying dephosphatation happened at flow ratio of fermentation liquid and municipal wastewater, 1:35.

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