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Analysis of phosphate-accumulating organisms cultivated under different carbon sources with polymerase chain reaction-denaturing gradient gel electrophoresis assay

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Abstract: To investigate the microbial communities of microorganisms cultivated under different carbon sources, three sequencing batch reactors were operated. They were supplied with sewage, glucose and sodium acetate as carbon sources respectively and showed high phosphorus removal performance. The results of denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction-amplified (PCR) 16S rDNA fragments demonstrated that β-*protebacteria*, *Actinomyces* sp. and γ-*protebacteria* only exited in 1 # reactor. The microbiological diversity of 1 # reactor exceeded the other two reactors. *Flavobacterium*, *Bacillales*, *Actinomyces*, *Actinobacteridae* and uncultured bacteria(AF527584, AF502204, AY592749, AB076862, AJ619051, AF495454 and AY133070) could be detected in the biological phosphorus removal reactors.

Keywords: 16S rDNA; carbon source; biological phosphorus removal; PCR-DGGE

Introduction

There have been many investigations attempting to explain biological phosphorus removal from biochemical point of view (Comeau, 1986; Wentzel, 1986; Satoh, 1992; 1996; Carucci, 1999), but all of the conceptual models have little matched microbial community description. The study was conducted to identify phosphate-accumulating organisms (PAOs) (Fuhs, 1975; Wagner, 1994; Sudiana, 1998; Wang, 1998; Philip, 1998; Daniel, 1998; Gregory, 2002; Julie, 2002; Che, 2003; Johwan, 2002), *Acinetobacter* was firstly separated and thought to be one of the important members of PAOs(Fuhs, 1975; Wentzel, 1986). However, some evidence showed that *Acinetobacter* might not be the dominant PAOs (Wagner, 1994; Sudiana, 1998; Wang, 1998; Philip, 1998; Daniel, 1998; Gregory, 2002), as it is easily isolated and identified based on culture dependent identification methods such as dilution or plating on various nutrient agar plates. Recently, with the development and usage of culture-independent methods such as fluorescent *in situ* hybridization (FISH), polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) assay in analysis of environmental samples, *Rhodocyclus* genus of β-*protebacteria* has gained increasing attention as the most possible PAOs candidates (Philip, 1998; Daniel, 1998; Gregory, 2002; Julie, 2002; Johwan, 2002; Che, 2003). Moreover, there are some other possible PAOs, like *Tetrasphaera* spp. and members of *Mcrolunatus* spp. (Eschenhagen, 2003), members of *Accumulibacter phosphatis*, the novel γ-*Porteobacterial* group and filamentous group resembling *Candidatus Nostocoidia* limicola (Liu 2001) and *Cytophagales* (Dabert, 2001).

Nonetheless, there are few studies investigating the difference between bacterial community structure and diversity in biological phosphorus removal systems supplied with different carbon sources. This paper reports on the microbial structure and the phylogenetic affiliation in three sequencing batch reactors supplied with different carbon sources, i.e. sewage water, glucose and sodium acetate with polymerase chain reaction-denaturing gradient gel electrophoresis assay (PCR-DGGE).

1 Materials and methods

1.1 SBR operation

Three laboratory-scale sequencing batch reactors (SBRs), each with an 18 L working volume, were operated and had gained good EBPR performance after about 90 d. All of them were seeded from a full-scale SBR, which was used to treat wastewater from a hotel and had achieved BPR when it was used to seed in this experiment. They were operated under sequencing anaerobic-aerobic conditions with an 8 h cycle, which consisted of 2.5 h anaerobic, 3 h aerobic and 2.5 h settling fill/draw and idle stages. During the anaerobic phase, nitrogen gas was purged into reactors for 15 min to reach anaerobic conditions.

Phosphorus removal efficiencies under different pH, temperature and SRT conditions were investigated in batch experiments. Data were not shown here. Results showed that different systems reached to optimal phosphorus removal at pH 7.2. It was also found that temperature had no obvious effect on phosphorus removal in the interval of 10 to 25℃. When sludge retention time(SRT) was on the day 7 and 20, all the systems had better phosphorus removal. Thus, the operation temperature was fixed at 22℃; pH values were controlled around 7.0 ± 0.2 by adding 0.5 mol/L HCl or 0.5 mol/L NaOH; extra sludge was withdrawn from system at the end of each cycle to maintain the SRT around 7 d and mixed liquid suspended solid(MLSS) around 3000 mg/L.

1.2 Wastewater

Domestic sewage and two kinds of synthetic domestic wastewater were used in this study. The synthetic water was prepared by mixing tap water with glucose and acetate respectively. To avoid fermentation, above components were added to water tank before the start of each cycle. The compositions of synthetic wastewater for three reactors are shown in Table 1.

Table 1 Composition of synthetic wastewater for three reactors

Compound, mg/L	1 # reactor	2 # reactor	3 # reactor
Sewage/glucose/ sodium acetate		589.5	741.4
P(KH_2PO_4)	4—7.2	15	15
NH_3-N(NH_4Cl, average)	35.1	40	40
COD	285.4—326.8	600	600
Mg($MgCl_2$)	/	10	10
Cu($CuSO_4$)	/	0.1	0.1
Ca($CaCl_2$)	/	5	5
Mn($MnSO_4$)	/	0.1	0.1
Zn($ZnCl_2$)	/	0.1	0.1
Peptone	/	10	10

Note: /. no data

The biological treatment units were run for over 3 months under controlled conditions to reach steady phosphorus removal performance. Then the test was carried out, and the performance of whole treatment system was analyzed.

1.3 Chemical analysis

Chemical oxygen demand (COD), PO_4^{3-}, NH_4-N,

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NO_3-N, MLSS and MLVSS were analysed according to Standard Methods (SEPAC, 1989). Total organic carbon (TOC) was measured by a TOC analyser (TOC-V_{CPN}, SHIMADZU). Dissolved oxygen(DO) was measured by a DO meter(JPB-607). pH was measured by an acidometer(PHB3-PH).

1.4 PCR-DGGE analysis

1.4.1 Sludge sample preparation and DNA extraction

Sludge samples were collected from each reactor at the end of aerobic phases, and treated as described by Johwan *et al*, (Johwan, 2002). After being concentrated by centrifugation at 9000 r/min(Anke GL-20G-II, Shanghai) for 5 min at 4℃, the samples were vortexed with 3 volumes of TE buffer(10 mmol/L tris-HCl, 1 mmol/L EDTA, pH 8.0) for 5 min. The process as described above was repeated 3 times, and then the samples were stored at −20℃. Total DNA was extracted from sludge samples by a rapid freeze-and-thaw method from Tsai(Tsai, 1991).

1.4.2 Primers and PCR amplification

16S rDNA fragments were amplified with the forward primer EUB341f(5'-CCTACGGGAGGCAGCAG-3') with a GC clamp (5'-CGCCCGCCGCGCGGCGGCGGGCGGGGCGGGCACGGGGGG-3') at the 5' end, and the reverse primer UNIV 907r (5'-CCCCGTCAATTCCTTTGAGTTT-3') (Johwan, 2002). PCR amplification was performed in an automated thermal cycler(BIO-RAD, USA). To minimize non-specific annealing of the primers to target DNA, a "hot start" technique(Muyzer, 1998) was carried out at 94℃ for 5 min, the denaturing step was followed by the addition of 1 μl of Taq DNA polymerase to the reaction mixture at a temperature of 80℃, annealing at 65℃ for 1 min and extension at 72℃ for 3 min. Then touchdown PCR(Muyzer, 1998) was performed as follows: denaturation for 1 min at 94℃, annealing for 1 min at 64℃, but the annealing temperature was lowered by 1℃ every second cycle until it was 55℃. 10 additional cycles were carried out at annealing temperature of 55℃. Primer extension was performed at 72℃ for 3 min. The final extension was 10 min at 72℃. The PCR mixture had a final volume of 50 μl that contained 5 μl of 10 × PCR buffer, 10 pmol of each primer, 4 μl of 25 mmol/L concentration of each dNTP, 50 ng template and 1 U polymerase. 3 μl of the PCR product was electrophoresed on 1%(wt/v) agarose gel in 0.5 × TAE for 30 min at 100 V, and then checked with ethidium bromide(EB) staining before being used for DGGE analysis.

1.4.3 DGGE analysis

25 μl PCR product was loaded onto a 6% (wt/v) polyacrylamide gel(acrlamide/bis-acrylamde ratio, 37.5:1) in 0.5 × TAE buffer(20 mmol/L Tris base, 10 mmol/L glacial acetic acid, 0.5 mmol/L EDTA, pH 8.0) for 24 h at 100 V and 60℃. The polyacrylamide gel contained a linear gradient ranging from 30% to 70% denaturant. After electrophoresis, the gel was stained for 10 min with EB, and then scanned with UV transilluminator(BIO RAD Gel Doc 1000) combined with a digital camera.

1.4.4 Sequencing of DGGE bands

Bands in the DGGE gel of 16S rDNA fragments were excised with a razor blade. The DNA was retrieved from the acrylamide block with Centriluter(Amicon) at 150 V for 3 h. The DNA was reamplified with GC-clamp-EUB341f and UNIV 907r. The clones were PCR amplified again with the same primers above and were analysed by DGGE. The clones that produced a DGGE band at the same position as the excised band were selected for subsequent sequence analysis. The sequence analysis of selected clones was performed on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, USA) according to the manufacture's guidelines.

2 Results and discussion

2.1 Phosphorus removal in three different carbon sources SBRs

After more than three-month operations, three SBRs got good EBPR performance (Fig. 1). As can be seen, the effluent phosphorus concentration of 1 # reactor supplied with sewage was the best for its relatively low influent phosphorus concentration. Its phosphorus removal efficiency was nearly 100% after 70 d operation, which may be due to the fluctuant phosphorus in influent. Most of the time in this experiment, phosphate concentration in sewage was below 5 mg/L. 3 # reactor supplied with sodium acetate did not achieve the best phosphorus removal as described by literature (Wang, 2001)(phosphorus removal efficiency was 90.94% after 98 d operation), whereas 2 # supplied with glucose gained better result (phosphorus removal efficiency was 96.9% after 94 d operation). The reason may be that the phosphorus-removing ratio between PAOs and EPS was different with various carbon sources(Liu, 2004).

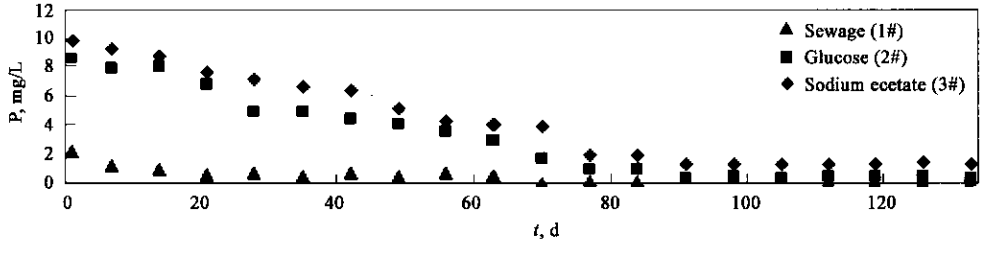

Fig.1 Phosphorus concentrations in effluent of three SBRs during experiment

2.2 Fingerprinting of reactor communities by DGGE

2.2.1 Sludge gene DNA amplification

Sludge was sampled at the end of anaerobic phase and was treated before being used for gene extracting.

The gene DNA from sludge was about 23 kb. Then the DNA was amplified with "touch down" PCR process. The gene fragments of predicted length of about 500 bp was obtained and used for the following DGGE analysis.

2.2.2 DGGE analysis of 16S rDNA PCR production

Bacterial community structures and phylogenetic affiliation of three SBRs were screened by DGGE of the PCR-amplified 16S rDNA (Fig. 2). The existing visible bands demonstrated the complexity and diversity of microbial ecology in sludge samples. It could be found from the DGGE profiles that the sludge sample from 1 # reactor that was supplied with sewage as carbon source had the most bands. Bands 1, 2, 3, 4 and 5 existed in all three reactors, bands 8, 9, 12 and 13 existed only in 1 # reactor, bands 6 and 7 only existed in 2 # reactor, band 11 only existed in 3 # reactor, while band 10 exited in both 1 # and 3 # reactors.

Bands 1—13 were excised from DGGE polyacrylamide gel and their sequences were analyzed. Comparison of 16S rDNA sequences of bands from DGGE with sequences available in GenBank database revealed high similarity values (Table 2). Sequence analysis revealed that DGGE bands 1, 2, 3, 4 are closely related to genus *Flavobacterium*(Fig.3). Furthermore, bands 3 and 4 are uncultured bacterium and related to the bacterium that were submitted by the

Table 2 Identification of bands originating from three reactors and their similarity values from GenBank

Reactor(crabon source)	Band	Organism	Similarity, %
1 #, 2 #, 3 #	1	*Chryseobacterium* sp.	97
1 #, 2 #, 3 #	2	*Chryseobacterium* sp.	97
1 #, 2 #, 3 #	3	Uncultured bacterium clone LPB28 16S ribosomal RNA gene	97
1 #, 2 #, 3 #	4	Uncultrued bacterium clone HP1B06 16S ribosomal RNA gene	96
1 #, 2 #, 3 #	5	Uncultured bacterium clone Napoli-4B-10 16S ribosomal RNA gene	94
2 #	6	*Bacillus* sp.	99
2 #	7	*Propionibacterium cyclohexanicum* gene for 16S rRNA	98
1 #	8	Uncultured beta proteobacterium gene for 16S rRNA clone: OS1L-4	96
1 #	9	Uncultured gamma proteobacterium partial 16S rRNA gene, clone MTAD19	99
1 #, 3 #	10	Uncultured eubacterium clone T19 16S ribosomal RNA gene	97
3 #	11	*Actinomyces* sp.	97
1 #	12	*Actinomyces suimastitidis* partial 16S rRNA gene, strain CCUG 39276	96
1 #	13	Uncultured gamma proteobacterium clone ces2170	99

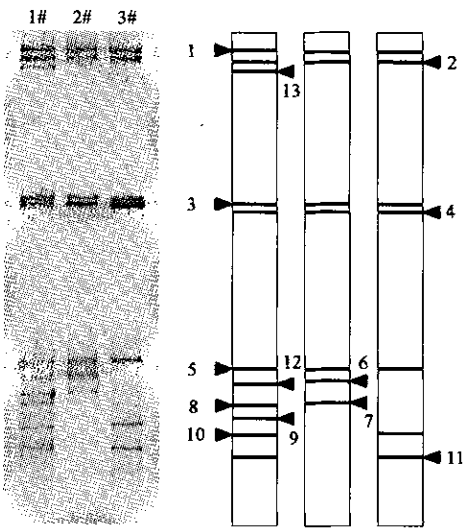

Fig.2 DGGE profiles of 16S rDNA fragments obtained from three reactors

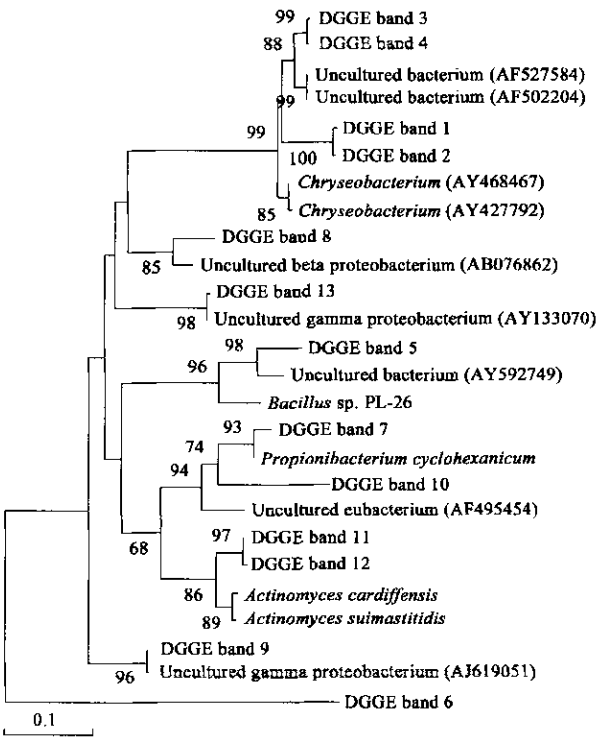

Fig.3 Phylogenetic tree of 16S rDNA excised from DGGE profiles. Evolutionary distance and parsimonious analyses were carried out in MEGA 3.0. The scale indicates 0.1 nucleotide substitution per nucleotide position. The tree was rooted on midpoint

Department of Chemical Engineering, University of California at Berkeley in 2002 from a laboratory scale sequencing batch reactor carrying out enhanced biological phosphorus removal. DGGE bands 5 and 6 have high similarity to genus Bacillales. Bands 7, 10, 11 and 12 are related to the genus *Actinobacteridae*. Band 8 is closely related to genus β-*protebacteria*, whereas bands 9 and 13 are related to genus γ-*protebacteria*. The uncultured bacteria (bands 3 and 4: AF527584 and AF502204) could be detected in all three reactors and were proposed to be PAOs (they needs to be verified with more experiments), so could *Bacillales* (bands 5). However, genus of β-*protebacteria* and γ-*protebacteria* (corresponding to bands 8, 9 and 13) only existed in sewage-fed reactor. There was several uncultured bacteria found in this study: bands 3, 4, 5, 8, 9, 10 and 13, the locus codes are AF527584, AF502204, AY592749, AB076862, AJ619051, AF495454 and AY133070 respectively.

2.2.3 Microbial community biomass analysis

Analysis of the DGGE gel profiles was carried out by software Gel-Pro Analyzer (version 3.1, Media Cybernetics, USA) to get the information of microbial communities diversity and their relative amount (Fig.4). As shown in Fig. 4, three reactors had common dominant bacteria that were submitted to GenBank as PAOs and had almost the same biomass (bands 3 and 4, linear gradient of denaturant: 42%—46%). 1 # reactor supplied with sewage had the most dominant microbial communities. *Bacillales* (linear gradient of denaturant: 54%—58%) was more dominant in 2 # reactor supplied with glucose than the other two ones, while *Flavobacterium* (linear gradient of denaturant: 30%—46%) was the most dominant in 3 # reactor supplied with sodium acetate as carbon source.

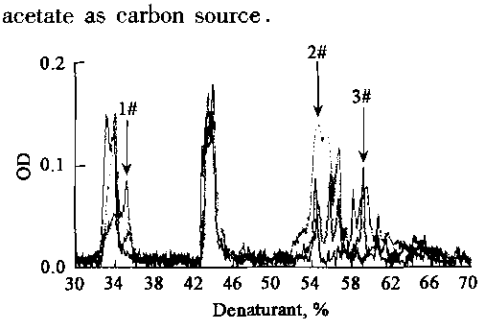

Fig.4 Comparison of different possible microbial biomass from three reactors

3 Conclusions

The diversity of bacteria in three SBRs supplied with different carbon sources was determined by PCR-DGGE, and

the identification was performed by sequence analysis. DGGE patterns of DNA extracted from three reactors sludge showed that the diversities of the bacterial communities in phosphorus removal systems fed on different carbon sources were different. The diversity of bacterial in sewage-fed reactor(1 #) was much higher than that in glucose-fed one(2 #) and sodium acetate-fed one(3 #). Several bands corresponding to uncultured bacteria were present in the DGGE profile(bands 3, 4, 5, 8, 9, 10 and 13). Among them, bands 3 and 4 have high similarity to PAOs submitted to GenBank, and existed in all three reactors dominantly. The determination of phylogenetic affiliation demonstrated that *Flavobacterium* (bands 1, 2, 3 and 4), *Bacillales* (bands 5) were found in all three reactors, while β-*protebacteria*, *Actinomyces* sp. and γ-*protebacteria* (corresponding to bands 8, 9, 12 and 13) only existed in sewage-fed reactor.

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