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Original Research Paper

Analysis of Microbial Community in the Anaerobic Phosphorus Sludge Using Molecular Techniques

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ABSTRACT

Through anaerobic culture test, from the six different anaerobic phosphorus removal sludge, it was found that Anaerobic Sequencing Batch Reactor (ASBR) sludge is the most appropriate sludge source to remove phosphorus in the liquid medium, followed by Expanded Granular Sludge Bed (EGSB) sludge and chicken manure. Microbial community structures in the six different sludge sources were investigated by 16S Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) method when the anaerobic systems operated steadily. The DGGE fingerprints were analysed by the software Quantity One to obtain the information of the microbial species in the six different sludge sources, which showed that there was a high diversity in the bacterial communities, and the richness value of ASBR sludge was 0.59 whose number occupied more than half of the total bands, while The richness value reached highest (0.61) when it was chicken manure. The community similarity between ASBR sludge and EGSB sludge is the highest 71.7. In the bands strength schematic diagram, the No. 1, No. 8 and No. 18 band that existed in ASBR sludge, EGSB sludge and chicken manure might represent the colony related to the anaerobic phosphorus microbe.

INTRODUCTION

Anaerobic biological phosphorus removal technique used in wastewater treatment that turns phosphide into phosphine gas is a new development direction, which has many advantages as low energy consumption, less excess sludge, recycled phosphorus, etc. At present, towards the study on biological reduction of phosphate, domestic and foreign scholars focus on the distribution and sources of phosphine gas, with few reports on the application in wastewater treatment. The objective of this study is to choose the most appropriate seed sludge through investigating the laws of total phosphorus (TP) removal in sewage with six different sludge sources.

In addition, on the study of microbial community structures, under some conditions, bacteria can enter a viable but non-culturable (VBNC) state and are therefore not enumerated by traditional culturing methods (Rompre 2002). Traditional microscope and isolated culture have some limitations as too many steps, heavy workload, hard to simulate the real condition of the microbial growth, cannot reflect the real situation of microbial environment, etc. These years, methods in molecular microbiology have become a valid support to traditional techniques (Ercolini 2004). Molecular techniques provide specificity, rapid detection, and the detection is independent of culturability of the bacteria (Lleo 2005). In this study, 16S ribosome DNA molecular biology techniques was applied to study the microbial community structures during the stable operation of the six anaerobic systems by the method of DGGE of touchdown PCR (TD PCR) amplified 16S rDNA gene fragments.

MATERIALS AND METHODS

Tested materials: There are six different sludge sources to be tested, i.e., swine manure (Conghua Pig Factory in Guangzhou), chicken manure (Beiting Food Market in Guangzhou Higher Education Mega Centre), EGSB sludge, ASBR sludge, the simultaneous nitrogen and phosphate removal sludge, and the secondary clarification sludge (Lijiao Sewage Plant in Guangzhou). The anaerobic reactor appliance is showed in Fig. 1.

The culture medium was composed of: Glucose: 500 mg/L, Sodium acetate: 500 mg/L, $MgSO_4 \cdot 7H_2O$: 200 mg/L, $CaCl_2$: 75 mg/L, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$: 40mg/L, KNO_3: 500mg/L, NH_4Cl: 500mg/L, K_2HPO_4: 500mg/L, Peptone: 500mg/L, Yeast extract: 250mg/L.

Content of water quality of the culture medium was: COD: 1200 mg/L, NH_4^+-N: 300 mg/L, NO_3^--N: 20 mg/L, TN: 320 mg/L, TP: 120 mg/L, pH: 7.0 ~7.5, Water temperature: 35°C

Experimental method: Swine manure and chicken manure were sieved by wire netting (mesh of the net: 0.4cm × 0.4cm) to eliminate the bigger particles while the other sludge did not need to be sieved. 600 mL high concentration of sludge

with graduated cylinder after 1h free sedimentation in 2500 mL conical-flask was taken in which 1800 mL culture medium was added and then with a glass rod the materials were mixed in the conical-flask well. After leaving it for a while, the culture medium samples were taken to determine the initial total phosphorus (TP) concentration, and then nitrogen gas was filled into the conical-flasks for 5 minutes to remove the oxygen. Plastic film and rubber band were used to seal the opening, and the plastic film was pricked to release the gas produced in the microbial metabolism. Finally, double layers of black plastic were used to cover the whole conical-flask to get rid of sunlight. The sludges were cultured at a constant temperature of 35°C, and at the end of a six-day period culture medium samples were taken to determine TP concentration, removed oxygen and covered the opening (as stated). It took ten periods for sludge culturing-and-domesticating. As the sludges were successfully domesticated, they were put into the appliance as shown in Fig. 1, and then went on for five periods (a period had six days), and culture medium samples were taken to determine TP concentration.

The measured index and the method used in the experiment were: TP concentration - molybdenum-antimony anti-spectrophotometric method; Temperature/pH - WTW measuring apparatus and the probe.

The combination of PCR amplification of 16S rDNA genes with denaturing gradient gel electrophoresis (DGGE) analysis was used to reveal the structures of bacterial communities in the sludge (Hesham 2011) which was successfully cultured and had gone into a stable running period. The DGGE fingerprints were analysed by the software Quantity One to gain the information of diversity and similarity of the sludge samples.

Recent development of several commercial DNA extraction kits for environmental samples, which simplify the extraction process and generate PCR quality DNA, have been adopted by most researchers (Lebuhn 2003, Lebuhn 2004, Rose 2003). In the study, total genomic DNA was isolated from sludge samples using 3S DNA Isolation Kit V2.2 for environmental samples (Shanghai, Shenergy Biocolor Co.), according to the manufacturer's instructions. The extracted DNA was electrophoresed on 1% agarose gel and photographed on a UV transillumination table to check the extraction efficiency.

The bacterial general primers were used to amplify the sludge samples by adding 1μL extracted DNA, 1μL Primer One, 1μL Primer Two, 22 μL ddH_2O to 25μL 2×Taq Master Mix which included Taq DNA Polymerase, dNTPs, $MgCl_2$, buffer solution, reaction enhancer, optimizer and stabilizer. The primers produced by Shanghai Biological Engineering

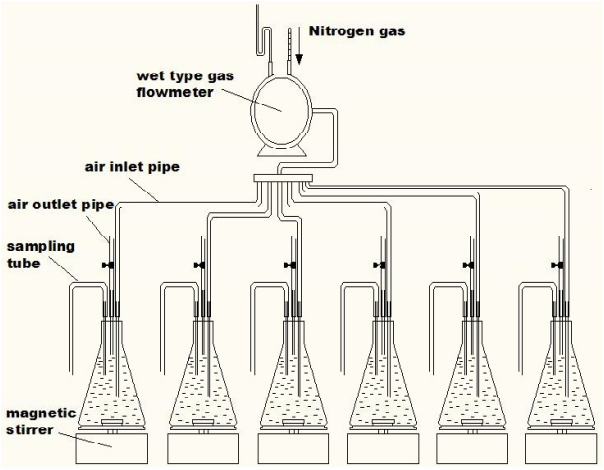


Fig. 1 The experimental appliance

Technology Service Company were:

5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCAGCAG-3',

5'-ATTACCGCGGCTGCTGG-3'

A touchdown technique was used for DNA amplification to increase specificity and sensibility of PCR: 94°C 7min (initial denaturation) → 94°C 40s (denaturation) → 65°C 40s (annealing) → 72°C 40s (extension, back to step 2, went on 21 cycles, there is a 0.5°C decrease of the annealing temperature in each cycle) → 94°C 40s → 65°C 40s → 72°C 1.5min (back to step 5, went on 10 cycles) → 72°C 10min → 12°C 20min → 4°C stop.

After PCR amplification, PCR product was electrophoresed on 1% agarose gel, and then photographed on a UV transillumination table to checked with ethidium bromide staining.

DGGE was performed with the Dcode System (USA, Bio-Rad Co.) with the following steps:

- Dip the two glass plates in the washing liquor made by sulphuric acid and potassium dichromate for more than 24h until the plates got clean, and then wash them by ddH_2O and dry at 60°C.
- Fix the glass plates onto the device following the manufacturer's instruction.
- With ice operation, make up 15mL 40% gel (8% acrylamide, 16% methylenebia-crylamide, 2.8M urea and 1×TAE) and 15mL 70% gel (8% acrylamide, 28% methylen-ebiacrylamide, 4.9M urea and 1×TAE) and stored them on ice.
- Add 140μL 10% ammonium persulfate and 10μL TEMED in the gel as stated, and then mix them up quickly.

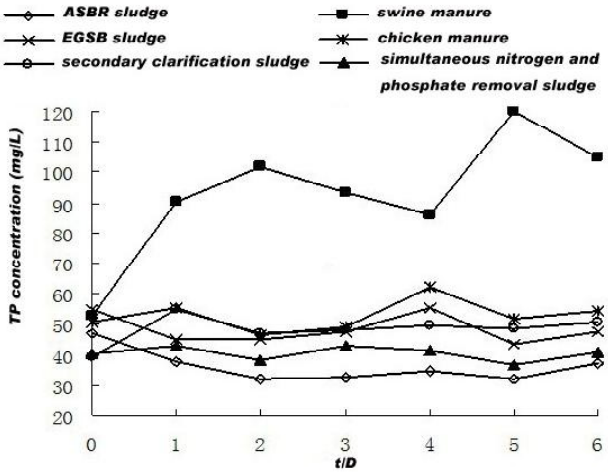
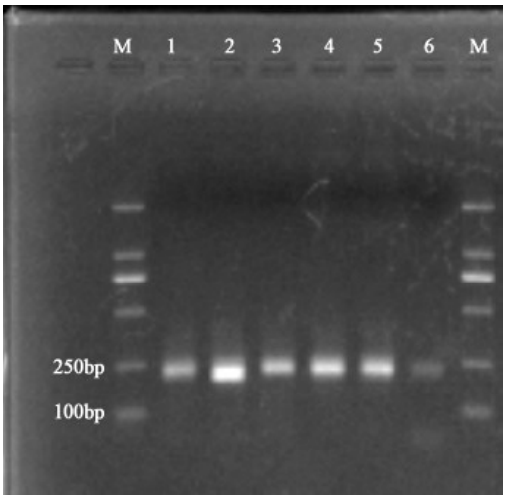


Fig. 2: TP concentration in the culture mediums of the six sludge sources.

- Take out the -20°C pre-cooling injectors to inhale 14.5mL of the gel of different concentration made in step (4) quickly, connect the rubber tube and the Y shape adapter and fasten the injectors onto the gradient hybrid device.
- Turn the wheel on the gradient hybrid device clockwise slowly at a constant speed to make sure there were no bubbles in the gel and then insert comb to make 1mm gel holes and polymerize the gradient gel in light for an hour at least.
- With the gradient gel set, pull out the comb and wash the gel holes with 1×TAE buffer solution.
- Take out the washed electrophoresis core onto which fasten the glass plates with gradient gel, and then put the whole device into the electrophoresis system in which there were 7L 1×TAE buffer solution (pH 7.4).
- Turn the switch on, start the pump and set the temperature 60°C, load 40μL samples (PCR product sample: loading buffer = 1:1) when it reached 60°C, and then close the sample cover, electrophoresis on 75V for 14 hours.
- After the gel cooled naturally, peel the gel from the plates which was then cleaned by ddH_2O three times and dyed for 10~20 minutes by Gelred (use ddH_2O to dilute 10000 × Gelred to 3300 times into 0.1M NaCl to produce the 3 × staining solution), and then wash the gel three times by tap water and three times by ddH_2O, photograph on a UV transillumination table to get the DGGE fingerprints.

RESULTS AND DISCUSSION

Results of sludge choice test: The effect of different sludge sources on the total phosphorus (TP) concentration in the culture mediums during the stable running period is shown



M: DL-2000 marker 1: ASBR sludge, 2: swine manure, 3: simultaneous nitrogen and phosphate removal sludge, 4: EGSB sludge, 5: chicken manure, 6: the secondary clarification sludge

Fig. 3: PCR products pattern of different sludge source samples.

in Fig. 2, which revealed that TP removal efficiency arrived maximum with EGSB sludge and ASBR sludge, followed by chicken manure, while the other sludge had inconspicuous removal efficiency of which the system running were unstable, either. Among them, the TP removal efficiency was zero with the swine manure of which the TP concentration had even been higher than that of original culture medium, for the reason that the phosphorus content in swine manure was high (about 21g/kg) which would release into the culture medium with time. Chicken manure had less phosphorus content than that of swine manure (about 17g/kg), but could also release phosphorus into the culture medium which led to the unstable phosphorus content in the culture medium even though the experiment showed the large production of pH_3. It is shown in Fig. 2 that the TP removal efficiency of the secondary clarification sludge and the simultaneous nitrogen and phosphate removal sludge was not good after anaerobic domestication. The TP removal amount of EGSB sludge and ASBR sludge could both reach more than 20mg/L and stable, which showed that they could be used to be the anaerobic seed sludge to remove phosphorus in wastewater. While the anaerobic granular sludge of EGSB sludge was not formed well to reach the normal running situation, bacteria in the sludge had not been fully developed to be used as the anaerobic seed sludge. Since ASBR sludge reactor was in stable running to have a full growth of bacteria, it was the best choice to be anaerobic phosphorus removal seed sludge.

Results of study on microbial community: DNA length was about 23 kb, agarose gel electrophoresis pattern of PCR products is obtained in Fig. 3. The length of PCR products

Table 1: Richness value (Rs) of bacterial community in different sludge source samples.

Lane	1	2	3	4	5	6
Number of bands	24	18	21	23	25	28
Rs	0.59	0.44	0.51	0.56	0.61	0.68

Table 2: Dice index of bacterial population in different samples with ASBR samples.

Lane	1	2	3	4	5	6
1	100	47.4	58.0	71.7	55.1	53.8

was about 250bp and the band is clear which meant that there were no non-specificity in the PCR products and the products could be used in DGGE, on the other hand, the method for DNA extraction and PCR was appropriate for the research.

The species of microbial community get more with the number of bands increases, while the quantity of bacteria get larger with the intensity of the bands become higher. Consequently, the relationship between species and quantity of bacteria will be determined in different kinds of anaerobic culture medium system to gain the information of microbial diversity (Cui 2009).

DGGE fingerprints were automatically scored by the presence or absence of co-migrating bands, independent of intensity (Liu 2007). As shown in DGGE fingerprints (Fig. 4a), the microbial community was very rich, while the bands strength schematic diagram (Fig. 4b) which was obtained by Quantity One with lane 1 (ASBR sludge) as a standard had detected 41 different bands.

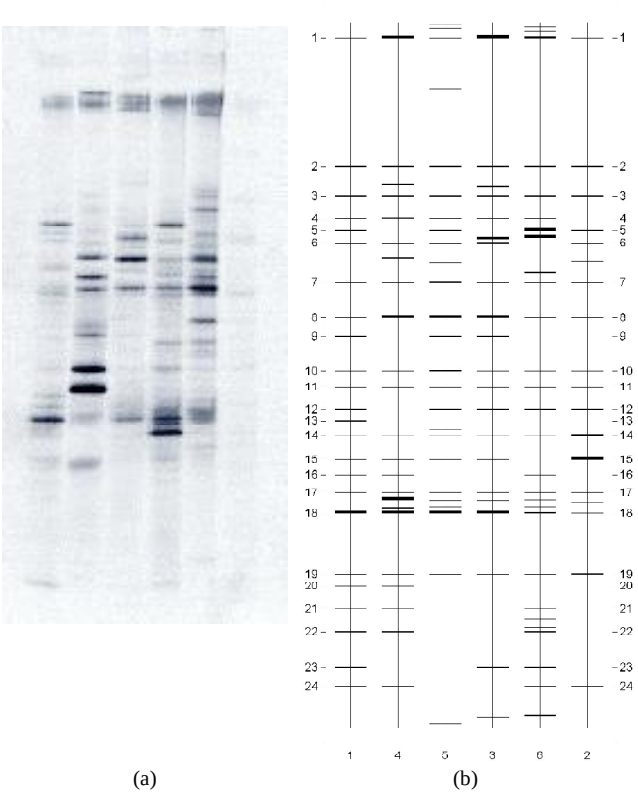
According to

$$Rsi = Li/L_T \quad ...(1)$$

Rsi - Richness value of lane i, Li-the number of bands of lane I; L_T - the number of all bands in the diagram.

The richness values of different sludge sources were determined in Table 1. The richness value of ASBR sludge was 0.59 whose number occupied more than half of the total. The swine manure had less species of microbial than others whose Rs was only 0.44. The Rs reached highest (0.68) when it was the secondary-clarification-sludge which verified that there were abundant microbes in it.

From the bands strength schematic diagram (Fig. 4b), not only the Rs of swine manure was lowest but the intensity of it was low, which indicated that the swine manure was unable to be cultivated to remove phosphorus in anaerobic environment. The quantity of bands of the ASBR sludge whose efficiency to remove phosphorus was the highest, but was not high with only one dominant band (No.18 band),



(a) (b)

1: ASBR sludge, 2: Swine manure, 3: The simultaneous nitrogen and phosphate removal sludge, 4: EGSB sludge, 5: Chicken manure, 6: The secondary-clarification-sludge

Fig. 4: DGGE fingerprints of the six sludge sources (a) and the bands strength schematic diagram (b).

may be the reason that the primary bacterial colony to remove phosphorus that the No.8 band represented was the one and only dominant colony in the sludge without any other competitors or antagonism. Moreover, a law existed in the Fig. 4b that the intensity of No.8 band decreased left to right which coincided with the phosphorus removal law. It can be concluded that the bacterial colony No. 8 band represented was very likely the one to remove phosphorus, the quantity and growth of which was the key factor to differentiate the removal efficiency.

Dice index of bacterial population in different samples with ASBR samples was quantified (Table 2) according to the Dice index (Cs) as:

$$Cs = 2j/(a+b) \quad ...(2)$$

Where, j - the number of bands common to samples A and B, a,b - the number of bands in samples A and B, respectively (Dice 1945).

It was found that the community similarity between ASBR sludge and EGSB sludge is the highest 71.7. Since EGSB sludge is the second better sludge to remove

phosphorus, the dominant colonies in it might be helpful for phosphorus removal. No. 1 and No. 8 band existed both in ASBR and EGSB sludge, which meant that the colony No. 1 represented might be related to the anaerobic phosphorus removal bacteria or the one could also remove phosphorus. No. 8 band existed in the six different sludge sources, and the intensity of it was high in chicken manure and EGSB sludge; it might also be related to the phosphorus removal. The community similarity between swine manure and ASBR sludge was the lowest 47.4; the No. 1, No. 8 and No. 18 band were all dim in swine manure whose phosphorus removal efficiency was not good, obviously, the No. 15 band had nothing to do with phosphorus removal.

CONCLUSIONS

1. It was found that ASBR anaerobic sludge and EGSB sludge were appropriate for anaerobic phosphorus removal by comparing the phosphorus concentration in culture mediums of the six different anaerobic sludge sources, and SABR sludge ran better and stably to be the seeding sludge for the experiment of anaerobic phosphorus removal. Chicken manure had certain effect to remove phosphorus but the system is not stable.
2. The DGGE fingerprints of the sludge analysed by Quantity One showed that there were abundant species in the six sludge sources. The community similarities were not so high, while ASBR sludge and EGSB sludge shared the most bands in the fingerprints, and the community similarity between them was the highest. The No. 1, No. 8 and No. 18 band that existed in ASBR sludge, EGSB sludge and chicken manure might represent the colony related to the anaerobic phosphorus microbe.

Since the sludge source to remove phosphorus in anaerobic environment was determined, the further research on anaerobic phosphorus removal could carry on in future. And the species the No. 1, No. 8 and No. 18 band represented should be sequenced and study the growth law of them during the periods by using real-time PCR in future. The microworld in the activated sludge would gradually emerge to our eyes in the future.

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