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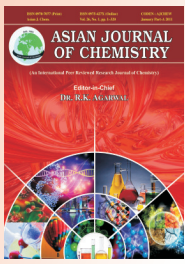

Microbial Community Analysis in Two-Phase Anaerobic Reactor by PCR-Temperature Gradient Gel Electrophoresis†

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The seed sludge and the anaerobic sludge for a two-phase anaerobic process that is used for the treatment of N-phenylglycinonitrile wastewater were analyzed by PCR-TGGE technology. Furthermore, the structural differences of the seed sludge and the anaerobic sludge were investigated and compared. Moreover, the Shannon diversity index, an important parameter for studying microbial community diversity, was calculated for each sample. The results showed that the sludge's microbial community structure was significantly different before and after domestication. The microbial species and structure in the different stages of a two-phase anaerobic reactor were also obviously different. The acidogenic sludge's diversity index was the highest. Finally, the sludge diversity index of the secondary methanogenic reactor was higher than that of the primary methanogenic reactor and its degradation efficiency of pollutants was also higher.

Keywords: PCR-TGGE, Two-phase anaerobic, Sludge, Microbial community.

INTRODUCTION

Microbial ecology is the ecology of microorganisms. Their relationship with one another and with their surrounding environment. However, the field primarily focuses on microbial community. The ability of a biochemical treatment of wastewater system to effectively degrade pollutants is based on the structural rationality of various engineering facilities as well as based on the microbial populations' variety and functionality. Therefore, studying microbial community structure and diversity contributes greatly to the understanding of microbial communities' degradation mechanisms and for improving wastewater and to improve the efficiency of wastewater treatment efficiency.

Temperature gradient gel electrophoresis (TGGE) is a DNA fingerprint technology. The underlying principle of TGGE is that double-stranded DNA of varying GC content require differing melting temperatures (Tm). Once the double-stranded DNA has been melted, its electrophoretic velocity in gel electrophoresis will sharply decrease. Therefore, DNA fragments of equal length are obtained by PCR amplification and then added to a gel that has a set temperature gradient and then DNA fragments of different sequences will be denatured by their respective corresponding Tm. Thus, their spatial configuration will change, leading to a sharp decline in their electrophoretic velocity and then, they remain in their corresponding positions at different Tm's. After dyeing, the separate bands in the gel can be observed[1]. The number of different bands represents the microbial community abundance and their colour depth represents the relative composition and structure of the different dominant communities. Thus, microbial community comparisons can be learned from the TGGE bands distribution through the macro level. Furthermore, 16S rDNA commonly exists in prokaryotic microorganisms, it consists of both a relatively conserved region and a variable region and its size is suitable for electrophoresis analysis. Therefore, a particular microbial community's 16S rDNA sequence can be separated by the TGGE method and thus, it has become a popular means of studying prokaryotic microbial communities[2-4]. Compared to other techniques, the TGGE method is highly advantageous. It has a high mutation detection rate, small sample volume, good repeatability and efficient operation[5]. Therefore, researchers have focused on the development and application of this method.

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In the study, the seed sludge and the anaerobic sludge for a two-phase anaerobic process that is used for the treatment of N-phenylglycinonitrile wastewater were analyzed by PCR-TGGE technology. The test is designed to study the multiplicity of microbe groups and community structure variation and to provide a theoretical molecular biology basis for the further optimization of a two-phase anaerobic process for similar wastewater.

EXPERIMENTAL

Experimental set-up: The entire system consists of a four grid ABR baffled acidification reactor, a primary UASB methanogenic reactor with a reflux device and a secondary UASB methanogenic reactor with a reflux device in series (Fig. 1)[6].

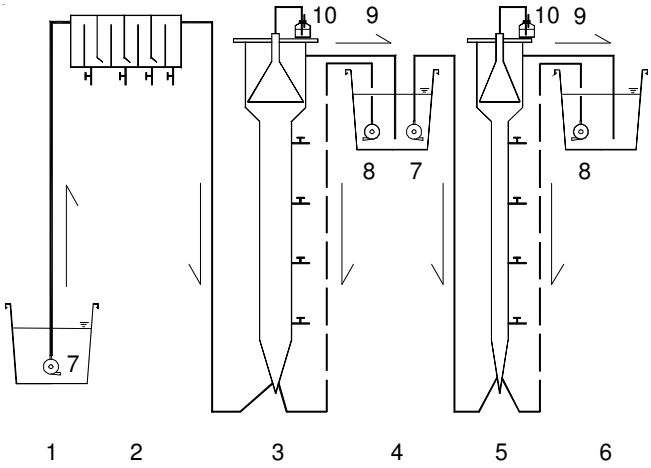

Fig. 1. Progress flow diagram. (1. The inflow tank; 2. ABR acidification reactor; 3. Primary methanogenic reactor; 4. Primary reflux tank; 5. Secondary methanogenic reactor; 6. Secondary reflux tank; 7. Inflow submersible pump; 8. Reflow submersible pump; 9. Outlet pipe; 10. Gas collecting device)

A DNA purification kit and an SK1131PCR product purification kit were obtained from Sangon Biotech (Shanghai) Co., Ltd.

A PCR reaction system agent and an ordinary rTaq enzyme suit were obtained from the TaKaRa Company and contained a 10x PCR buffer, a $MgCl_2$ solution, 2.5 mmol/L dNTP, a 6x loading buffer and a 50 μ rTaq DNA polymerase. Finally, TGGE membranes (Maxi) were obtained from the German Biometra Company. A high speed refrigerated centrifuge (Model 236HK), made by the American HERMLE Company. An automatic PCR amplification (Model TProfessional 196), made by the German Biometra Company. A gel imaging system (UVP) (Model Bio SpectrumAC+Gel Camera), made by the American UVP Company and a temperature gradient gel electrophoresis (TGGE) (Model Maxi TGGE), made by the German Biometra Company.

Sludge sampling: Two-phase anaerobic reactors were seeded with activated sludge taken from the facultative tank of a coking wastewater processing station. Additionally, the seed sludge had a slight coal tar odor and was a dark black colour. Sludge was inoculated into the three reactors in a proportion of 50, 60 and 60 %, respectively. The active sludge was domesticated for 62 days before the successful start-up of the reactors. After the load operation, the system retained a stable removal efficiency because the microbial community had adapted to the water quality changes. While the influent COD was in the 5000-7000 mg/L range and the COD removal efficiency was higher than 50 %, the sludge was taken out of the two-phase anaerobic reactors to serve as the samples. For COD analysis, the HACH method was applied and the COD value was measured every two days.

The sludge from each reactor unit and the seed sludge from the facultative tank of a coking wastewater processing station served as the experiment's samples. The sludge samples are numbered as follows:

Sample No. 1 was collected from the facultative tank of a coking wastewater processing station from the Chongqing Iron & Steel Company.

Sample No. 2 to No.5 were collected from the four grid ABR baffled acidification reactors, respectively.

Sample No. 6 was collected from the primary UASB methanogenic reactor.

Sample No. 7 was collected from the secondary UASB methanogenic reactor.

DNA of the sludge samples extraction: A sample was collected from the anaerobic sludge and the precipitation was collected *via* centrifugation. This was repeated twice. Using a clean centrifugal tube (7 mL), a 1 mL sample was taken in sterile water was added to the tube and it was then centrifuged at 12,000 r/min for 10 min. After centrifugation, the supernatant was discarded. Then, 2 mL buffer and 40 μL protease were added and mixed and the tube was immersed in a constant 37 ºC temperature bath and shaken horizontally for 0.5 h. Next, 2 mL 5 % SDS was added to the tube; it was then heated in a 65 ºC water bath for 90 min. It was centrifuged at 8000 rpm for 10 min again and the supernatant was collected in another sterilized centrifuge tube for storage at -20 ºC. 1.5 mL supernatant was collected in another new sterilized centrifugal tube and 1.5 mL phenol-chloroform was added to it. The tube was then centrifuged at 9000 rad/min until the white matter dissolved into the solution. The supernatant was collected and equal amounts of chloroform were added and then the tube was centrifuged at 9000 rad/min for 10 min. The supernatant was wastewater carefully added to a new sterile centrifugal tube and 2 mL 95 % ethanol was added. The tube was mildly oscillated and then frozen at -20 ºC for 40 min. When the temperature maintained 4 ºC, the tube was centrifuged at 1000 rad/min for 5 min and the supernatant was discarded. Next, 70 % ethanol was added to the tube, then it was centrifuged at 1000 rad/min for 5 min and the supernatant was discarded. The precipitation was carefully blow-dried. Then, the precipitation was dissolved in 100 μL 1xTE buffer or sterile ultrapure water and transferred to a PCR tube for storage at -20 ºC until use[7,8].

16S rDNA V3 district amplification: DNA samples were purified using a DNA purification kit and the samples' purities and concentrations were tested by routine agarose gel electrophoresis. If the results met expectations, the 16S rDNA V3 District was amplified. Otherwise, the DNA extraction was performed again. At this time, the DNA length was between 1 and 2 Kpb.

The universal primer for the 16S rDNA V3 District in most bacteria was used. The primers were F341-GC and R518. After pilot-scale experiments, the PCR reaction system was confirmed as the following:

2.5 μL 10xPCR buffer	25 μL in total
1.5 μL MgCl2	
2.0 μL 2.5 mmol/L dNTP	
1.2 μL Upsteam primer (F341-GC) 25 μL in total	
1.2 μL Downstream primer (R518)	
3.0 μL template	
0.6 μL rTaq polymerase	

The reaction conditions are as follows[9,10]: pre-deformation at 95 ºC for 5 min, deformation at 94 ºC for 1 min, then reaction at 56 ºC for 1 min, further annealing at 72 ºC for 1.5 min and followed by extension at 72 ºC for 10 min. Additionally, every cycle of the 35 total cycles decreases the annealing temperature by 0.1 ºC until 15 ºC and finally heating preservation at 15 ºC for 1 h.

Finally, gel electrophoresis was used for extraction detection. At this time, the DNA length was about 300 pb.

Temperature gradient gel electrophoresis: The TGGE program was divided into three steps: (1) Electrophoresis was run at 300 V for 10min. The determined temperature was set between 20-30 ºC. (2) Electrophoresis was run at 0 V for 10 min. The determined temperature was set between 42-60 ºC. (3) Electrophoresis was conducted at 155V for 18 h. The determined temperature was set between 42-60 ºC.

The TGGE membrane was fixed with 10 % glacial acetic acid after the electrophoresis. It was then silver stained for 25 min and was finally visualized with a developer for 12-15 min.

Microbial community structure analysis: The TGGE profile was analyzed using the Quantity One V4.5 software made by the Bio-rad Company (USA) and from which, the band lane image was obtained. The band structure distribution of the anaerobic sludge was compared with that of the seed sludge, which indicates that the activated sludge acclimation greatly influenced the microbial community structure[11,12]. A comparison between different anaerobic sludge structures indicated that the structural difference also occurred in the same seed sludge that was domesticated by different unit reactors. Bands' similarity coefficient matrix obtained from the Quantity One software was stored in the ecological statistical software PC-ORD. Using the NMD technique, computer-simulated 2D graphics were used to study the microbial community structure. Meanwhile, the similarity of a particular microbial population could be speculated and illustrated[13].

Microbial community diversity analysis: Each band's amount represents the microbial quantity of the different sludge samples. According to the band quantity, a sample's Shannon diversity index (S) can be calculated. This index(S) was used to evaluate microbial community diversity. For example, the higher the S value is, the richer the microbial population. The diversity index consists of two main factors: (1) a sample's species quantity or diversity; (2) the species distribution or species evenness. The Shannon diversity index formula can be written as[14]:

$$S = -\sum_{i=1}^{N} p_i \ln p_i \quad (1)$$

where S is the Shannon diversity index; p_i is the proportion of the ith microbial quantity and i is the total microbial quantity in the samples.

RESULTS AND DISCUSSION

Reactor performance: From day 80 to 98, the influent COD concentration was gradually increased from 5199 to 7058 mg/L. Furthermore, the total COD removal efficiency in a two-phase system was maintained at (46.57 ± 1.54) %. Among the system's various components, the COD removal efficiency in the acidification reactor was (10.26 ± 1.78) %, in the primary methanogenic reactor it was (16.15 ± 1.16) % and in the secondary methanogenic reactor it was (20.16 ± 1.29) %. The denitrification process took place in the acidification reactor; however, methanogenesis did not occur, so the effluent acidification rate was 35 %. In the secondary methanogenic reactor, insignificant amounts of methane were produced.

16S rDNA V3 district amplification: The PCR amplification products were examined by agarose gel electrophoresis. The DL2000 DNA Ladder made by TaKaRa was composed of six bands, which are 2000, 1500, 1000, 500, 250 and 100 bp from the top to bottom. The electrophoresis results sow that each sample has a clear, specific target band between 190 and 230 bp. The amplification products were a mixture of different bacterial 16S rDNA V3 fragments. Although, since mixed templates (total genomic DNA) were used in the PCR reaction and the sequence similarities among these templates were very high, the 16S rDNA amplification resulting from the total genomic DNA template was, consequently, very error-prone and generated some "16S rDNA" sequences that would not have originally existed in the environment. These "16S rDNA" sequences were referred to as artifact in literatures[15]. Because of this error, the environmental microbial diversity will consequently be overestimated, leading to wrong conclusions. This influence can be eliminated through a method known as "Reconditioning PCR"[16] and furthermore, the primers to templates ratio should maintain a high level in the amplification process. This would ensure that annealing between primers and templates is superior to that between different templates, therefore reducing errors.

Comparative study on samples bands lane identification figure: The lanes were slightly distorted, but they can be modified by analysis software, which will not have a significant impact on the results.

The TGGE band patterns can be identified by the trace tracking method in the Quantity One software. According to the bands' amount and their relative degree of lightness or darkness among the different samples, a bands lane identification image for each sample was identified and automatically generated by the software.

The acidification reactor and the methanogenic reactor were seeded with identical sludge. Compared to the original seed sludge, the dominant microbial community in the two-phase system dramatically changed in various environmental and functional conditions.

As shown in Fig. 4, the 11th, 16th and 26th seed sludge bands were the dominant microbial communities. However, the original dominant bands had been converted into ordinary

bands in the acidification reactor. Only the 11th band in the third and fourth grid was still a dominant microbial community. This could possibly be because most of the microbial communities in the original seed sludge were eliminated by the acclimation condition because they did not adapt to early acid-forming conditions. However, after the initial hydrolysis process and entering the acid-forming stage, a small portion of the microbial communities in the original seed sludge survived and became the dominant microbial communities.

Compared to the microbial communities in the unique seed sludge, the microbial communities in the methanogenic reactor varied remarkably; namely, the original dominant bacteria had disappeared or converted to ordinary communities. Methanogenesis is a strict anaerobic process, whereas the seed sludge was from a facultative tank. Two different sludges' living conditions are quite different; thus, the screening and elimination were obvious during domestication, when then generated a significantly different community structure in the two different sludges.

According to the Dice Coefficient (Cs) and compared to the original seed sludge, the similarity coefficients of acidification reactors #2, #3, #4 and #5 were 48.0, 48.2, 59.7 and 56.8 %, respectively; the similarity coefficients of primary and secondary methanogenic reactors were 50.6 and 50.2 %, respectively. Seed sludge was domesticated by different functional reactors and its group structure significantly changed. Meanwhile, surviving in the differing environments of the acidification reactor and the methanogenic reactor made the microorganism' structural differences apparent.

In the acidification reactor' first and second grids, the microorganisms' structual differences were apparent and the similarity coefficient between the two grids was only 45.2 %. The results are shown in Table-1. From Fig. 4, it can be seen that the main bands in the two lanes did not remain in the same place. This suggests that the wastewater may have negatively impacted the microorganism, which had a significant screening effect on the microbe in the plug flow process. The microbe in the first grid was impacted the worst by the shock loading, whereas the microbe in the second grid suffered less. Then, the microbial activity of the third and fourth grids was gradually restored. The similarity coefficient between the second and third grid was 63.3 % and between the third and fourth grid, it was 73.4 %. Additionally, from Fig. 4, it can be seen that the dominant bands in lane four and five were in the same position and other bands were also in a similar position.

TABLE-1
SIMILARITY COEFFICIENTS

Lane	1	2	3	4	5	6
1	100	–	–	–	–	–
2	48.0	100	–	–	–	–
3	48.2	45.2	100	–	–	–
4	59.7	60.2	63.3	100	–	–
5	56.8	53.3	60.2	73.4	100	–
6	50.6	59.8	57.3	58.1	62.6	100
7	50.2	57.1	55.9	61.1	57.3	54.1

Some dominant microbial communities in the methanogenic reactors' two levels stayed in the same location, demonstrating that the two levels had the same type of microbial communities present. The 7th, 10th, 12th and 24th bands in the primary methanogenic reactor were the main bands. In the secondary methanogenic reactor, only the 10th band and the bands between the 9th and 10th were the main bands. Compared to the primary methanogenic reactor, the secondary methanogenic reactor's microbial environment became more suitable for survival for microbes that are at a fermented terminal stage. However, in the primary methanogenic reactor, there was a greater abundance of substrates than in the secondary methanogenic reactor, but it simultaneously had some non-degradable substances that were very toxic to the microbes. After substrate consumption, the number of microbial communities that lived on these substrates gradually reduced. Therefore, they were the non-dominant microbial communities in the secondary methanogenic reactor.

Two peaks can be seen at point 0.08 and point 0.18 for the No. 6 sample (lane 6), whereas the peaks for the No. 7 sample (lane 7) migrated backwards at the corresponding place. Furthermore, the peak values for lane 7 were lower than that for lane 6. The peak at point 0.88 for lane 6 coincided with the same peak for lane 7, but the former value was still higher than the latter. The total peak quantity for lane 6 was small, but the total peak value was high. Conversely, there was a large total peak quantity lane 7, but its total peak value was low and the dominant microbial communities only focused on a few bands. The original dominant floras in the primary methanogenic reactor were transformed into normal microbial communities, which agrees well with the previous conclusions.

Microbial community structural changes analyzed by NMDS: The samples' distance represents the similarity among samples. The NMDS analysis chart shows that there was no overlap among the samples, which means that not all the samples were exactly the same. The samples were grouped into three classes: lane 4 was similar to lane 5; lane 1 and lane 6 were similar to lane 7; and lane 2 and lane 3 were of poor similarity because they were at a greater distance. From the first dimension, Axis one from left to right may represent the primary sludge that was domesticated from the acidification reactor to the methanogenic reactor. The left and right sides of the first dimension show two different species and the difference between the acid-producing bacteria on the left and the methanogens on the right is obvious. The second dimension (Axis two) may represent the degree to which the microorganism tolerates shock loading during domestication. Because the lane 2 sample was in the acidification reactor's first grid, it had the greatest impact on the sample. Lane 4 and lane 5 were at the end of the hydrolysis acidification process; first, the wastewater was treated by the acidification reactor's first and second grids, followed by the acidification reactor's third and fourth grids and finally they were partially hydrolyzed into small molecules, which contributed to a more complete acid production. Wastewater treated by the hydrolysis acidification process entered into the methanogenic reactor and some inert or refractory materials still had a high impact on the methanogens; thus, the microbes still needed to adapt to their environments.

Microbial community diversity analyzed by Shannon index: The Shannon index reflects the species' richness in the whole community, which is an important parameter for

studying microbial community diversity. The bands quantity in samples can approximately represent the number of microbial species.

Table-2 shows that the variety of domesticated sludge significantly improved . In the early stage of the acid-producing process, the diversity consistently increased, but in the late stage, it declined slightly. Some analysts believe that different microflora exist in the hydrolysis process and the acidification process, along with differing substrates consumption and a change in living conditions, which then causes a change in the microbial community structure. The microbial diversity was similar at the methane-producing stage, but the biological abundance was higher in the secondary methanogenic process. It can be inferred that, the primary methanogenic reactor could provide a better environment for microbial growth. Therefore, the degradation efficiency of pollutants should be higher. The theoretical analysis results are in accordance with the reactor performance data.

TABLE-2
SHANNON INDEX

Lane	1	2	3	4	5	6
No. of bands	26	29	31	33	32	29
Index	0.372	0.394	0.407	0.419	0.413	0.394
Lane	1	2	3	4	5	6

Conclusion

Through regular agarose gel electrophoresis analysis, the length of extracted DNA fragments by PCR amplification was between 190bp and 230bp, illustrating that the PCR amplification was successful. Through similarity analysis of a band lane identification image, the similarity coefficient between the seed sludge and the sludge from the acidification reactor's four grids were 48.0, 48.2, 59.7 and 56.8 %, respectively. The similarity coefficient between the seed sludge and the sludge from the primary and the secondary methanogenic reactors were 50.6 and 50.2 %, respectively. The microbial population structures of the seed sludge and of the domesticated sludge had significant differences. The sludge structures in the first and second grid and in the third and fourth grid in the acidification reactor were obviously different. Additionally, the sludge structures in the primary and secondary methanogenic reactors were significantly different. Due to different reactor environments and functions, the sludge structure was significantly different after being domesticated. The domesticated sludge's diversity index revealed a tendency to increase and the 4th sample had the highest diversity index. The sludge diversity index of the primary methanogenic reactor was lower than that of 3rd and 4th acidogenic sludge samples. However, the secondary methanogenic reactor's sludge diversity index was higher than the former and its degradation efficiency of pollutants was also higher.

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