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Original scientific paper

DIVERSITY OF METHANE-PRODUCING MICROBIAL COMMUNITY STRUCTURE WITH 16S-V3 rDNA PCR-DGGE

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The method of 16S-V3 rDNA PCR-DGGE was utilized to study the diversity of methane-producing microbial community structure under different methane-producing microbial community structure. Results showed that community structure of the methanogens under different environmental condition was significantly different. The number of bands about community under extreme condition was less than normal conditions, but the main bands were obvious. The sequence analysis showed that the predominant strains mainly were uncultured bacteria that could not be identified in the level of species .Z2 was *Clostridia*, Z8 was *Clostridiales*, D5 was *Eubacteriaceae*, and D8 was *Flavobacteriaceae*. This study laid some extent foundation for future research about establishing a methane-producing bacterial system with high gas yield in winter.

Keywords: community structure, PCR-DGGE, uncultured bacteria

INTRODUCTION

As problems of energy shortage and environmental pollution to be more serious, human survival and development is under serious threat. In recent years, lots researching focus on the anaerobic fermentation of micro-organisms, especially methanogens (DIMITAR *et al.*, 2005; WIWAT *et al.*, 2005). The development of methane producing is an effective way to solve the problems of energy shortage and environmental pollution, sustainable development of agriculture. Environmental parameters, such as pH, volatile fatty acids, and methane gas production are key factors in the process of anaerobic fermentation, and methane yield is always used as a functional process parameter, ignoring to study the entire microbial community structure, especially the dynamic changes in the structure of process (MCMAHON *et al.*, 2001;

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GRIFFIN *et al.,* 1998). DEARMAN *et al.,* (2005) point out the relationship of methane gas yield is closely related with bacterial community structure, not *archaea*; and acetogenic bacteria, producing acetic acid as substrate of methanogens, is closely related with the growth of methanogens and the dynamic changes community structure. The methane-producing system is a complex ecosystem with a variety of bacteria and methanogenic *archaea*. As microbial community structure determines its ecological functions, the ultimate goal of methane-producing engineering and process improvement are to optimize the microbial population structure, and give full play to the ecological functions of the various groups, so that the efficient degradation and transformation of organic waste can be gotten. In winter, most methane-producing bacterial system under natural condition can not make the full degradation of the fermentation of raw materials, resulting in low efficiency of gas. Establishing low-temperature methane-producing bacteria system that can be inoculated into methane-producing reactor to be the dominant species of the winter is a good way to improve the efficiency of gas yield. Therefore, studying the microbial community structure is particularly important. As about 99% of micro-organisms in nature can not be isolated and cultured, so the conventional researching methods are can not get good results about the microbial community structure (AMANN *et al.,* 1995).

Bacterial 16S ribosomal DNA genes contain nine "hypervariable regions" (V1 – V9) that demonstrate considerable sequence diversity among different bacteria. Species-specific sequences within a given hyper variable region constitute useful targets for diagnostic assays and other scientific investigations. V3 were most suitable for distinguishing most of bacterial species to the genus level (SOUMITESH *et al* 2007).

Denaturing gradient gel electrophoresis (DGGE) has been used as a molecular tool for exploring microbial community structure, the mainly strains, and dynamic changes community structure that is a good way for us to study on the low-temperature methanogens. At the same time, studying the main bacterial population structure in the methane-producing process to control the microbial activity with key role effect is very important to improve gas yield under low-temperature condition in winter.

MATERIALS AND METHODS

Sample collection

Four samples were collected from one dual-chamber reactor invented by our laboratory; and the 5th sample was collected from the four-in-one reactor. The samples were placed in sterilized vials and stored at − 20°C until extraction. No.1 was sampled from the reactor in the acidification stage of the methane-producing process at 60ºC, pH 5.5, using pig manure as substrate. No 2 was sampled in the stage of gas production at 30 ºC, pH 7.5, and pig manure as substrate. Sample No 3 was collected in the step of gas production at 20 ºC, pH 7.5, and pig manure as substrate. No.4 was sampled in the start stage, room temperature, pH 7.5, and pig manure as substrate. Sample No.5 was obtained during the stage of gas production at room temperature, pH 7.5 using pig manure as substrate.

DNA extraction

Approximately 10 ml of leachate was drawn via the sample tap from methane-producing reactor. Leachate was placed in sterilized vials and stored at −20°C until extraction. For extraction, 1 ml of sample was taken and placed in a micro centrifuge tube and centrifuged at 13000 rpm for 5 min. The supernatant was poured off, and the procedure was repeated using

another 1ml sample, equivalent to a total of 2 ml volume. The pellet from the 2ml leachate sample was then re-suspended in 300µl of leachate using a vortex. DNA extraction was carried out using Soil DNA Extraction Kit according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene V3 variable region

The target gene fragment was amplified by polymerase chain reaction using universal primers, 341F(GC) and 534R (MUYZER *et al.*, 1997). PCR tubes having total volume of 25µl comprised of 1µl of Taq polymerase (5U/µl), 2.5µl $MgCl_2$ (25mmol/L), 2.5µl of 10×PCR buffer, 2µl dNTP(10umol/L), 1µl each primer, 2µl DNA template and 13µl of double distilled water. The PCR reaction was performed on the thermal cycler by the following program; initial denaturation at 95ºC for 5min, and then 35 cycles of 94 ºC for 1 min, 55 ºC for 1min, 72 ºC for 1min, and final extension at 72 ºC for 10 min. Amplified fragments were analyzed by 1% agarose gel electrophoresis, photographed under U.V. light.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis polyacrylamide gels (8%) was used with a denaturing gradient of 40%. The gel was subjected to constant voltage of 200V, 60ºC for 6h. The gel was silver stained and photographed with Bio-Rad gel imaging analysis system.

Cloning and Sequencing of DGGE bands

SCHWIEGER & TEBBE (1998) method was used to get the specific bands, and amplified with the same primers. The amplified products were further purified using purification kit. The target gene fragment was ligated with pMD18-T vector and transformed into DH5α competent cells, coated tablet, cultured overnight for blue-white screening and PCR. The PCR product was sequenced by Sangong Inc (Shanghai China).

Phylogenetic analysis

The results were identified based on sequence similarities to homologous 16S rDNA gene fragment in the Gene Bank and their phylogenetic analysis was done by MEGA4.1 software to construct phylogenetic tree.

Statistical analysis

QuantityOne software (Version 4.5) was used for digital analysis of the DGGE bands. Shannon-Wiener diversity index H of bacterial community structure was estimated by the following equation;

$H=-\sum (Pi)(\ln Pi)$

$Pi=ni/N$

Where ni is the value of one band, N is the total value of all band). Quantity One software (Version 4.5) was used for cluster analysis based on unweighted pair group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

16S V3 variable region of rDNA PCR results

In this study diversity of methane producing microbial community structure was assessed by amplification of 16S V3 variable region of rDNA. The total DNA was used as

template, and the size of product was found about 200bp that consistent with the expected size that be used for PCR-DGGE analysis directly.

The number, position and lightness of bands represent different microbial species in the DGGE fingerprinting. By using same amount of DNA, the band position and lightness corresponds to an abundance of specific species (HU *et al.*, 2004). The content of DNA is proportional to the lightness of band (YANG and CROWLEY, 2000). Figure 1 represents the number, position and lightness of bands for microbial community structure sampled at different environmental conditions. There were several obvious main bands about every samples, some bands were similar while some bands were different that indicated that some diversity in microbial community structure exist among these samples. The main bands of sample No 1 and sample No 3 were obvious that showed some microbial species were dominant in the microbial community.

The diversity index of samples could be known from Fig. 2 and Fig. 3; and the result showed sample No 5 had the highest diversity index, followed by sample No 2, and the sample No 4 had the lowest diversity index. These results indicated that despite there were kinds of bacteria in each samples, but every sample had obvious main species and the main species in difference samples were also difference. Cluster analysis was done based on Dice coefficient using UPGMA from PCR-DGGE profiles of the bacterial community structure from different environments. Results (Fig. 3) revealed that sample number 5 and 2 contained rich bacterial diversity. The bacterial community diversity of No 1 and the No 3 sample were relatively small and the dominant species were very obvious. So the difference of bacterial community structure was significant in different environmental condition that indicated that the impact of environmental conditions on the bacterial community structure was great.

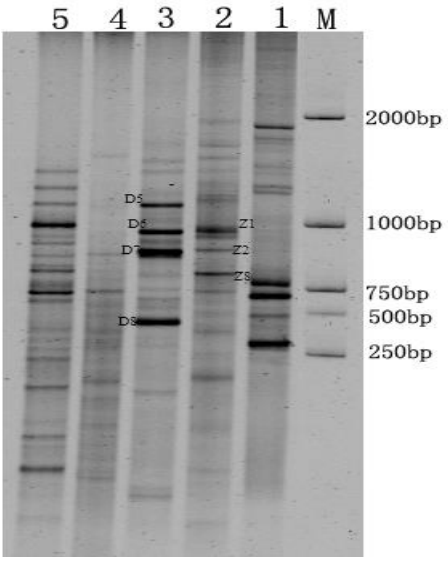

Fig. 1 DGGE fingerprint map of samples obtained under different conditions of reactor. Only labelled bands were excised and sequenced.

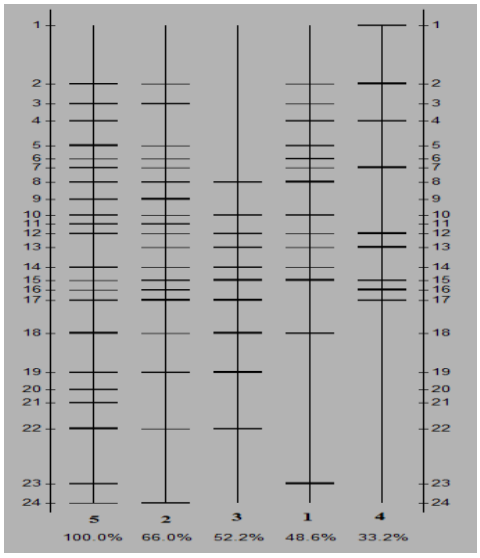

Fig. 2 Samples' bands tree under different environment conditions.

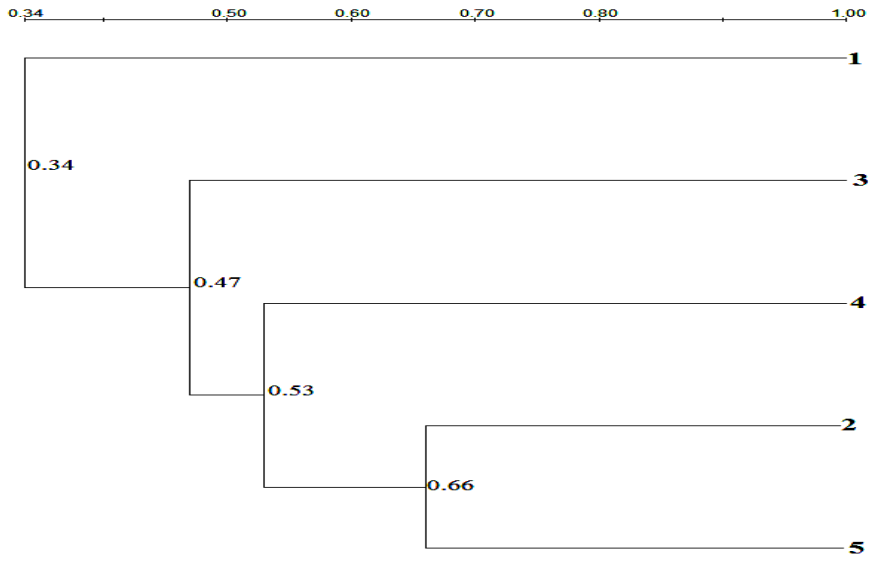

Fig. 3 Cluster analysis of sample under different environment conditions using UPGMA method.

The Shannon-Wiener diversity index (H) of methane producing microbial community structure was found in range of 2.25-3.12 from all samples (table 1). Sample number 5 contained highest Shannon-Wiener diversity index (H) among all samples. So our findings were very similar to previous reports. The Shannon-Wiener diversity index (H) range of 2.86-3.23 for V3 region was reported for microbial communities in paddy soils (LI *et al* 2009). YING *et al* (2008) reported highest Shannon-Wiener diversity index (2.605) of photohydrogen producing microbial community on 15th day of production.

Table 1. Shannon diversity index of different samples calculated from DGGE profile.

Sample No.	Shannon Diversity Index (H)
1	2.31297
2	3.03286
3	2.62714
4	2.25096
5	3.11569

Phylogenetic tree

DGGE maps showed that the main bands among these samples showed variation indicating that the environment had significant impact on the microbial community structure. Many researchers focused on intermediate temperate and low temperature for methane production, so seven bands, D5, D6, D7, D8, Z1, Z2, and Z8 in the No 2 and No 3 samples were chosen for cloning, sequencing and phylogenetic tree construction (Fig. 4).

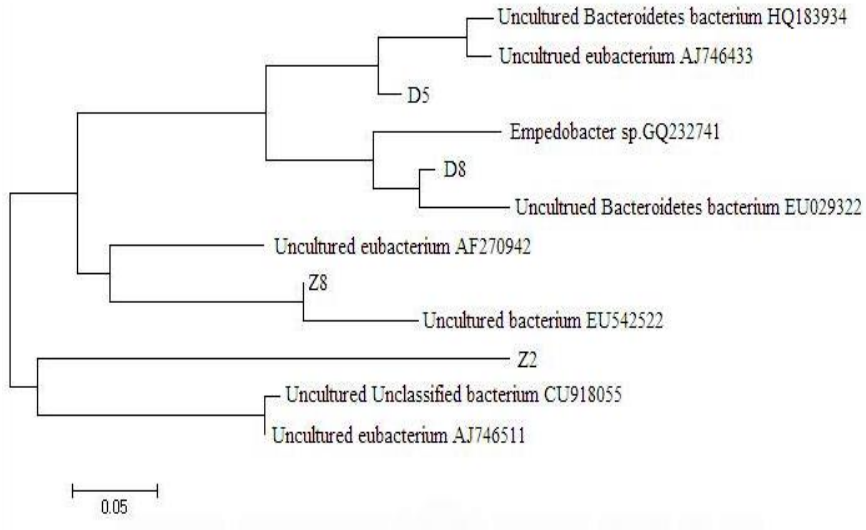

Fig. 4 Phylogenetic position of isolated bacteria using neighbor joining method.

The main bands from the DGGE map were selected for sequencing and these sequences were compared with NCBI database. The results showed that D6, D7, and Z1 had low similarly with identified bacteria from blast results. The sequence of Z8 had high similarity of 96% with the sequence of uncultured bacterial Er-the LLAYS-67, and these sequences of D5, D8, and Z2 exhibited more than 97% sequence similarity with uncultured bacteria. Z2 was identified as *Clostridia*; Z8 was identified as *Clostridiales*; D5 was identified as *Eubacteriaceae*, and D8 was identified as *Flavobacteriaceae* (Table 2).

Table 2. Results about the homology comparison.

Band	Partial 16S rRNA gene sequence		
	Accession number	Best match	Similarity
D5	HQ183934	Uncultured Bacteroidetes bacterium clone De162	98%
D8	EU029322	Uncultured Bacteroidetes bacterium clone M180	100%
Z2	AJ746511	Uncultured eubacterium clone LKB116	98%
Z8	EU542522	Uncultured bacterium clone Er-LLAYS-67	96%

PCR-DGGE fingerprinting method could quickly analyze a large number of samples without having to know any specific sequences in the sample, not only make it possible to resolve complex ecosystem, but also make the dynamic changes of the microbial community as possible, but studies have found that the same a band also has polymorphism (SEKIGUCHI *et al.*, 2001). Therefore, the method is a useful way to study microbial community. This study laid some extent foundation for future research about establishing a methane-producing bacterial system with high gas yield in winter.

CONCLUSION

In this study, the method of PCR-DGGE was used to analysis the diversity of methane bacteria under different environmental conditions. Results showed that the No 5 (sampled in the step of gas producing, room temperature, pH 7.5, and pig manure as substrate) had the highest diversity while No 2 and No 5 had low diversity. The bacterial diversity of the community of No 1 and No 3 were relatively small and the dominant community in these samples was the most obvious. These findings showed that the differences of bacteria in methane-producing microbes reactor were significant, indicating that environmental conditions had a greater impact on the methane-producing bacterial community structure.

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UTVRĐIVANJE RAZLIČITOSTI STRUKTURE MIKROORGANIZAMA KOJI PROIZVODE METAN KORIŠĆENJEM 16S-V3 rDNA PCR-DGGE

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Izvod

Korišćen je metod 16S-V3 rDNA PCR-DGGE u ispitivanju divergentnosti strukture zajednice mikroorganizama proizvođača metana. Dobijeni rezultati su pokazali da je struktura metanogenih zajednica različita u različitim uslovima spoljne sredine. Analiza sekvenci je pokazala da su bili predominantni sojevi negajeni u kulturama koje ne mogu biti identifiovane na nivou vrste. Z2 je identifikovan kao *Clostridia*, Z8 kao *Clostridiales*, D5 kao *Eubacteriaceae*, i D8 kao *Flavobacteriaceae*. Ova ispitivanja su osnova za dalja istraživanja i uspostavljanje sistema bakterija koje proizvode metan sa visokim prinosom gasa u toku zime.

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