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Spatial dynamics of bacterial communities in rural household biogas digesters in different climatic regions of Yunnan plateau, China

B. Yang[1,2,3], Q. Li[1,2,3], Y. Wu[4], G. Tian[1,2,3], M. Dong[4], F. Yin[1,2,3], X. Zhao[1,2,3], Y. Wang[4], W. Xiao[4], W. Zhang[1,2,3*], X Cui[4*]

[1]*Yunnan Research Center of Biogas Technology and Engineering, Yunnan Normal University, Kunming 650500, People's Republic of China*

[2]*Engineering and Research Center of Sustainable Development and Utilization of Bioenergy, Ministry of Education, Yunnan Normal University, Kunming 650500, People's Republic of China*

[3]*Yunnan Key Laboratory of Rural Energy Engineering, Yunnan Normal University, Kunming 650500, People's Republic of China*

[4]*Yunnan Institute of Microbiology, State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Kunming 650091, People's Republic of China*

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In order to reveal correlations between spatial dynamics, metabolic pathways and environmental factors of bacterial communities present in household biogas digesters in different climatic regions of Yunnan Plateau, bacterial communities were characterized based on phylogenetic analysis of 16S rRNA sequences and redundancy analysis (RDA) from 4 representatives of the biogas digesters located in 4 different climatic regions. Four 16S rRNA gene clone libraries were constructed from sampled activated sludges, which resulted in a total of 14 bacterial phyla, dominated by *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*. These are responsible for completion of hydrolysis, fermentation, hydrogenesis and acetogenesis in the fermentation metabolic process. RDA analysis identified a positive correlation between the abundances of bacterial communities and environmental temperatures as well as volatile solid contents of the four samples. The results demonstrate that biogas digesters were promoted in tropical and subtropical regions, while organic wastes containing a high VS were utilized as raw materials, because under these conditions the most diverse bacteria are predicted to improve the efficiency of the biogas fermentation system.

Keywords: biogas digester, bacterial community, 16S rRNA gene clone library, spatial dynamic, redundancy analysis (RDA), metabolic pathway.

INTRODUCTION

The biogas fermentation technology has become increasingly popular for mitigation of environmental, agricultural and energy issues [16]. An investigation by the authors towards development and investments in the biogas industry in Yunnan, China, showed that up to 2013, about 3,000,000 rural households made use of a biogas digester, illustrating the increasing role of biogas fermentation for solving rural environmental issues [18], prevention of deforestation [14] and increasing the rural energy supply [3].

The fermentation process to produce biogas depends on microbial activity, with a large fraction of uncultured bacteria typically present in a biogas fermentation system [6], which makes the process a bit like a "black box" [5]. Limited knowledge is available about the basic ecology such as microbial composition, population structure and metabolic pathways of the bacterial communities. The correlations between bacterial community and environmental factors also remain largely obscure, which makes optimization of the fermentation process difficult, for instance by selection of the right bacterial groups and environmental factors. Traditional microbiological technologies based on growth of pure cultures failed to reveal the typical biological processes of biogas fermentation [19]. Although in recent years microbiologists have utilized methods and technologies traditionally applied to molecular ecology to study the basic principles of biogas fermentation to design high-efficiency systems [25], the understanding of the basic ecological issues of bacterial communities in a biogas fermentation system remains largely unknown, and the biogas digesters of the Yunnan Plateau, which are subject to a variable local climate, are no exception.

Here, for the first time the 16S rRNA gene library technology was utilized to study the diversity and metabolic pathways of bacterial communities in rural household biogas digesters in the Yunnan Plateau. Four samples were analyzed at depth, taken from digesters located in four different climatic regions. A redundancy analysis (RDA) was

To whom all correspondence should be sent:
E-mail:wootichang@163.com；xlcuiynu@aliyun.com

performed to analyse the relationship between the detected bacterial communities and environmental factors. The resulting data present a theoretical basis for design of high-efficiency rural household biogas fermentation systems in Yunnan Plateau.

MATERIALS AND METHODS

Sampling locations

Sampling was performed in four counties of the Yunnan Plateau with different local climates: Jinghong County represents a north tropical tropic climate zone, Jianshui County is south subtropical, Yulong County is in the south temperate zone and Shangri-La County is located in a northern temperate climatic region. In all these four zones, initially activated sludges were sampled from a total of 26 plateau rural household biogas digesters in July and August, 2012. The digesters re-sampled 3 times and samples were stored in 50mL sterile sampling tubes at -80°C in the laboratory prior to analysis.

Pilot experiments assessed the diversity of prokaryotic micro-organisms by denaturing gradient gel electrophoresis (DGGE), which showed that differences in the microbial diversity were not significant for various samples within every climatic region. Therefore, one biogas digester was selected to represent each climate zone, and the active sludge samples of these four digesters were used to construct four 16S rRNA gene clone libraries. Details of these four sampling sites are provided in Table 1.

Analysis of abiotic factors (physical and chemical parameters)

The following physical and chemical parameters of the collected samples have been determined and given in Table 2: pH; total solid (TS) and volatile solid (VS) content as determined by weight [22]; total phosphorus (TP) content measured by the vanadium ammonium molybdate spectrophotometric method [12]; ammonia nitrogen (NH_3-N) content by Nessler's reagent colorimetric method [21]; and chemical oxygen demand (COD) by the potassium dichromate method [9].

DNA extraction, PCR amplification and purification

Total bacterial DNA was extracted from the samples by using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) [27] following instructions of the manufacturer. For PCR amplification of the bacterial 16S rRNA gene, forward primer 27F [24] and reverse primer 1541R [11] were used. The amplification reaction contained 5μL 10×PCR buffer (including 1.5mmol/L Mg^{2+}), 4μL dNTP mix (2.5mmol/L each), 5μL BSA (0.1%), 2μL primer 787F (with GC clips, 10pmol/L), 2μL primer 1059R (10pmol/L), 2.5 U Taq DNA polymerase (5 U/μL) and 3μL DNA solution in a total volume of 50μL. The PCR amplification conditions were as follows: pre-degeneration at 94°C for 5 min, followed by 16 cycles of 45 s at 94°C, 45 s at 60-52°C (steps of 0.5°C decrease per cycle), 2 min at 72°C followed, by 9 cycles with 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. A final extension was performed for 10 min at 72°C.

Table 1. Geographical data of the four sampled rural household biogas digesters in Yunnan Plateau, China.

Sample No.	Sampling site	Annual mean temperature (°C)	Longitude	Latitude	Elevation (m)
YN1	Jinghong County	21.9	101°6′E	22°51′N	840
YN2	Jianshui County	18.4	102°89′E	23°70′N	1296
YN3	Yulong County	12.6	100°14′E	26°85′N	2451
YN4	Shangri-La County	5.5	99°77′E	37°78°N	3218

Table 2. Physical and chemical parameters of 4 rural household biogas digesters samples in Yunnan Plateau.

Sample No.	pH	TS (%)	VS (%)	TP (mg/L)	NH_3-N (mg/L)	COD (mg/L)
YN1	7.37	11.18	75.10	190.19	231.9	6989.4
YN2	7.68	8.33	74.14	260.15	265.77	17172
YN3	7.27	3.32	68.35	320.63	167.90	13356
YN4	7.63	9.68	53.73	433.28	249.85	25948.8

Amplicons were analysed using DNA gels, excised and purified with SanPrep DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions.

Construction of 16S rRNA gene clone library

Purified PCR amplification products were ligated into pMD® 19-T Vector (TaKaRa, Dalian, China) using a cloning kit and transformed into competent E. *coli* DH5α [4]. A total of 500 white colonies from each sample were selected randomly for sequence analysis, which was performed by Sangon Biotech (Shanghai) Co., Ltd.

Sequence analysis

Detection of possible chimeras was carried out utilizing Mallard software [1]. Operational Taxonomic Units (OTU) were attributed using DOTUR software [26] with a similarity cutoff ≥97% within an OTU. Sequences were then aligned with the best hit found in the GenBank database by Blast [23]. The coverage rate was used to evaluate the sequencing depth of the clone library, which was calculated by utilizing SPADE software [10].

Analysis of the correlation between bacterial communities and environmental factors

A redundancy analysis (RDA) was carried out to determine correlations between bacterial communities and environmental factors by use of Canoco software [2].

RESULTS

The number of sequences, OTUs, and coverage rate of the samples are presented in Table 3. A total of 1285 bacterial sequences were obtained, which could be divided into 430 OTUs. The average coverage rate of the library was 82.6%. This high coverage indicates that the sequencing depth was sufficient to cover the majority of bacteria present, allowing an indepth analysis of the main bacterial populations present. The results indicate that clear geographical spatial differences exist between the 4 samples.

The obtained sequences were used to query the GenBank database by BLAST. The highest proportion of sequences that could be attributed to a bacterial group was found for YN1; this sample also contained the most diverse population, from which 13 different bacterial phyla were detected, compared to 10 phyla from YN2, 6 from YN3 and 7 from YN4. Approximately 10 to 25% of the sequences could not be attributed to a known phylum (unclassified bacteria). The results are summarized in Table 4

Table 3. OTU identification in the four samples.

Sample No.	Sequence number	OTU number	Coverage rate (%)
YN1	330	108	81.5
YN2	350	127	77.9
YN3	269	80	84.4
YN4	336	115	86.6
Total	1285	430	82.6 (mean)

Table 4. Abundance of bacterial phyla detected in the four samples.

Bacterial phylum	YN1 (%)	YN2 (%)	YN3 (%)	YN4 (%)
Bacteroidetes	23.62	16.88	11.5	11.1
Chloroflexi	21.50	26.07	3.4	0.5
Firmicutes	13.94	13.76	27.5	65.5
Proteobacteria	8.76	10.61	9.1	4.4
Armatimonadetes	2.12	1.44	ND	ND
Synergistetes	1.81	1.44	ND	ND
Planctomycetes	1.20	3.46	ND	3.8
Actinobacteria	1.20	0.57	0.7	1.9
Thermotogae	0.60	0.86	ND	ND
Spirochaetes	ND	0.29	2.6	2.5
Chlorobi	0.30	ND	ND	ND
Verrucomicrobia	0.30	ND	ND	ND
Fusobacteria	0.30	ND	ND	ND
Deinococcus-Thermus	0.30	ND	ND	ND
Unclassified bacteria	24.23	24.92	45.4	10

ND: not detected

The major bacterial phyla detected in all four samples were *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*, which were found in all four samples and also were the most abundant. In total the phylum *Firmicutes* was the most abundant bacterial group detected and included the most OTUs (148), accounting for 34.42% of the total OTUs. However, their relative numbers were only highest for YN3 and YN4. *Firmicutes* are typical inhabitants of anaerobic digested sludge, wastewater treatment bioreactors and faeces [13]. They are responsible for cellulose degradation, hydrolysis of organic matter and degradation of long-chain fatty acid, and often act together with methanogens in anaerobic digestion processes as they produce acetate on which methanogens feed to produce CH_4[13]. The abundance of *Firmicutes* in YN3 and YN4 particularly suggests that the anaerobic process with production of CH_4 based on acetate metabolism occurs in these rural household biogas digesters.

Bacteroidetes were the second most abundant bacteria in the samples. They were the most abundant detected bacteria in this phylum in YN1 and YN2. These organisms are typical for anaerobic environments such as seabeds, intestines and anaerobic reactors where they are responsible for degradation of acids from macromolecular carbohydrates [7]. The third major bacteria detected belonged to the phylum *Chloroflexi*, though their abundance was only high in YN1 and YN2. *Chloroflexi* use carbohydrates such as glucose and co-occur with hydrogenotrophic methanogens (to which the *Chloroflexi* supply hydrogen)[15]. In turn, these hydrogenotrophic methanogens can produce CH_4 from H_2 and CO_2[15]. It is likely that similar process, resulting in a net reaction of $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ occurs in the sampled rural household biogas digesters YN1 and YN2.

The phylum *Proteobacteria* was the fourth major bacterial group, which are primarily isolated from soil, faeces, anaerobic activated sludge, etc [17]. They are responsible for hydrolysis of starch, long-chain fatty acids, amino acids and so on[17].

DISCUSSION

Metabolic pathways for dominant bacterial groups

The metabolic degradation of organic matter in a typical biogas fermentation process can be divided into 3 stages [24]. During the first stage, bacteria are responsible for hydrolysis and fermentation of organic macromolecules; the second stage is responsible for hydrogenesis and acetogenesis for which other bacteria are responsible, while during the third stage methanogenesis is performed by methanogens [22]. In view of this "3-stage theory" the metabolic pathways for the dominant bacteria detected in the rural household biogas digesters were predicted, as shown in Figure 1.

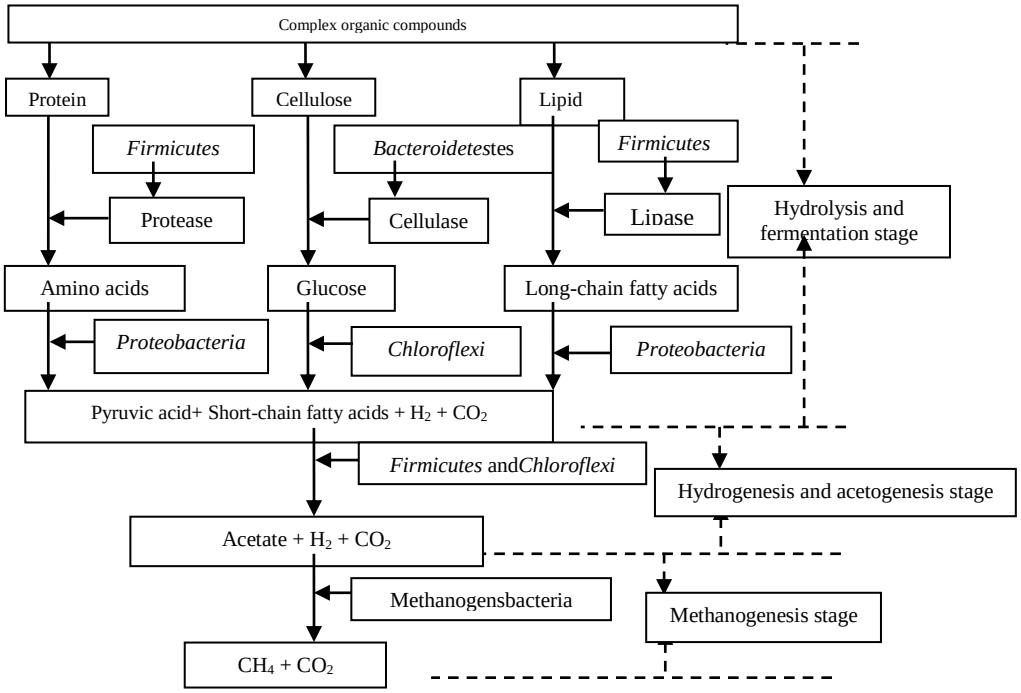

Fig. 1. Predicted metabolic pathways for dominant bacterial groups in rural household biogas digesters in Yunnan Plateau.

The figure illustrates that the fermentation process is complex and depends on multi-flora cooperative metabolism to completely decompose organic matter into CH_4 and CO_2. Those dominant

bacteria detected in the rural household biogas digesters in Yunnan Plateau are mainly responsible for degradation of complex organic matter in the first stage (hydrolysis and fermentation) and the second stage (hydrogenesis and acetogenesis). These processes would supply the necessary substrates, such as acetate, H_2 and CO_2, for biogas (CH_4) production by methanogens.

Correlation between bacterial communities and environmental factors

A redundancy analysis (RDA) was performed and the findings were biordered with respect to physical and chemical characteristics of the samples, producing a diagram as shown in Figure 2. Now it can be seen that of the 14 detected bacterial phyla, 10 phyla (*Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, *Armatimonadetes*, *Synergistetes*, *Thermotogae*, *Chlorobi*, *Verrucomicrobia*, *Fusobacteria* and *Deinococcus-Thermus*) positively correlated to the annual mean temperature as well as to VS content. The abundance of *Firmicutes* and *Spirochaetes* positively correlated with the TP content as well as to the elevation of the sampled digesters. Further, two phyla (*Actinobacteria* and *Planctomycetes*) positively correlated with COD, pH, TS and NH_3-N content. Restricting the findings to the 4 major bacteria groups, a strong positive correlation was found between abundance of *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* with annual mean temperature as well as VS, while a strong negative correlation existed between the abundance of *Firmicutes* and temperature and VS. The detected correlations between bacterial groups and environmental factors shows that, of the analysed parameters, atmospheric temperature and VS are the major environmental factors influencing compositions of bacterial communities in rural household biogas digesters in Yunnan Plateau.

Metabolic activities of bacterial groups in biogas digesters are closely related to the local atmospheric temperature [20]. The higher this temperature is, the more robust the metabolism of bacterial groups in a certain temperature range would be [20]. At higher temperatures, growth and enrichment of various bacteria will be stimulated, which could explain the high number of 13 phyla in YN1, which was situated in the tropics (annual mean atmospheric temperature: 21.9°C) compared to 10 phyla in YN2 (from subtropical Jianshui County with an anannual mean temperature of 18.4°C) and relatively few phyla from the digesters located in temperate zones. The abundance of various bacterial groups may effectively improve the efficiency of any biogas fermentation system. Thus, we suggest that biogas digesters should be promoted in regions in the tropics and subtropics.

The VS are organic substances found in the raw materials, which support the catabolism of bacterial groups [8]. A high content of VS may promote growth and enrichment of various bacteria groups [8]; it seems to have resulted in more abundant bacterial groups in YN1 and YN2 (VS content 75.10% and 74.14%, respectively), compared to YN3 and YN4 (VS content 68.35% and 53.73%, respecitively). Thus, we suggest that biogas fermentation raw materials with a high VS content are optimal for biogas digesters.

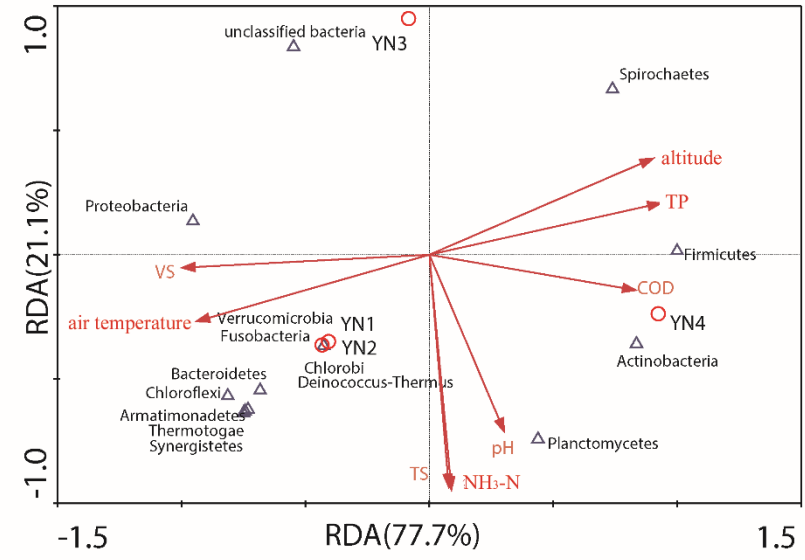

Fig. 2. RDA biordered diagram of the correlation between bacterial groups and environmental factors.

CONCLUSION

The bacterial groups detected in rural household biogas digesters in four different climatic regions of Yunnan Plateau belong to 14 bacterial phyla, of

which *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* are dominant. However, a considerable proportion of detected bacterial sequences belonged to unclassified bacteria. Geographical spatial differences correlated with observed diversities of bacterial groups; the bacterial diversity was significantly higher in samples obtained from digesters located in the northern tropics and southern subtropics than from the south and north temperate zones. In a biogas fermentation microecological system, *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* are the dominant bacterial groups, which are mainly responsible for the hydrolysis and fermentation stage and the hydrogenesis and acetogenesis stage. The atmospheric temperature and VS content were found to be the major environmental factors that influence composition and diversity of bacteria in rural household biogas digesters in Yunnan Plateau.

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