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# Comparative study of reactor performance and microbial community in psychrophilic and mesophilic biogas digesters under solid state condition

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**Psychrophilic (15°C) and mesophilic (35°C) reactor performance and microbial community dynamics were compared when the biogas fermenters were performed at high altitude and solid state condition using animal manure and highland barley straw as substrate. Longer biogas fermentation time, higher peak methane content and lower volatile fatty acids (VFA) accumulation were found at psychrophilic condition compared to that of at mesophilic condition although the biogas production in both temperature conditions was similar. The cumulative biogas production at 35°C and 15°C were 246 (±5) and 225 (±7) ml/g volatile solids, respectively. The highest total VFA concentration under 35°C was 10,796 (±310) mg/kg total solid, while it only reached to 2346 (±87) mg/kg total solid at the condition of 15°C. Additionally, the variation of pH, soluble chemical oxygen demand and total ammonia nitrogen during the anaerobic digestion under psychrophilic condition were much smaller than that of under mesophilic condition. Polymerase chain reaction and denaturing gradient gel electrophoresis analysis followed by 16S rDNA sequencing showed that bacteria of genera *Bacillus* and *Clostridium* and archaea of genera *Methanosarcina* and *Methanosaeta* played a pivotal role during the biogas production.**



[**Key words:** Psychrophilic and mesophilic; Solid state anaerobic digestion; Biogas; Microbial community; High altitude]

Anaerobic digestion (AD) is a proven technology for the effective treatment of organic wastes, and is increasingly applied around the world including in the high altitude regions. According to the concentration of total solids of substrate, AD can be classified as wet (<10%), semi-solid (10–15%), and solid state (>15%) (1). Solid state AD is a promising alternative for high solid content waste management in many parts of the world due to several advantages. Firstly, the reactor volume of solid state AD is greatly reduced, and the digested residues is much more easily handled due to lower water content. Secondly, there are almost no problems of floating fibrous materials in solid state treatment, whereas substrate floating is a common phenomenon observed in wet and semi-solid digestion processes (2). Certainly, solid state AD is not perfect, but has disadvantages. For instance, it needs a lot of inocula to achieve fast start-up of reactor, and is more sensitive due to the easy accumulation of toxic or inhibiting matter like ammonia and volatile fatty acids (VFAs) (3).

Temperature is one of the factors that could strongly affect the microbial diversity and the performance of AD (4). Presently, most AD systems in China and other countries are operated at mesophilic (25–40°C) or thermophilic (55–70°C) condition (5). Nevertheless, interest in psychrophilic (<20°C) AD has increased over the past several years. One of the main advantages of psychrophilic AD would be due to the lower energy input required for heating the bioreactor and thus greatly reduces the operating cost. Psychrophilic AD is now being investigated extensively focusing on aspects of waste water and some easily degradable solid waste treatment (6). However, many researches showed that the biogas production under psychrophilic condition was mostly lower than that of under mesophilic and thermophilic conditions (7,8). One of the important reasons might be due to the low microbial activity resulted from the low temperature condition. Therefore, using high-activity psychrophilic methanogenic communities as inoculum and keeping the microbial community in high activity during the biogas fermentation are considered to be effective ways to increase biogas production (9). However, the activity of microbial community could be affected by many factors besides temperature, such as the component of feedstocks and the environment conditions. Therefore, comprehensive analysis of the structure and function of microbial community in biogas fermentation system is important to enhance biogas production under psychrophilic condition. However, as far as the authors know, little information about microbial community under solid psychrophilic AD condition has been available up to now.

Biogas fermentation is commonly divided into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (10). During these four stages, complex organic substrates are firstly degraded into simple organic compounds by hydrolytic microorganisms; and the metabolites are converted into various VFAs and other small molecular compounds by fermentative bacteria in the second stage; then homoacetogenic or syntrophic bacteria produce acetate at the third stage; lastly, the small molecular metabolites produced by bacteria, like $H_2$, $CO_2$, acetate, formate and simple methylated compounds are converted to methane by methanogenic archaea. Although the methanogens are very diverse, the

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substrates they can only utilize must be processed by anaerobic bacteria or eukaryotes (11). Therefore, syntrophic cooperation between bacteria and methanogens is critical for efficient and successful conversion of organic matter into methane (12).

High altitude region is characterized by low atmospheric pressure, which is a proven factor that could affect the performance of AD (13). However, the mechanism remains largely unknown. One of the possible reasons is that low atmospheric pressure decreases the partial pressure of $CH_4$ and $CO_2$ content in the biogas, and thus reduces acidification and affects methanogenesis. For example, Jiang et al. (13) observed a higher pH in low pressure reactor during the whole experiment compared with the system under normal pressure. Oppositely, significant pH dropping was found during the pressurized AD process (14,15). To date, the effect of high pressure on the performance of AD (14–16) and microorganism (17,18) has been extensively studied, while the information about AD at high altitude, namely low atmospheric pressure condition is still poor.

Previous experiments that have studied the reactor performance and microbial community dynamic, however, were conducted at mesophilic or thermophilic conditions, with no published study investigating the microbial community dynamic at psychrophilic (≤15°C) temperatures and solid state condition. The objective of this study was to compare the performance of reactors operated at two different temperatures, i.e., 15°C (psychrophilic) and 35°C (mesophilic), under solid state and low atmospheric pressure condition, and to characterize the dynamics of the microbial community during the whole biogas fermentation stage by using polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) method based on 16S rDNA sequences. Both reactors were continuously analyzed with regard to biogas production, the changes of pH, soluble chemical oxygen demand (SCOD), total ammonia nitrogen (TAN), free ammonia nitrogen (FAN) and VFAs.

## MATERIALS AND METHODS

**Feedstock and inoculum** The substrate used for biogas production in this study was the mixture of highland barley straw (BS), Tibetan pig (TPM) and cattle manure (CM) according to desired proportion. The material was prepared as previously described (19). The inocula were obtained from two laboratory scale (about 30 L) psychrophilic (15°C) and mesophilic (35°C) anaerobic digesters fed with TPM, CM and BS, and operated as sequencing batch reactors. The physico-chemical characteristics of manures, BS and inocula are listed in Table 1. The altitude of the laboratory is 3000 m, and the mean air pressure is 74.5 kPa.

**Experimental design** Digesters with a total volume of 0.52 L and working volume of 0.30 L were fed with a mixture of TPM, CM and BS at a ratio of 1: 1: 2, based on volatile solids (VS) content. Each digester was seeded with inoculum at an inoculum to feedstock ratio of 2: 1 (also based on VS), then was added with distilled water to obtain a final total solid (TS) content of 20%. The mixing ratio of substrate and inoculum to feedstock ratio were based on our previous results (19). The digesters then were flushed with nitrogen gas to provide an anaerobic environment and were placed under controlled 15°C or 35°C for biogas fermentation. Reactors with only inoculum were run as controls. Each test was repeated twice. All bottles were manually mixed once a day for about 1 min during the biogas fermentation period. At predetermined time intervals (day 0, 10, 20, 30, 40, 50 and 90 for psychrophilic AD and day 0, 1, 3, 6, 10, 15 and 29 for mesophilic AD), all the content in each digester was taken out, thoroughly mixed and stored at −20°C for the later composition and microbial analysis. Before experiment, the inoculum was kept under anaerobic conditions at 15°C or 35°C for about one week to reduce non-specific biogas generation.

**TABLE 1.** Characteristics of feedstocks and inocula used in this study.

| Item | TPM | CM | BS | Inoculum | |
|---|---|---|---|---|---|
| | | | | 15°C | 35°C |
| TS (%) | 92 ± 2 | 91 ± 2 | 93 ± 2 | 18 ± 2 | 19 ± 2 |
| VS (%TS) | 51 ± 2 | 79 ± 2 | 94 ± 2 | 62 ± 1 | 56 ± 3 |
| pH | 7.5 ± 0.1[a] | 7.2 ± 0.1[a] | 6.7 ± 0[a] | 8.17 ± 0.1 | 8.27 ± 0.1 |
| SCOD (g/kgTS) | 102 ± 4 | 53 ± 4 | 58 ± 5 | 68 ± 2 | 98.1 ± 0.9 |
| TAN (mg/kgTS) | 501 ± 41 | 333 ± 61 | / | 969 ± 158 | 1557 ± 39 |
| Total VFA (mg/kgTS) | 31 ± 12 | 25 ± 9 | / | 234 ± 7 | 835 ± 109 |

[a] Adding distilled water until TS 20%, then measured.

**Analytical methods** Methane content was measured using a gas chromatograph (GC122, China) and the volume of biogas was measured using water displacement and corrected to standard temperature and pressure (273 K, 1 atmosphere) (19). In order to avoid absorption, the water used for displacement was flushed with biogas obtained from other experiments under similar conditions for 1 min before the biogas volume test. Samples for SCOD, TAN and VFAs measurements were prepared by suspending 5 g of digestate in 10 mL of distilled water, thoroughly mixing it, and then separating the solids by centrifugation (10,000 rotations per minute, 10 min). The supernatant was directly filtered through a 0.45-μm membrane for SCOD and TAN analysis or acidified to pH 2–3 by adding hydrochloric acid then filtered for VFAs analysis. TS, VS, SCOD and TAN were measured according to the standard analysis methods of China (20). VFAs were detected using another gas chromatograph (Agilent GC6890N, Agilent Technologies Inc., USA) equipped with a flame ionization detector and a 30 m × 0.32 mm I.D. × 0.50 μm capillary column (DB-FFAP, Agilent Technologies Inc.) according to the procedure described by Qu and Liu (21). Total VFAs were calculated by summing up each individual VFA after which was transformed as the equivalent mg/kgTS of acetic acid. pH was measured by a pH meter (PHS-3C, REX, Shanghai, China). Free ammonia concentrations were calculated according to the following equation (22,23):

$$[NH_3] = \frac{[T\_NH_3]}{1 + 10^{(pK_w - pK_b - pH)}} \quad (1)$$

where $[NH_3]$ is the free ammonia concentration, $[T\_NH_3]$ is the total ammonia concentration, $K_w$ is dissociation constant of water and $K_b$ is ionization constant of ammonia.

**Microbial analysis** The fermentation samples were sent to Beijing Centre for Physical and Chemical Analysis (Beijing, China) for PCR-DGGE and sequencing analysis. The DNA of samples was extracted using soil DNA isolation kit (Tiangen Biotech, Beijing, China). The extracted DNA was then used as template for 16S rDNA (V3 region) amplification by PCR. The universal primers for bacteria were 357F (5′-CCTA CGGG AGGC AGCA G-3′) and 517R (5′-ATTA CCGC GGCT GCTG G-3′), for archaea were 344F (5′-GACG GGGH GCAG CAGG CGCG A-3′) and 522R (5′-GWAT TACC GCGG CKGC TG-3′) (24,25). A GC clump (CGCC CGCC GCGC GCGG CGGG CGGG GCGG GGGC ACGG GGG) was added to the forward primers to facilitate DGGE analysis. The PCR was run on a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) and the product was subjected to DGGE analysis using a DCode Universal Mutation Detection System (Bio-Rad) after verified. DGGE gel was visualized using SYBR Green. The prominent bands were excised from the gel, purified using TaKaRa MiniBest DNA fragment purification kit (Takara Bio, Shiga, Japan), and then followed by PCR reaction with the same DGGE primers but without GC clump and under the same condition, after cloned the PCR products were sequenced using ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting sequences were submitted to the BLASTn search program (Basic Local Alignment Search Tool) (26) (http://www.ncbi.nlm.nih.gov/BLAST) of the National Center for Biotechnology Information (NCBI) to find similar sequences. Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu) was used for taxonomic affiliation of the identified 16S rDNA sequences (27).

**Accession numbers of DNA sequences** All the raw DGGE sequences in this study were deposited in GenBank under the accession numbers KY458130 to KY458156 except that some of archaea DGGE sequences were too short.

**Data analysis** Two-tailed *t*-tests were performed to compare the difference in daily biogas production, cumulative biogas production, methane content and SCOD removal rate between the two temperature conditions by using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA), and statistical significance was determined at the 95% confidence level ($P < 0.05$). The DGGE gel images were processed using Bio-Rad software Quantity One. The band patterns were subjected to principle component analysis (PCA), which was conducted on the binary matrices derived from the archaea or bacteria DGGE profiles. To analyze the potential influence of environmental variables (temperature, VFA, pH, SCOD, TAN) and the performance of reactor (daily biogas production, methane content) on archaea and bacterial community composition, redundancy analysis (RDA) with Monte-Carlo permutation tests were performed. Both PCA and RDA analysis were conducted using Canoco for Windows version 4.5 (28).

## RESULTS AND DISCUSSION

**Biogas production** The difference in biogas process between psychrophilic (15°C) and mesophilic (35°C) condition was obvious, although biogas could be produced immediately from the first day at both temperature conditions (Fig. 1A and B). Under mesophilic

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