Construction of biogas metabolic pathway in a low-temperature biogas fermentation system

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Abstract
The main objective of this study was to establish the metabolic pathway of a biogas fermentation system active at low temperature, and to describe the bacterial and archaeal methanogenic species responsible. A biogas fermentation inoculum adapted to be active at 9°C was used in a 10-L batch-type temperature-controlled fermentor, using pig manure as the raw material. Fermentation at 9°C was followed for 120 days. A combination of nonbiological and biological factors was analyzed. Species were identified from OTU analysis based on 16S rDNA amplicon sequencing, and correlation analysis was used to study the effect of nonbiological factors, bacterial communities, and archaea communities. Biogas production was most effective between days 50 and 90, where gas production was above 0.70 L/d and the CH4 yield was above 0.36 L/g-volatile solid. The relative abundance of the first dominant bacterial OTU with 99% identity to Clostridium cellulovorans varied between 16.17% and 27.30%. These bacteria typically degrade cellulose and hemicellulose. The second dominant bacterial OTU (relative abundance 15.23%-27.15%) was 99% identical to Terrisporobacter petrolearius, a typical fermentative species. The most abundant (4.15%-37.14%) archaeal OTU was 98% identical to Methanocorpusculum sinense, which is a typical hydrogenotrophic methanogen. A low-temperature biogas metabolic pathway was constructed, based on abundant bacterial and methanogen OTUs. The most suitable metabolic pathway describes that hydrogen is produced by one part of the community and then used to reduce the produced carbon dioxide into CH4; this seems to be the major pathway by which biogas is produced under low-temperature conditions.

KEYWORDS
archaeal OTU, bacterial OTU, biogas fermentation, metabolic pathway

1 | INTRODUCTION

Biogas technologies make use of a variety of organic wastes to produce renewable energy, providing a solution to the energy crisis and reducing environmental pollution. In China, in 2015 the number of rural household biogas digesters reached 41.933 million, with an annual production capacity of 13.575 billion cubic meter biogas beneficially serving 200 million farmers. China has become the country with the largest biogas development globally, and biogas
Biogas generation is more efficient at elevated temperatures, but about 75% of the biosphere on the earth’s surface is located in a cold environment; low temperatures are a major limiting factor for large-scale biogas expansion. Studies have shown that more than 75% of household biogas digesters in China are operated at low temperatures. Therefore, the research and development of high-efficiency biogas technology operating at low temperatures has become a topic of high concern. Since biogas fermentation depends on biological metabolism, its performance is closely related to the microorganisms being present. Microorganisms that are active in low-temperature biogas fermentation systems are therefore intensively researched, in particular, the initial community structure. However, not only the initial community but also the succession of dominant populations and the correlation of fluctuations with environmental factors need to be taken into account. Metagenomics has been applied to identify the functional genes in a biogas production system, and this uncovered the initial biochemical reaction processes. However, studies toward the relationship among bacterial and archaeal communities and their metabolic pathways are still rare.

To fill this knowledge gap, in this study, a biogas fermentation inoculum was domesticated at a low temperature of 9°C, after which the population was inoculated into a batch fermentor based on pig manure as the raw material. This model system was allowed to form biogas by fermentation at 9°C. Comprehensive tools including nonbiological and biological factor analysis (based on 16S rDNA amplicon sequencing) were applied to investigate the process. We aimed to establish the active biogas metabolic pathways based on the levels of bacterial and archaeal species being present. This work helps to clarify the key microbial species in a biogas fermentation system operating at low temperatures and provides guidance for the design of efficient low-temperature biogas production systems in the future.

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**FIGURE 1** Schematic diagram of the test setup for low-temperature biogas systems (1 Chiller; 2 Inlet pipe; 3 Outlet pipe; 4 Water bath; 5 Batch fermentation reactor; 6 Sampling tube; 7 Gas valve; 8 Gas pipe; 9 Gas storage cabinet; 10 Exhaust pipe)
nitrogen, and volatile fatty acids (VFAs) in the mixture were determined from samples drawn every 10 days. Samples drawn for determination of biological factors (see below) were also drawn every 10 days and stored at −80°C prior to analysis, as described under point 2.5.

2.4 | Analysis of nonbiological factors

The biogas was collected in a gas storage cabinet of which the bell jar was equipped with a volumetric metering scale. The biogas production was measured every day, and its composition (methane, CH₄, vs carbon dioxide, CO₂) was determined by gas chromatography (GC-6890A; Lunan Analytical Instrument Co., Ltd.). An air-drying oven with a thermostat (DHG-9070A type; Shanghai Yiheng Scientific Instrument Co., Ltd.) was used to dry samples for TS determination, and a box-type resistance furnace (SX-5-12 type; Tianjin Taisite Instrument Co., Ltd.) was used for combustion to determine VS. The pH was measured with a pH meter (PHS-25 type; Shanghai Jinmai Instrument Co., Ltd.). The amounts of sCOD, ammonia nitrogen, and VFAs were determined using a COD analyzer (CODmax II type, Hach Company), an ammonia nitrogen analyzer (Amtax Compact II type; Hach Company) and by gas chromatography (GC-9790 type II; Zhejiang Fuli Analytical Instrument Co., Ltd.), respectively. All instruments were operated according to their instruction manuals.

2.5 | Biological factors and their determination

2.5.1 | DNA extraction and PCR amplification

The total DNA of each sample was extracted by magnetic bead DNA extraction kit (DP328; Tiangen Biochemical Technology Co., Ltd.). Purity and concentration of the DNA were determined by 1% agarose gel electrophoresis. DNA was then diluted to 1 ng/μL with sterile water and stored at −20°C. Using the diluted genomic DNA as a template, the V3-V4 variable region of the bacterial 16S rDNA gene and the V4-V5 variable region of the 16S rDNA of archaea were amplified by PCR. For this, bacterial primers 341F/806R with barcode and the archaeal primer Arch519F/Arch915R were used. All PCRs were carried out in 30 μL solutions containing 15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs), forward and reverse primers (10 μM, 2 μL of each), and template DNA (1 ng/μL, 2 μL), and ddH₂O (9 μL). The samples were initially denatured at 98°C for 1 minutes, followed by 30 cycles of denaturing at 98°C for 10 seconds, annealing at 50°C for 30 seconds, and elongation at 72°C for 30 seconds. Finally, the samples were held at 72°C for 5 minutes. PCR products were analyzed by 2% gel electrophoresis, and the main band sized between 400 and 450 bp was excised and purified using the GeneJET Gel Extraction Kit (K069; Thermo Fisher Scientific) prior to sequencing.

2.5.2 | Library construction and sequencing

A DNA library was constructed using the Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs), followed by library quality assessment (Qubit and Q-PCR quantification). After the library was qualified, HiSeq2500 PE250 was used for sequencing.

Paired-end reads were acquired from the Illumina HiSeq sequencing platform. After removal of the barcode and primer sequences, the reads of each sample were spliced by FLASH, and the obtained sequences produced Raw Tags. By referring to the quality control process of Qiime’s Tags, Raw Tags were filtered to obtain high-quality Tags (Clean Tags). These were compared with the GOLD database to detect and subsequently remove chimeric sequences using UCHIME software. All remaining sequences were then combined per sample to produce the final Effective Tags.

2.5.3 | OTU clustering and species annotation

All Effective Tags sequences were clustered by Uparse software, and the sequences were binned into operational taxonomic units (OTUs) with 97% identity. Representative sequences of OTUs were selected to perform species annotation using the Mothur method and SILVA’s SSUrRNA database. Relevant taxonomic information was obtained to compare the community composition of each sample at each classification level.

2.6 | Correlation analysis

Correlations were analyzed between abiotic factors, abundant bacterial and archaeal OTUs, and bacterial and archaeal communities, in various combinations. All correlation analyses were based on two-side t test and Pearson correlation analysis, and the relevant data were statistically processed using SPSS software (Version 22.0).

3 | RESULTS

3.1 | Time dynamics of abiotic factors

The dynamic changes of abiotic factors during the low-temperature biogas fermentation over time are summarized in Table 1. After initiation of fermentation, the TS and VS contents decreased gradually, indicating that organic material was consumed by the microorganisms. On day 10 after the
| Fermentation times | Total solid (%) | Volatile solid (%) | Soluted chemical oxygen demand (mg/L) | NH$_3$-N (mg/L) | pH | Acetic acid (mg/L) | Propionic acid (mg/L) | Butyric acid (mg/L) | Isobutyric acid (mg/L) | Pentanoic acid (mg/L) | Isopentanoic acid (mg/L) |
|-------------------|-----------------|--------------------|----------------------------------------|----------------|----|---------------------|-----------------------|-------------------|---------------------|----------------------|-------------------------|
| Day 0             | 4.30 ± 0.10     | 65.72 ± 1.08       | 7415 ± 92                              | 423 ± 143      | 7.42 ± 0.05 | 1487 ± 176         | 244 ± 30              | 390 ± 38            | 48 ± 4               | 32 ± 6                 | 74 ± 3                  |
| Day 10            | 4.74 ± 0.10     | 63.24 ± 0.89       | 15163 ± 446                            | 709 ± 29       | 7.02 ± 0.04 | 1936 ± 33          | 279 ± 32              | 347 ± 33            | 51 ± 10              | 29 ± 10                | 67 ± 30                 |
| Day 20            | 4.73 ± 0.52     | 65.56 ± 1.34       | 9260 ± 99                              | 622 ± 77       | 6.95 ± 0.10 | 2046 ± 376         | 305 ± 26              | 300 ± 26            | 89 ± 9               | 56 ± 8                 | 115 ± 8                 |
| Day 30            | 4.62 ± 0.25     | 65.27 ± 0.7        | 10030 ± 21                             | 683 ± 88       | 6.85 ± 0.04 | 1812 ± 282         | 321 ± 38              | 204 ± 20            | 97 ± 18              | 47 ± 4                 | 113 ± 9                 |
| Day 40            | 4.51 ± 0.13     | 74.16 ± 3.49       | 8605 ± 219                             | 600 ± 84       | 6.74 ± 0.03 | 2286 ± 239         | 408 ± 28              | 150 ± 13            | 95 ± 8               | 45 ± 2                 | 122 ± 10                |
| Day 50            | 4.28 ± 0.20     | 64.59 ± 1.25       | 11155 ± 92                             | 609 ± 40       | 6.64 ± 0.04 | 2616 ± 151         | 495 ± 44              | 118 ± 14            | 92 ± 11              | 44 ± 7                 | 122 ± 10                |
| Day 60            | 4.36 ± 0.18     | 69.64 ± 2.62       | 12325 ± 1662                           | 729 ± 114      | 6.53 ± 0.02 | 2823 ± 455         | 696 ± 25              | 126 ± 1             | 116 ± 3              | 58 ± 4                 | 145 ± 6                 |
| Day 70            | 3.24 ± 0.15     | 55.12 ± 2.12       | 9280 ± 368                             | 462 ± 8        | 6.61 ± 0.02 | 2260 ± 424         | 815 ± 132             | 84 ± 13             | 124 ± 18             | 63 ± 6                 | 152 ± 6                 |
| Day 80            | 3.90 ± 0.08     | 66.53 ± 1.63       | 9660 ± 14                              | 579 ± 30       | 6.84 ± 0.06 | 1009 ± 119         | 857 ± 110             | 25 ± 3              | 125 ± 15             | 51 ± 4                 | 159 ± 6                 |
| Day 90            | 3.86 ± 0.14     | 63.66 ± 0.99       | 8385 ± 262                             | 605 ± 98       | 6.91 ± 0.10 | 738 ± 252          | 925 ± 17              | 20 ± 4              | 103 ± 2              | 20 ± 6                 | 129 ± 9                 |
| Day 100           | 3.90 ± 0.05     | 65.03 ± 0.95       | 7570 ± 184                             | 618 ± 82       | 6.91 ± 0.11 | 670 ± 53           | 742 ± 98              | 18 ± 10             | 51 ± 11              | 20 ± 15                | 114 ± 11                |
| Day 110           | 3.70 ± 0.09     | 64.47 ± 0.96       | 6480 ± 474                             | 485 ± 17       | 6.93 ± 0.09 | 561 ± 134          | 137 ± 100             | 18 ± 9              | 46 ± 26              | 18 ± 10                | 46 ± 20                 |
| Day 120           | 3.46 ± 0.17     | 64.46 ± 0.24       | 5405 ± 35                              | 476 ± 22       | 6.94 ± 0.09 | 473 ± 95           | 124 ± 20              | 13 ± 19             | 42 ± 33              | 14 ± 11                | 40 ± 28                 |
| Day 130           | 3.59 ± 0.14     | 63.04 ± 0.14       | 5400 ± 226                             | 508 ± 30       | 6.91 ± 0.08 | 476 ± 106         | 256 ± 55              | 14 ± 7              | 43 ± 25              | 12 ± 6                 | 38 ± 15                 |
| Day 140           | 3.65 ± 0.13     | 63.14 ± 0.86       | 5120 ± 354                             | 530 ± 26       | 6.94 ± 0.09 | 456 ± 187          | 237 ± 79              | 12 ± 16             | 40 ± 15              | 9 ± 8                  | 36 ± 19                 |
| Day 150           | 3.18 ± 0.17     | 61.97 ± 1.27       | 6480 ± 269                             | 716 ± 26       | 7.03 ± 0.07 | 579 ± 150         | 296 ± 62              | 14 ± 6              | 26 ± 7               | 6 ± 10                 | 41 ± 10                 |
| Day 160           | 3.47 ± 0.17     | 63.89 ± 3.33       | 4650 ± 240                             | 585 ± 4        | 6.98 ± 0.14 | 449 ± 100         | 211 ± 101             | 9 ± 11              | 28 ± 9               | 6 ± 13                 | 51 ± 6                  |
start of fermentation, the sCOD content reached a maximum (15.16 g/L). This maximum was reached when organic solids (insoluble macromolecular organic matrix) were hydrolyzed to smaller and water-soluble organic molecules, leading to a rapid increase in sCOD content, while these products were not yet fermented further. The ammonia nitrogen remained relatively constant around 600 mg/L. The average concentration of acetic acid in the fermentation liquid accounted for more than 60% of the total acid concentration. This acetic acid gradually increased after the start of fermentation, reaching a maximum of 2.82 g/L on day 60. The source of this acetic acid is the water-insoluble organic matrix that was hydrolyzed and further fermented with acetic acid as one of the final products. This resulted in acidification of the liquid, as was visible by the constant decrease in pH. The total volatile organic acid content also peaked at day 60 of fermentation.

After that, the pH tended to increase and then stabilized at day 90 and beyond at a value of around 7.

Gas production was monitored for 160 days, and the relative content of CH₄ and CO₂ was determined every 10 days. The result was summarized in Figure 2. Biogas production was above 0.70 L/d, with a yield of CH₄ above 0.36 L/g-VS during four intervals, between days 51 and 90, which represented peak performance. The highest peak of gas production was observed between 71 and 80 days.

### 3.2 Bacterial community structure and relative abundance

Based on ribosomal DNA sequences as determined from PCR amplicons, OTUs of bacteria and archaea were identified. The present microbial community displayed a rich diversity, with
between 853 and 1111 bacterial OTUs and between 561 and 967 archaeal OTUs. The top 35 bacterial OTUs (representative species level) in terms of average relative abundance (r.a.) were selected to examine the dynamic changes in r.a. during the fermentation process (see Figure 3).

The average accumulative abundance of these top 35 bacterial OTUs ranged between 76.36% and 87.75%. Among them, 25 OTUs belong to Firmicutes, 7 to Bacteroidetes, 2 to Synergistetes, and 1 OTU represented a Proteobacteria. These four bacterial phyla were main participants of the hydrolytic fermentation process. A BLAST analysis was performed on the representative sequences of these 35 OTUs. The best hit in GenBank was chosen to identify the highest similarity to known species, and the literature on metabolic functions of these species was consulted to generate a table that compared the species results (see Table 2).

The analysis for 10 OTUs with an average r.a. over 1% is summarized below (note: when the sequence similarity is ≥97% to a described strain or species, and when the comparison result is the closest, the group represented by the sequence is considered to equate the known strain or species. Its metabolic functions were then inferred based on this assumption).

First, we zoom in at members of *Clostridium* species. The r.a. of the most dominant OTU, B-OTU1 (B stands for bacteria), was between 16.17% and 27.30%. Its 16S sequence presents, a similarity to *Clostridium cellulovorans* of 99%. These bacteria are typical decomposers of cellulose and hemicellulose. Its metabolites mainly include H₂, CO₂, acetic acid, butyric acid, formic acid, and lactic acid, products of hydrolysis, and fermentation. These bacteria are also anaerobic fermentative hydrogen producers. After initiation of fermentation, the r.a. of B-OTU1 had increased gradually until day 140 of fermentation. The second most dominant OTU, B-OTU2, had a r.a. gradually increased up to day 70 of fermentation, with a small peak on day 30. Their abundance dynamics in the first 70 days were similar to that of B-OTU1. These two main species seemed to compete and metabolize the available substrates at the initial stage of fermentation at equal numbers. From day 70 onwards, B-OTU2 gradually increased to reach its highest peak (27.15%) on day 110 of fermentation, lagging behind the highest abundance peak of B-OTU1 (100 days of fermentation). This finding suggests that B-OTU2 ferments soluble sugars produced by B-OTU1 when that already hydrolyzes hemicellulose. Therefore, the r.a. of B-OTU2 showed a gradual decline, although there was another peak at day 140, when the bacteria again profited from B-OTU1 hydrolyzing cellulose to produce soluble sugars. Similar observations were made for B-OTU3 (r.a. 3.74%-10.76%), B-OTU318 (r.a. between 4.36% and 7.83%), B-OTU5 (r.a. between 0.73% and 1.30%, numbers 7 and 10, respectively, in order of abundance) produced sequences that were 100% identical to *Clostridium beijerinckii* and 98% identical to *Clostridium butyricum* and 98% identical to *Clostridium beijerinckii*, respectively. These are typical amylolytic bacteria, mainly producing metabolites such as butyric, acetic, and formic acid, together with the gases H₂ and CO₂.

The second most dominant OTU, B-OTU2, had a r.a. between 15.23% and 27.15%, and its variable 16S region was 99% identical to *Terrisporobacter petrolearius*. These bacteria can use monosaccharides such as glucose, fructose, and maltose. Their metabolites are mainly acetic acid and CO₂, which is typical for fermentative bacteria. Their r.a. gradually increased up to day 70 of fermentation, with a small peak on day 30. Their abundance dynamics in the first 70 days were similar to that of B-OTU1. These two main species seemed to compete and metabolize the available substrates at the initial stage of fermentation at equal numbers. From day 70 onwards, B-OTU2 gradually increased to reach its highest peak (27.15%) on day 110 of fermentation, lagging behind the highest abundance peak of B-OTU1 (100 days of fermentation). This finding suggests that B-OTU2 ferments soluble sugars produced by B-OTU1 when that already hydrolyzes hemicellulose. Therefore, the r.a. of B-OTU2 showed a gradual decline, although there was another peak at day 140, when the bacteria again profited from B-OTU1 hydrolyzing cellulose to produce soluble sugars. The relative abundance of B-OTU5 is between 4.63% and 8.47%, and it is the fourth dominant OTU. It has an identity to *Clostridium saudii* of 99%, again a species with cellulose and hemicellulose decomposing properties. B-OTU5 (r.a. between 1.15% and 1.72%) and B-OTU1141 (r.a. between 0.73% and 1.30%, respectively, in order of abundance) produced sequences that were 100% identical to *Clostridium beijerinckii*, *Clostridium butyricum*, and *Terrisporobacter petrolearius*. These bacteria can use monosaccharides such as glucose, fructose, and maltose. Their metabolites are mainly acetic acid and CO₂, which is typical for fermentative bacteria. Their r.a. gradually increased up to day 70 of fermentation, with a small peak on day 30. Their abundance dynamics in the first 70 days were similar to that of B-OTU1. These two main species seemed to compete and metabolize the available substrates at the initial stage of fermentation at equal numbers. From day 70 onwards, B-OTU2 gradually increased to reach its highest peak (27.15%) on day 110 of fermentation, lagging behind the highest abundance peak of B-OTU1 (100 days of fermentation). This finding suggests that B-OTU2 ferments soluble sugars produced by B-OTU1 when that already hydrolyzes hemicellulose. Therefore, the r.a. of B-OTU2 showed a gradual decline, although there was another peak at day 140, when the bacteria again profited from B-OTU1 hydrolyzing cellulose to produce soluble sugars. Similar observations were made for B-OTU3 (r.a. between 4.36% and 7.83%), B-OTU318 (r.a. between 4.36% and 7.83%), B-OTU6 (0.21%-2.87%), and B-OTU7 (0.01%-7.22%). B-OTU3, the third dominant OTU, produced 99% identity to *Turicibacter sanguinis*. B-OTU318, number 5 in terms of abundance, was 99% identical to *Romboutsia timonen-sis* sequences. Number 8, B-OTU6 produced 96% identity to *Ruminococcus gauvreauii*, and finally, B-OTU7, the ninth dominant OTU, produced a hit with 96% identity to *Bacteroides graminisolvens*.

The sixth dominant OTU is also of interest to point out here. The relative abundance of B-OTU4 was between 0.47% and 8.38%, and its sequences were 100% identical to *Streptococcus galloyticus*. This species is typical proteolytic. The relative abundance of B-OTU4 peaked around day 30 and then showed a rapid decline to only 0.47% by the
| B-OTU   | Relative abundance (%) | Closest species (similarity) | Accession number | Main substrates/main products                                                                 | Classification (Phylum/Genus) |
|--------|------------------------|-----------------------------|------------------|------------------------------------------------------------------------------------------------|-----------------------------|
| B-OTU1 | 16.17-27.30            | *Clostridium cellulovorans* (99%) | KF528156.1       | Cellulose, pectin, glucose, maltose/H₂, CO₂, acetic acid, butyric acid                        | Firmicutes/Clostridium     |
| B-OTU2 | 15.23-27.15            | *Terrisporobacter petrolearius* (99%) | NR_137408.1      | Glucose, fructose, maltose, xylose/CO₂, acetic acid                                         | Firmicutes/Terrisporobacter |
| B-OTU3 | 3.74-10.76             | *Turicibacter sanguinis* (99%) | HQ646364.1       | Maltose/lactic acid                                                                           | Firmicutes/Turicibacter     |
| B-OTU555 | 4.63-8.47             | *Clostridium saudii* (99%) | NR_144696.1      | Cellulose, hemicellulose, glucose/acetic acid, butyric acid                                  | Firmicutes/Clostridium     |
| B-OTU318 | 4.36-7.83              | *Romboutsia timonensis* (99%) | KT835017.1       | Protein, cellulbiose, fructose, glucose/lactic acid                                          | Firmicutes/Streptococcus   |
| B-OTU4 | 0.47-8.38              | *Clostridium butyricum* (100%) | CP013239.1       | Starch, glucose and sucrose/butyric acid, acetic acid, H₂, CO₂                                 | Firmicutes/Clostridium     |
| B-OTU6 | 0.21-2.87              | *Ruminococcus gauvreuili* (96%) | NR_044265.1      | Glucose, galactose, fructose/acetic acid                                                      | Firmicutes/Ruminococcus    |
| B-OTU7 | 0.01-7.22              | *Bacteroides graminisolvens* (100%) | KT321286.1      | Pectinose, xylose, glucose/acetic acid, propionic acid                                       | Bacteroidetes/Bacteroides  |
| B-OTU1141 | 0.73-1.30            | *Clostridium beijerinckii* (98%) | NR_113388.1      | Starch, glucose and sucrose/butyric acid, acetic acid, H₂, CO₂                                | Firmicutes/Clostridium     |
| B-OTU10 | 0.75-1.34              | *Cellulosilyticum lentocellum* (98%) | NR_074536.1      | Cellulose, xylose and maltose/formic acid, acetic acid, CO₂                                  | Firmicutes/Cellulosilyticum |
| B-OTU16 | 0.70-1.14              | *Clostridium chartatabidum* (98%) | NR_029239.2      | Cellulose, sucrose, fructose, glucose/acetic acid, butyric acid, H₂                          | Firmicutes/Clostridium     |
| B-OTU8 | 0.51-1.43              | *Tangfeifania diversioriginum* (87%) | NR_134211.1      | Starch, ribose, xylose, fructose/VFAs                                                        | Bacteroidetes/Tangfeifania |
| B-OTU13 | 0.62-1.03              | *Clostridium lavalense* (98%) | EF564278.1       | Glucose, fructose, lactose/acetic acid, lactic acid, fumaric acid                             | Firmicutes/Clostridium     |
| B-OTU9 | 0.16-2.19              | *Sunxiuqinia faeciviva* (89%) | NR_108114.1      | Tyrosine/VFAs                                                                                | Bacteroidetes/Sunxiuqinia  |
| B-OTU11 | 0.01-5.44              | *Atopostipes suiciolaclis* (98%) | NR_028835.1      | Glucose, lactose, maltose/lactic acid, propionic acid, acetic acid, formic acid               | Firmicutes/Atopostipes     |
| B-OTU22 | 0.06-1.73              | *Lactobacillus reuteri* (100%) | KP317691.1       | Ribose, xylose, fructose, lactose/lactic acid                                               | Firmicutes/Lactobacillus   |
| B-OTU15 | 0.11-1.45              | *Saccharicrinis marinus* (87%) | NR_137404.1      | Cellulbiose, maltose, lactose/VFAs                                                           | Bacteroidetes/Saccharicrinis |
| B-OTU17 | 0.08-2.05              | *Cloacibacillus porcorum* (93%) | CP016757.1       | Amino acid/acetic acid, propionic acid, formic acid                                          | Synergistetes/Cloacibacillus |
(Continues)
end of fermentation. This is because the bacteria degraded proteins until this substrate became in short supply. It was further noticed that lipolytic bacteria, such as B-OTU28, were also found in the top 35 OTUs, with a r.a. between 0.02% and 0.69%, and an identity to *Pseudomonas caeni* of 100%.

### 3.3 Archaeal community structure and relative abundance

For archaeal species found in the fermentation mixture, OTUs representing methanogens with average relative abundance greater than 0.1% were selected. The results are shown in Table 3.
Figure 4 demonstrates the abundance of OTUs with an average r.a. of greater than 1% as a function of fermentation time.

A-OTU1 (r.a. between 4.15% and 37.14%) was the most dominant archaeal OTU, and this was 98% identical to Methanocorpusculum sinense, based on rDNA sequences. This species is a typical hydrogen consumer, metabolizing this and CO₂ to CH₄. This high abundance indicates that the production of CH₄ in the system was primarily due to reduction in carbon dioxide. From the beginning of fermentation on to day 60, the r.a. of A-OTU1 was maintained at around 30%, as the archaea profited from H₂ and CO₂ produced by the hydrolytic activity of fermentative bacteria. Subsequently, the r.a. of A-OTU1 decreased rapidly to its lowest value on day 80. A gradual recovery followed, with a peak at day 150 with its highest abundance. By this time, the archaea could utilize...
the fermentation products of hemicellulose and cellulose. A-OTU8 (99% identity to Methanobrevibacter millerae) is also a hydrogen-using methanogen but it was less abundant (r.a. 0.11%-9.31%) than M. sinense.

The second most abundant archaea was A-OTU2 (r.a. 1.45%-23.21%) which belongs to uncultured MCG archaea. This group of uncultured archaea that typically can degrade aromatic compound is widely distributed. Members of this group can also degrade chitin, cellulose, and protein. After initializing fermentation, the relative abundance of A-OTU2 showed a gradual decline and reached the lowest value on the 80th day of fermentation. Subsequently, a trend of rapid recovery began with a peak at days 110-120 and again at days 140-150 of fermentation. The first of these two peaks indicates that MCG archaea were benefiting from catabolism of hemicellulose, and the latter peak may be driven by decomposition of cellulose. A-OTU1026 (r.a. 0.41%-2.68%) and A-OTU16 (r.a. 0.37%-2.30%) also belong to the group of MCG archaea.

The fifth most abundant archaea, A-OTU9 (r.a. 0.37%-3.25%, reported 99% identity with Methanosarcina soligeldi), is a facultative vegetative methanogen, which can not only produce methane from acetic acid, but also metabolize H2 and CO2 to CH4. From the start of fermentation to day 70, its relative abundance was stable at about 1%. Subsequently, it increased rapidly, with peaks on days 90, 120, and 150. Presumably, the products of hemicellulose and cellulose degradation supported its growth.

Among the 11 methanogen OTUs with an average relative abundance of more than 0.1%, there were eight OTUs belonging to the hydrogen-trophic methanogens, and their similarity with known methanogens was between 98% and 100%. These in combination sum up to an average relative abundance of 23.68%. There were two OTUs belonging to facultative methanogens, with DNA sequence similarities between 98% and 99%, and average relative abundances of 1.78%. We identified one OTU belonging to a methanogen using acetic acid as the main substrate, with 100% identity to a known species (see Table 3) but an average relative abundance of only 0.24%.

3.4 Correlation between environmental factors

A strongly significant positive correlation was found between B-OTU1 (responsible for decomposition of hemicellulose and cellulose) and the two fermentative acidogenic species B-OTU2 and B-OTU318 (c.c. 0.799**, and 0.676**, respectively). In addition, the major methanogens A-OTU1, A-OTU8, the hydrolyzed bacteria, and the fermentative acid-producing bacteria all produced a significant or extremely significant positive correlation between each other, implying the significance of these dominant hydrogenotrophic methanogens to degrade and ferment the used matrix.

3.5 Biogas metabolic pathway–based dominant bacterial species and archaeal species

The theoretical explanations for converting biomatter into biogas by fermentation either include four stages, as proposed by Eckenfelder and colleagues in 1961, two stages as proposed by Mckinney in 1962, or three stages as proposed by Lawrence and McCarty in 1967. At present, the three-stage theory is generally recognized and accepted by most scholars. For this reason, the dominant bacterial groups and methanogenic bacteria obtained in this study were mapped to the crucial steps in the three-stage theory of biogas fermentation to construct a low-temperature biogas metabolic pathway based on OTU descriptions at the species level. The result is shown in Figure 5.

The first stage covers the hydrolysis and fermentation stage. During the hydrolysis stage, cellulose and hemicellulose are hydrolyzed by bacteria, in our case for example B-OTU1 and B-OTU555; protein is hydrolyzed by bacteria such as B-OTU4, fat might be hydrolyzed with B-OTU28 as a suitable candidate, and starch hydrolysis might be performed by B-OTU5 and B-OTU1141. These hydrolyzing bacteria secrete extracellular hydrodrolases that hydrolyze the complex organic matters (water-insoluble carbohydrates, proteins, and lipids) to produce small water-soluble organic molecules (carbohydrates are hydrolyzed into soluble sugars, protein into amino acids, and lipids into long-chain fatty acids). In the fermentation stage, bacteria ferment monosaccharides (eg. by B-OTU2, B-OTU3, B-OTU318, B-OTU7) and produce acid. Likewise, amino acids are fermented by B-OTU32, B-OTU19, B-OTU18, and long-chain fatty acids by B-OTU5; this constitutes a bacterial community with fermentative, acidogenic, and hydrogen production functions. These bacteria take up short-chain fatty acids (such as acetic, propionic, butyric, or lactic acid) to produce CO2 by fermentation. Hydrolyzing bacteria and fermentative bacteria together play a key role in hydrolytic fermentation, and their function is to "start" the biogas metabolic pathway, so that they are the initial rate-limiting factor for biogas fermentation.

The second stage is that of hydrogen production and acetic acid production. During this stage, hydrogen-producing bacteria further degrade the short-chain fatty acids produced during the hydrolysis and fermentation stages, to produce acetic acid and H2. In some cases, CO2 can be generated from the reaction. In this study, we found a propionic acid-degrading bacterium B-OTU291 (96% identity to Pelotomaculum schinkii, accession number NR_119207.1) and butyric acid/pentanoic acid-degrading bacteria B-OTU53 (95% identity to Syntrophomonas zeihnderi, accession number NR_112642.1). These and other members of the bacterial community are likely responsible for hydrogen production and acetogenic activity, although the named candidates were not very abundant (r.a. of B-OTU291 was between 0.00% and 0.03%, that
of B-OTU53 between 0.01% and 0.44%). The conversions of the propionic acid, butyric acid, isobutyric acid, and isopentanoic acid to acetate acid need hydrogen-producing acetogens. Although the abundance of hydrogen-producing acetogens was low in bacterial community structure in our research, the functions on substrate conversion could not be ignored. Based on the data in Table 1, the VFAs (except acetate acid) varied in a relatively wide extent, demonstrating the function of hydrogen-producing acetogens. So, the hydrogen and acetic acid production stage is still important for low-temperature digestion. But why is the abundance of hydrogen-producing acetogens so low in this study? We think it is mainly due to ambient temperature. In our study, *S. zehnderi* is the most abundant bacteria in hydrogen-producing acetogens. Sousa et al.\(^2^2\) have found that the growth temperature range of *S. zehnderi* is 25-40°C during the pure culture of this strain. The most suitable growth temperature for this strain is 37°C. But the fermentation temperature of our experiments is 9°C, which is not in the growth temperature range of the strain, which leads to the growth of the strain is very slow, and which leads to a low abundance. However, the low abundance does not mean that the strain cannot play a metabolic role.

During the third, methanogenic stage, methane is actually produced. This is performed by methanogens that convert various metabolites (in this case mainly from the hydrolytic fermentation stage rather than from the hydrogen production stage) to CH\(_4\) through various pathways. Hydrogenotrophic methanogens such as A-OTU1, A-OTU8, and facultative methanogen A-OTU9 would use H\(_2\) and CO\(_2\) to form CH\(_4\).\(^{23}\) The nutrient-producing methanogens, such as A-OTU28, and facultative methanogen A-OTU9 use acetic acid to form CH\(_4\).\(^{23}\) The methanogens become active toward at the end of the biogas metabolic pathway, and they are the core biogas fermentation microorganisms. In this study, hydrogenotrophic methanogens accounted for the highest proportion of methanogens, suggesting low temperature favors growth of hydrogenotrophic methanogens. This also further indicates that CH\(_4\) production mainly stems from H\(_2\) and CO\(_2\).

### 4 | DISCUSSION

The results on low-temperature biogas fermentation reported here are in agreement with works by others, who have investigated the advantages of hydrolyzing fermentative bacteria in such systems. Seib et al.\(^2^3\) conducted a study on municipal wastewater in a 10°C biogas fermentation system and found *Clostridium* as the main dominant bacterial genus. The same genus was reported as being dominant when Dai et al.\(^2^5\) used wetland soil of the Qinghai-Tibet Plateau as inoculum to ferment cellulose and chitin at 15°C\(^\pm^\)\(^\circ\). Bialek et al.\(^7\) used dairy farm wastewater as raw material to carry out low-temperature biogas fermentation at 10°C and reported that *Clostridium aminobutyricum* was the most abundant bacterial species. Tian et
al$^{25}$ conducted a study on pig manure in a 15°C batch biogas production system and found *Clostridium sartagoforme* (decomposes cellulose and chitin) was dominant at the hydrolysis stage. *Clostridium* species thus seem to be dominant when the raw material is rich in macromolecular carbohydrates such as cellulose and hemicellulose. However, *Clostridium* is not exclusively responsible for low-temperature fermentation, as it can also be abundant in medium-temperature biogas fermentation systems, demonstrated, for example, by Rui et al$^{26}$ with 13 medium-temperature pig manure biogas engineering systems studied. This shows that members of this genus are strongly adaptable to the environment and that similar bacterial communities are active in medium-temperature and low-temperature biogas fermentation systems.

Reduction in carbon dioxide by consumption of hydrogen is the major pathway of CH$_4$ production in this study. This result is consistent with the research by others. O'Reilly et al$^{27}$ used a 15°C biogas fermentation system with glucose wastewater and found that the hydrogenotrophic methanogen *Methanocorpusculum* was most abundant. McHugh et al$^{28}$ used wastewater containing sucrose and VFAs as raw material and varied the fermentation temperature from 37°C to 16°C. This revealed that hydrogen-consuming methanogens were gradually replaced by acetic acid-consuming methanogens as the fermentation temperature declined. Tian et al$^{25}$ used pig manure as raw material to carry out low-temperature biogas fermentation at 15°C and found that *Methanobacterium formicicum* which can convert CO$_2$ and H$_2$ into methane in the methanogenesis stage was the most abundant archaeal species. Xing et al$^{29}$ fermented algae as raw material at 15°C and found that hydrogen-consuming Methanomicrobiales and Methanobacteriaceae were dominant. These findings may be related to the ability of hydrogenotrophic methanogens to adapt to a cold environment. For example, *Methanocorpusculum* has a growth temperature range of 15-45°C, and some strains have a minimum growth temperature between 3.6°C and 5°C$^{15}$; on the other hand, it may be related to the lower energy required for the hydrogenotrophic methanogenesis pathway at low temperatures. Lettinga et al$^{30}$ studied the Gibbs free energy of the main biochemical reactions during fermentation at 37°C and 10°C. This revealed that at 10°C, the pathways using nutrients that require lower energy for CH$_4$ production are preferred, related to the relatively higher solubility of H$_2$ and CO$_2$ at low temperatures (causing H$_2$ and CO$_2$ to be more easily utilized by methanogens). For example, at 10°C, 1 volume of water at standard atmospheric pressure can dissolve about 1.2 volumes of CO$_2$, while at 35°C, only about 0.6 volumes of CO$_2$ can be dissolved. At 10°C, the pathway using acetic acid requires more energy. This is because the low temperature causes an increase in the viscosity of the liquid, which reduces the diffusion coefficient of soluble compounds such as acetic acid. For example, diffusion coefficient of acetic acid is 1.26 at 40°C and only 0.57 at 10°C. Therefore, Lettinga et al used thermodynamics to show that hydrogen consumption is a more favorable methanogenic pathway than acetic acid consumption at low temperature.

Not all publications agree with our findings. Dong et al$^{31}$ collected fermented sludge from a rural household biogas digester in Shangri-La, Yunnan Province, which operated on pig manure at 12°C, and identified the dominant methanogen to be *Methanosarcina*. The reason for this different finding to our study may be related to the ammonia nitrogen concentration in the raw material. During our experiment, the ammonia nitrogen concentration ranged from 423 to 729 mg/L, which was 2.6-4.4 times higher than reported by Dong and colleagues (165 mg/L). McCarty$^{32}$ described that ammonia nitrogen is beneficial to biogas fermentation at concentrations between 50 and 200 mg/L. Beyond this range, it hampers fermentation. Angelidaki and Ahring$^{33}$ found that acetic acid-consuming methanogens are more sensitive to ammonia nitrogen than hydrogen-consuming methanogens because the latter can use NH$_3$ as the sole nitrogen source. Rui et al$^{26}$ reported the tolerance of different nutrient types methanogens to ammonia nitrogen to be in the order hydrogen consumers > facultative hydrogen and acetic acid consumers > acetic acid consumers. Thus, a constant low ammonia nitrogen concentration is related to the fermentation mode. Most household biogas digesters are semi-continuous fermentation that are daily discharged and resupplied with raw material, a practice that can actually result in regular removal of ammonia nitrogen. In contrast, ammonia nitrogen accumulated in the studied batch fermentor used in this study, with consequences to the microorganism communities.

5 | CONCLUSION

In a low-temperature biogas production system, we detected a rich microbial diversity, with between 853 and 1111 bacterial OTUs and between 561 and 967 archaeal OTUs, but only a few dominant species played key metabolic roles in hydrolytic fermentation and subsequent methanogenesis: only 10 bacterial and three archaea OTUs had an average relative abundance greater than 1%. The properties of the inferred species to which these OTUs most likely belonged were all relevant to the fermentation process.

From inferred function derived from 16S rDNA identity to reference organisms, we conclude that hydrolysis of cellulose/hemicellulose was mainly performed by B-OTU1 and B-OTU555, while protein was hydrolyzed by B-OTU4, fat by B-OTU28, and starch by B-OTU5 and B-OTU1141. The bacterial population responsible for fermentation was composed of members fermenting monosaccharides (B-OTU2, B-OTU3, B-OTU318, B-OTU7), amino acids (B-OTU32,
B-OTU19, B-OTU18), and long-chain fatty acids (B-OTU5). Methane was produced from carbon dioxide by archaea that mainly consumed hydrogen (A-OTU1, A-OTU8) and by facultative nutrient methanogens (A-OTU9).

We constructed the biogas metabolic pathway based on OTU levels, which indicated that the hydrogen-consuming methanogens such as A-OTU1 and A-OTU8 were mainly responsible for CH₄ production under low-temperature conditions.

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