Effects of environmental factors on low temperature anaerobic digestion of pig manure

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Abstract

In this study, we investigated the inhibitory effect of low temperature on biogas fermentation system. Biogas fermentation inoculum was domesticated at 4 °C, and subsequently inoculated into pig manure feedstock in a batch biogas fermentation process. A low-temperature biogas fermentation system was maintained at 9 °C, and its abiotic factors, bacterial community, and archaeal community were determined. The results showed that (1) the biogas fermentation lasted for 160 days, with a total gas production of 19,150 ml, including N\(_2\) production of 11,370 ml, CO\(_2\) production of 3,534 ml, and CH\(_4\) production of 3,031 ml. (2) The average relative abundance of the primary dominant bacterium operational taxonomic unit (OTU) was 30%, with 100% similarity to Pseudomonas caeni, atypical denitrifying bacterium. The average relative abundance of the secondary dominant bacterium OTU was 20.36%, with 99% similarity to Clostridium cellulovorans, a typical cellulose- and hemicellulose-degrading bacterium. The average relative abundance of the primary dominant methanogenic archaeon OTU was 7.22%, with 99% similarity to Methanosphaera cuniculi, a typical methylotrophic methanogen, and the secondary dominant methanogenic archaeon OTU had an average relative abundance of 4.15%, with 98% similarity to Methanocorpusculum sinense, a typical hydrogenotrophic methanogen. (3) The primary dominant bacterial genus Pseudomonas exhibited highly significant negative correlations with the dominant biogas-producing microorganisms such as Clostridium, Terrisporobacter, Turicibacter, Methanosphaera, and Methanocorpusculum. We concluded that (1) high concentrations of abiotic factors such as ammonia nitrogen, acetic acid, and propionic acid were important indicators of relatively poor operation of low-temperature biogas fermentation systems, while (2) high numbers of MCG archaea and Pseudomonas were important biotic factors showing comparatively poor operation of the system.

1. Introduction

Biogas technology can use a variety of organic wastes to produce clean energy and organic fertilizer, so it is widely used in the world to solve the problems of energy crisis, environmental pollution and sustainable agricultural development. In terms of high value utilization of biogas, Europe is in the forefront of the world. There are 17783 biogas projects in Europe, with a total installed capacity of 10532 MW [1]. Among them, Germany has built 10971 biogas projects, with an annual biogas output of more than 20 billion cubic meters, making it the largest biogas development country in Europe [1]. The production of biogas in Germany mainly comes from agricultural wastes and municipal solid wastes. Italy and France ranked second and third in the number of biogas projects in Europe, with 1655 and 742 respectively [1]. The rapid development of biogas in
Europe benefits from advanced biogas utilization technology, the mode of adapting measures to local conditions and relevant incentive policies [2]. 2116 biogas projects have been completed in the United States, including 239 biogas projects in livestock and poultry farms, 636 biogas projects in garbage landfills, and 1241 biogas projects in sewage treatment plants [3]. The United States is the world leader in the field of landfill waste recycling biogas, and the biogas output of landfills accounts for 43% of the biogas output of the United States [3].

Currently, biogas fermentation technology is one of the major methods in the treatment of livestock manure, converting organic matter into CH₄, a clean fuel, thereby eliminating environmental pollution problems. However, the biogas fermentation process is affected by numerous environmental factors, such as low temperature. Temperature is closely related to the metabolic capacity of microorganisms, and is an important process parameter to control the biogas fermentation performance. Within a certain range, the higher the temperature, the more vigorous the metabolism of biogas microorganisms, and the more conducive to improving the biogas production. According to different fermentation temperatures, biogas fermentation can be divided into low temperature fermentation (4 °C–20 °C), near medium temperature fermentation (20 °C–30 °C), medium temperature fermentation (30 °C–40 °C) and high temperature fermentation (50 °C–60 °C) [4, 5].

In the low-temperature biogas fermentation system, the low-temperature environment will not only inhibit the metabolic activity of the biogas bacteria, reduce the rate of biochemical reaction, but also increase the viscosity of the fermentation broth and reduce the degradation efficiency of organic matter [6]. Low temperature is the main limiting factor for the development of biogas fermentation technology in high latitudes [7]. In China, for example, high latitude provinces such as Heilongjiang, Jilin, and Liaoning have abundant livestock manure and crop straw resources; however, the rate of progress of biogas projects in these areas has been far behind that at the national level. For instance, Jilin province only has 59 biogas generation projects, whereas the total number of biogas projects in the country is 7737 [8]. To improve the efficiency of biogas fermentation in low-temperature environment, various heating processes, such as solar power heating, gas boiler heating, and heat pump heating, have been used in engineering practice to increase the operating temperature of the biogas fermentation system [9]. Although these approaches improved the temperature of the system during anaerobic digestion and increased the CH₄ yield, they raised the project investment and operation cost to a great extent. Meanwhile, as the heat treatment of low-temperature biogas fermentation system could only lessen the problem and is not an ultimate solution, the negative effects of low temperature on biogas fermentation has not been completely eliminated.

Over 70% of the biosphere is in a cold environment [10], which provides low-temperature habitats for a wide variety of psychrophilic and psychrotrophic microorganisms adapting to various cold environments. In recent years, psychrophiles and psychrotrophs have become a hot research topic in the study of extremophiles [11], including psychrophilic anaerobic microorganisms [12]. Song [13] isolated a psychrophilic cellulolytic bacterium CD-2 (the strain was 99.1% similar to *Clostridium papyrosolvens* and grew in the temperature range of 5 °C ~ 40 °C) from the muddy environment of Zoige meadow in Tibet, which was cultured, multiplied, and applied to an experimental low-temperature (15 °C) pig manure biogas fermentation system. The results showed that the strain had promoted the low-temperature biogas fermentation to a certain extent. Li et al [14] isolated a psychrophilic methanogenic strain LN-cl (99% similarity to *Methanocorpusculum labreanum*, with a growth temperature range of 4 °C ~ 45 °C) from the bottom sludge of a reservoir in Liaoning in winter, and found that the strain could undergo normal fermentation at 9 °C to produce CH₄.

In the normal low-temperature biogas fermentation system, researchers found that *Clostridium*, hydrogen trophic methanogens *Methanobacterium* are important biological factors indicating the good operation of low-temperature biogas fermentation system [15, 16], but it is not clear which biological factors indicate the poor operation of low-temperature biogas fermentation system, and it is necessary to further explore. Since biogas fermentation is a biological metabolic process [17], it is essential to elucidate the biotic factors that play an inhibitory role in low-temperature biogas fermentation systems to attenuate or even eliminate the inhibitory effect of low temperature on biogas fermentation. Therefore, in this study, biogas fermentation inoculum was domesticated at a low temperature of 4 °C, and further inoculated into pig manure feedstock in a batch fermentation process. A low-temperature biogas fermentation system was maintained at 9 °C. Abiotic factor analysis, biotic factor analysis (16 S rDNA amplicon sequencing), and SPSS correlation analysis were used to examine biotic and abiotic factors in the tested low-temperature biogas fermentation system and investigate the inhibitory effects of low temperature on pig manure biogas fermentation. The purpose of this study is to identify the biological factors that play an inhibitory role in the low-temperature biogas fermentation system, and then provide a basis for the selection of inoculum.
2. Materials and methods

2.1. Experimental setup

The experimental device of this study is shown in figure 1. The experimental device consists of three parts: a biogas fermentation system, a gas storage system and a water bath refrigeration system. The fermentation device of the anaerobic digestion system is a glass batch fermentation reactor with an effective volume of 10 l; the device of the gas storage system is a plexiglass low-pressure wet gas storage cabinet with a volume of 5 l; the refrigeration system consists of a refrigerator (MA-01 type, Jiaxing Xinmaqino Machinery Co., Ltd, China) and stainless steel water bath.

2.2. Raw materials and inoculum

The biogas fermentation raw material used in this study was fresh pig manure, which came from a large pig farm in Fumin County, Kunming City, China. The total solid (TS) of the pig manure was determined to be 29.76%, with the 81.11% of volatile solid (VS) on TS basis. The biogas fermentation inoculum used in this study was the anaerobic sludge stored at 4 °C for 1 year, and its TS was 6.40% and VS was 65.29% (TS basis). pH values of inoculum and raw material were 7.12 and 7.50 respectively.

2.3. Experimental methods

The biogas fermentation process used in this study was a fully mixed batch process with an anaerobic digestion temperature of 9 °C. In this study, the biogas fermentation feed solution was prepared according to the ratio of raw materials to microorganisms (F/M) [18], that was, the ratio of volatile solids of pig manure to inoculum (VS/VS) of 0.75 [19]. The total volume of the feed liquid was 10 l, in which the amount of inoculum was 200 g (calculated by VS), the amount of pig manure was 150 g (calculated by VS), and water was added to 10 l.

After the start of the experiment, for abiotic factors, the gas production was recorded regularly every day, and samples were taken every 10 days to measure gas composition, pH, TS, VS, soluble chemical oxygen demand (sCOD), ammonia nitrogen, and volatile fatty acids (VFAs); After the biogas fermentation experiment was over, the feed liquid samples of different biogas fermentation periods were tested for biological factors.

2.4. Abiotic factors and related analyses

The gas was collected by the gas storage cabinet, and the volume measurement scale was marked on the bell of the gas storage cabinet, and the gas output was measured regularly every day. The instruments for measuring TS and VS were electric heating constant temperature blast drying oven (DHG-9070A, Shanghai Yiheng Scientific Instrument Co., China) and box-type resistance furnace (SX-5-12, Tianjin Test Instrument Co., China), Determination methods were drying method and combustion method [20]. Gas chromatograph (Model GC-6890A, Lunan Analytical Instrument Co., China) was used to measure the gas composition, pH meter (Model PHS-25, Shanghai Jinmai Instrument Co., China) was used to measure pH, and sCOD was measured by COD analyzer (CODmax II, Hach Company, USA), ammonia nitrogen analyzer (Amtax Compact II, Hach Company, USA), and gas chromatograph (GC-9790 II, Zhejiang Fuli Analytical Instrument Co., China) for the determination of VFAs, the instrument operation procedures for measuring various indicators refer to the instruction manual.

Figure 1. Schematic diagram of the test setup for low-temperature anaerobic digestion system. (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).
2.5. Biotic factors and related analyses

2.5.1. DNA extraction and PCR amplification
The sample DNA was extracted using a magnetic bead method DNA extraction kit (DP328, Tiangen Biochemical Technology Co., China), and the extraction was carried out according to the steps listed in the kit’s instruction manual. Then use 1% agarose gel electrophoresis to detect the purity and concentration of DNA. After passing the test, take an appropriate amount of DNA sample into a centrifuge tube and dilute to 1 ng μl⁻¹ with sterile water [21].

Using the diluted genomic DNA as a template, the V3 ~ V4 variable regions of bacterial 16S rDNA and the V4 ~ V5 variable regions of archaeal 16S rDNA were selected as amplification regions, and bacterial primers with Barcode 341F/806R and archaeal primers were used. PCR amplification was performed with Arch519F/915R [22, 23].

Mix equal amounts of samples according to the concentration of PCR products and mix them well, then used 1 × TAE running buffer to make agarose gel (gel concentration is 2%) and conducted electrophoresis, and selected the main band size between 400 and 450 bp [24]. The target bands were recovered by gel tapping, and the PCR products were recovered and purified using The GeneJET Gel Extraction Kit (K069, Thermo Fisher Scientific, USA).

2.5.2. Library construction and sequencing
The library construction kit Ultra™DNA Library Prep Kit for Illumina (New England Biolabs, USA) was used for library construction, and the constructed library was used for library quality assessment (Qubit and Q-PCR quantification). After the library was qualified, HiSeq2500 PE250 was used On-board sequencing was performed on the platform.

For the off-machine data (PE reads) obtained by the Illumina HiSeq sequencing platform, after removing the barcode and primer sequences, FLASH was used to splicing the reads of each sample, and the obtained splicing sequence was the original Tag data (Raw Tags), refer to Qiime’s Tags in the quality control process, the spliced Raw Tags were filtered to obtain high-quality Tags data (Clean Tags); the Clean Tags were compared with the database (Gold database), the chimera sequences were detected, and the chimera sequences were removed by UCHIME Algorithm software. Combine the sequence to get the final effective data (Effective Tags) [25].

2.5.3. Operational taxonomic unit (OTU) clustering and taxonomic annotation
Uparse software was used to cluster all the Effective Tags of all samples, and the sequences were clustered into OTUs (Operational Taxonomic Units) with 97% identity. At the same time, the representative sequences of OTUs were selected, and species annotation was performed on the representative sequences of OTUs., using the Mothur method and SILVA’s SSUrRNA database to perform species annotation analysis to obtain taxonomic information and count the community composition of each sample at each taxonomic level [26].

2.6. Correlation analysis
In this study, the correlations between bacterial communities, between archaeal communities, and between bacterial communities and archaeal communities were analyzed [15]. The related data were statistically processed by using SPSS software (Version 22.0) based on two-sided T test and Pearson correlation analysis.

The systematic diagram for the methodology of our study is shown in figure 2.

3. Results and analysis

3.1. Dynamics of abiotic factors at different biogas fermentation stages
Figures 3–8 show the dynamic changes in abiotic factors in the low-temperature biogas fermentation system at different stages of anaerobic digestion in this study. The TS content of the fermentation broth generally showed a decreasing trend after the onset of fermentation, and the TS content decreased by approximately 1% after fermentation. Meanwhile, the VS content of the fermentation broth exhibited no noticeable decreasing trend, indicating that the water-insoluble macromolecular organic compounds in the system could be degraded by the biogas fermentation microorganisms, but the utilization degree was low. After fermentation was started, the sCOD content showed a gradual increase and reached a peak at the 100th day of fermentation. Thereafter, the sCOD content remained approximately 20,000 mg l⁻¹, which was double the content at the onset, and finally a maximum content of 1,400 mg l⁻¹ was reached. The average concentration of acetic acid and propionic acid jointly accounted for 90.81% of the average total acid concentration, indicating that acetic acid and propionic acid were the main
Figure 2. The flowchart for the methodology of the study.

Figure 3. Variation curve of TS and VS with fermentation time in the system.
Figure 4. Variation curve of sCOD and NH₃-N with fermentation time in the system.

Figure 5. Variation curve of pH with fermentation time in the system.

Figure 6. Variation curve of acetic acid with fermentation time in the system.
volatile fatty acids of the system. The concentration of acetic acid showed a rapid increase after the onset of fermentation and reached the maximum value (6,308.13 mg l\(^{-1}\)) at the 60th day of fermentation. Thereafter, the acetic acid concentration was stable at approximately 5,000 mg l\(^{-1}\), indicating that the biogas-producing microorganisms in the system did not subsequently utilize acetic acid. The propionic acid concentration underwent a rapid increase after the onset of fermentation and reached the maximum value (1,501.49 mg l\(^{-1}\)) at the 70th day of fermentation. Then, the propionic acid concentration fluctuated at approximately 1,200 mg l\(^{-1}\) with no more obvious decreasing trend, indicating the ineffective utilization of propionic acid in the system.

After the fermentation started, the pH of the fermentation broth rapidly declined and then remained between 6.4 and 6.6, without any obvious upward trend, indicating that the fermentation broth was already in a weakly acidic state and no longer conformed to the neutral pH (between 6.5 and 7.5) suitable for normal biogas fermentation.

Through the 160 d gas production process, the yield and ratio of CH\(_4\), CO\(_2\) and N\(_2\) were determined every 10 days, as shown in figures 9 and 10. The cumulative production of all gases peaked between the 11th and 20th day of fermentation, followed by a gradual decline; among the gases produced by the system, N\(_2\) was the major component, accounting for 43.52 ± 1.78% to 75.50 ± 1.11% of the total production, whereas the percentage of CH\(_4\) was only 6.15 ± 0.22% to 32.96 ± 0.64%, indicating severe inhibition of methane production in the fermentation system.

Figure 7. Variation curve of propionic acid with fermentation time in the system.

Figure 8. Variation curve of butyric acid, isobutyric acid, pentoic acid and isopentanoic acid with fermentation time in the system.
3.2. Structure and relative abundance of the bacterial community

The representative sequences of the 19 OTUs with an average relative abundance greater than 0.5% were subjected to BLAST analysis with known sequences in GenBank to identify the strains with the highest homology, and then a literature review of the metabolic functions of the strains was conducted to tabulate the species comparison results, as shown in table 1. The sum of the average relative abundance of the 19 OTUs was 83.12%, with 11 OTUs from Firmicutes, 5 from Bacteroidetes, 2 from Proteobacteria, and 1 from Synergistetes. The specific comparison results revealed that four OTUs could be successfully classified at the taxonomic level (100% similarity), and six OTUs achieved 99% similarity, with the remaining OTUs scoring between 86 and 98% similarity to their respective compared species.

The primary dominant bacterial species was B-OTU1 (B for bacteria) with 100% similarity to Pseudomonas caeni, indicating that denitrifying bacteria were the most dominant bacterial group in the system. Pseudomonas caeni is mainly involved in the degradation of proteins, and it also participates in denitrification under anaerobic conditions, reducing nitrate and nitrite to N2 [27]. After the onset of fermentation, the relative abundance of Pseudomonas caeni showed a gradual increase and reached the first peak during 30–50 d of fermentation, reflecting their active involvement in the catabolism of proteins in the fermentation broth and the denitrification process. Then, the relative abundance decreased rapidly and reached the minimum value at the 70th day of fermentation, followed by a rapid increase in its relative abundance, which stabilized around 35%, reaching the maximum value (43.17%) at the 140th day of fermentation.

The secondary dominant bacterial species was B-OTU2 with 99% similarity to Clostridium cellulovorans, indicating that macromolecular carbohydrate (hemicellulose and cellulose)-degrading bacteria were the second dominant bacterial taxon in the system [28]. From the onset to 50th day of fermentation, the relative abundance of Clostridium cellulovorans showed a gradual decrease, while remaining at a relatively high abundance level.
| B-OTU   | Mean relative abundance (%) | Closest species (similarity)                  | Accession number | Main substrates/main products                                      | Classification (Phylum/Genus)                  |
|---------|-----------------------------|-----------------------------------------------|------------------|--------------------------------------------------------------------|-----------------------------------------------|
| B-OTU1  | 30.00                       | *Pseudomonas caeni* (100%)                    | KX354320.1       | Protein, capric acid, malic acid/Amino acid, N₂                      | Proteobacteria/Pseudomonas                     |
| B-OTU2  | 20.36                       | *Clostridium cellulovorans* (99%)             | KF528156.1       | Cellulose, pectin, glucose, maltose/H₂, CO₂, acetic acid, butyric acid | Firmicutes/Clostridium                        |
| B-OTU3  | 10.54                       | *Terrisporobacter petrolearius* (99%)         | NR_137408.1      | Glucose, fructose, maltose, xylose/CO₂, acetic acid                 | Firmicutes/Terrisporobacter                   |
| B-OTU1520 | 2.89                   | *Clostridium saudii* (99%)                    | NR_144696.1      | Cellulose, hemicellulose/Acetic acid, butyric acid                 | Firmicutes/Clostridium                        |
| B-OTU657 | 2.22                | *Romboutsia timonensis* (98%)                 | NR_144740.1      | Sucrose, glucose, fructose/acetate acid, lactic acid, H₂, CO₂       | Firmicutes/Romboutsia                        |
| B-OTU4  | 1.95                        | *Streptococcus galloxyticus* (100%)           | KT835017.1       | Protein, cellobiose, fructose, glucose/lactic acid                  | Firmicutes/Streptococcus                     |
| B-OTU5  | 1.77                        | *Turbibacter sanguninis* (99%)                | HQ646364.1       | Maltose/lactic acid                                                 | Firmicutes/Turbibacter                       |
| B-OTU10 | 1.58                        | *Petrimonas sulfuriphila* (93%)               | KT183420.1       | Glucose, arabinose/Acetic acid, H₂, CO₂                             | Bacteroidetes/Petrimonas                     |
| B-OTU8  | 1.57                        | *Sedimentibacter saulensis* (96%)             | NR_025498.1      | Pyruvate/Acetic acid, butyric acid                                  | Firmicutes/Sedimentibacter                   |
| B-OTU6  | 1.54                        | *Advenella kashmirenensis* (99%)              | KF528154.1       | Nitrite/N₂                                                         | Proteobacteria/Advenella                     |
| B-OTU7  | 1.41                        | *Alkaliflexus imhonenkii* (93%)               | NR_117198.1      | Cellobiose, xylose and maltose/propanoic acid, acetic acid          | Bacteroidetes/Alkaliflexus                   |
| B-OTU9  | 1.24                        | *Clostridium montiflorae* (99%)               | KY079341.1       | Peptone, arginine/H₂, acetic acid                                   | Firmicutes/Clostridium                       |
| B-OTU930 | 1.11               | *Sedimentibacter saulensis* (98%)             | NR_025498.1      | Pyruvate/Acetic acid, butyric acid                                  | Firmicutes/Sedimentibacter                   |
| B-OTU12 | 1.02                        | *Clostridium butyricum* (100%)                | CP013239.1       | Starch, glucose and sucrose/butyric acid, acetic acid, H₂, CO₂      | Firmicutes/Clostridium                       |
| B-OTU16 | 0.94                        | *Proteobacterium acetogenes* (95%)            | NR_043154.1      | Protein, pyruvate, glycine/acetic acid, propionic acid, NH₄          | Bacteroidetes/Proteobacterium                |
| B-OTU11 | 0.89                        | *Caloramator australicus* (86%)               | HM228392.1       | Glucose, fructose, xylose, maltose, sucrose/ethanol and acetic acid | Firmicutes/Caloramator                       |
| B-OTU14 | 0.84                        | *Petrimonas sulfuriphila* (94%)               | KT183420.1       | Glucose, arabinose/Acetic acid, H₂, CO₂                             | Bacteroidetes/Petrimonas                     |
| B-OTU13 | 0.63                        | *Sphingobacterium thermophilum* (87%)         | NR_108120.1      | Glycerin/-                                                         | Bacteroidetes/Sphingobacterium               |
| B-OTU15 | 0.59                        | *Cloacibacillus porcorum* (93%)               | CP016757.1       | Amino acid/acetic acid, propionic acid, formic acid                 | Synergistetes/Cloacibacillus                 |
above 20%, which was because of the further acidogenic metabolism of the hydrolysis products of the
macromolecular organic substrates in the fermentation feedstock carried out by this bacterium; subsequently,
the relative abundance rebounded and reached the first peak (34.86%) at the 60 day, resulting from its activity in
the decomposition of hemicellulose in the fermentation broth; the relative abundance remained above 25%
from the 70th to the 100th day of fermentation; the second peak (36.76%) appeared at the 110th day owing to the
iractivity in the decomposition of cellulose in the fermentation broth; the relative abundance was maintained at
approximately 20% at the last stage of fermentation. The tertiary dominant bacterial species was B-OTU3 with
99% similarity to Terrisporobacter petrolearius, indicating that fermentative (soluble sugars) and acidogenic
(organic acids) bacteria were the third dominant bacterial taxon in the system [29]. The relative abundance of
Terrisporobacter petrolearius gradually decreased after fermentation initiated, while remaining above 6% owing
to its effective utilization of hydrolysis products in the fermentation broth; subsequently, the relative abundance
gradually increased to reach the peak (18.24%) at the 70th day of fermentation, owing to the utilization of soluble
monosaccharides such as glucose for fermentative and acidogenic processes mediated by this bacterium. The
second peak occurred at the 110 day of fermentation, resulting from its use of hydrolysis products of cellulose;
the relative abundance remained above 8% at the latter stage of fermentation.

3.3. Structure and relative abundance of the archaeal community

Based on the species annotation and abundance information at the OTU level for samples from different
fermentation periods, the top 9 ranking OTUs in terms of average relative abundance were selected from each
dominant archaeabacterial phylum that had been identified and compared using BLAST. These OTUs bore 94 to
100% similarity to the compared archaeabacterial species, which were mainly classified as methylotrophic
methanogens, hydrotropic methanogens, Nitrososphaera, and MCG archaea (table 2).

The primary dominant archaeabacterial species was A-OTU5 (A for archaea) with 99% similarity to
Methanosphaera curvata, and the peak relative abundance of this methanogen occurred between the onset and
40 days of fermentation. Since this archaea was a methylotrophic methanogen [30], its metabolic substrates were
H₂ and methanol, indicating that the production of CH4 in the system was mainly derived from H₂/methanol.

The secondary dominant archaeal species was A-OTU4 with 98% similarity to Methanocorpusculum sinense,
a hydrogenotrophic methanogen [31]. The highest peak of relative abundance occurred at the 70th day owing to
the peaking of hemicellulose decomposition at the 60 day of fermentation, which produced the hydrolytic
products, H₂ and CO₂, as the metabolic substrate.

The tertiary dominant archaeabacterial species was A-OTU9 (98% similarity to Methanobrevibacter beijingerse)
and the quinary dominant archaeabacterial species was A-OTU28 (99% similarity to Methanobrevibacter millerae), both of which were hydrogenotrophic methanogenic genera [32, 33], indicating that the second source of CH₄ production was H₂/CO₂ in the system.

3.4. Correlation between biotic factors

3.4.1. Correlation between bacterial taxa

In the system, the respective correlation between the primary dominant bacterial genus Pseudomonas and other
dominant bacterial genera Clostridium, Terrisporobacter, and Turicibacter had correlation coefficients of
−0.671 **, −0.840 **, and −0.602 *, uniformly showing highly significant negative correlations. These indicated
that an environment suppressing hydrolytic fermentative bacteria was instead conducive to the growth and
multiplication of denitrifying bacteria. There was a highly significant positive correlation between Clostridium
and Terrisporobacter (correlation coefficient of 0.772 **), indicating a symbiotic relationship between the two
with Terrisporobacter utilizing the hydrolysis products of Clostridium.

3.4.2. Correlation between archaeal taxa

The correlation coefficients between Methanosphaera and other archaea such as Methanobacterium, unidentified
MCG, and Methanobrevibacter were 0.911 **, 0.659 **, and 0.945 **, respectively, all of which showed highly
significant positive correlations, indicating a symbiotic relationship between these dominant archaeal taxa.

3.4.3. Correlation between bacterial and archaeal taxa

The correlation between the primary dominant bacterial genus Pseudomonas and the dominant methanogenic
taxa Methanosphaera, Methanocorpusculum, and Methanobacterium, had correlation coefficients of −0.028,
−0.796 **, and −0.046, respectively. They were significantly negative, indicating that Pseudomonas did not
provide metabolic substrates for methanogens. Significant positive correlations between the dominant bacterial
genus Clostridium and the dominant methanogenic taxa Methanosphaera, Methanocorpusculum and
Methanobacterium, were observed with correlation coefficients of 0.496 *, 0.272 and 0.525 *, respectively.
Similarly, the correlations between Terrisporobacter and the dominant methanogenic taxa such as
| OTU  | Mean relative abundance (%) | Closest species (similarity) | Accession number | Main substrates/main products | Classification (Phylum/Genus) |
|------|-----------------------------|------------------------------|------------------|-------------------------------|-----------------------------|
| A-OTU5 | 7.22 | Methanosphaera cuniculi (99%) | NR_104874.1 | H₂, methanol/CH₄ | Euryarchaeota/Methanosphaera |
| A-OTU4 | 4.15 | Methanocorpusculum sinense (98%) | NR_117149.1 | H₂, CO₂/CH₄ | Euryarchaeota/Methanocorpusculum |
| A-OTU9 | 1.89 | Methanobacterium beijingense (98%) | KP109878.1 | H₂, CO₂/CH₄ | Euryarchaeota/Methanobacterium |
| A-OTU18 | 1.07 | — | — | — | MCG |
| A-OTU20 | 0.99 | Nitrososphaera viennensis (94%) | NR_134097.1 | O₂, NH₃/Nitrous acid | Thaumarchaeota/Nitrososphaera |
| A-OTU28 | 0.91 | Methanobrevibacter millerae (99%) | KP123404.1 | H₂, CO₂/CH₄ | Euryarchaeota/Methanobrevibacter |
| A-OTU44 | 0.80 | Nitrososphaera viennensis (94%) | NR_134097.1 | O₂, NH₃/Nitrous acid | Thaumarchaeota/Nitrososphaera |
| A-OTU46 | 0.51 | Nitrososphaera viennensis (94%) | NR_134097.1 | O₂, NH₃/Nitrous acid | Thaumarchaeota/Nitrososphaera |
| A-OTU39 | 0.50 | Nitrososphaera viennensis (94%) | NR_134097.1 | O₂, NH₃/Nitrous acid | Thaumarchaeota/Nitrososphaera |
Methanosphaera, Methanocorpusculum and Methanobacterium were 0.367, 0.636** and 0.414, respectively. There also existed significant positive correlations, indicating that the metabolic substrates of predominant methanogens were mainly derived from hydrolytic fermentative bacteria.

4. Discussion

4.1. Effect of denitrifying bacteria Pseudomonas and MCG archaea on low-temperature biogas fermentation systems

The denitrifying bacterium Pseudomonas caeni was the primary dominant bacterium in this system, where, P. caeni produced \( \text{N}_2 \) through denitrification. Meanwhile, as P. caeni was in an environment with high ammonia nitrogen concentration, it was worth discussing whether this strain also utilized ammonia nitrogen (correlation analysis showed that the change in ammonia nitrogen concentration was correlated with P. caeni, and ammonia nitrogen concentration was significantly positively correlated with \( \text{N}_2 \) production). Hu et al. [34] isolated a strain of denitrifying bacteria P. mendocina from an anaerobic ammonia oxidation reactor after five years of research, which used both nitrate and nitrite to produce \( \text{N}_2 \), and also effectively used ammonia, demonstrating that the denitrifying bacteria was also able to anaerobically oxidize ammonia (\( \text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O} \)). The known anaerobic ammonia-oxidizing bacteria were relatively limited and the multiplication time of anaerobic ammonia-oxidizing bacteria could be as long as 11 days [35], resulting in slow growth and reproduction. If P. caeni was confirmed to have anaerobic ammonia-oxidizing activity, based on the study of Hu et al, it would be possible to further explore the strains with anaerobic ammonia-oxidizing ability and provide more microbial resources for the engineering application of anaerobic ammonia-oxidizing process.

In this system, the relative abundance of MCG archaea was second only to Euryarchaeota and Thaumarchaeota. MCG archaea were the most widely distributed class of uncultured archaea, widely present in marine, soil, and low-temperature environments [36]. Some scholars have also found MCG archaea in biogas fermentation systems; Yuan et al. [37] detected MCG archaea in a mixed biogas fermentation system of corn straw and pig manure, and Chen [38] found them in an anaerobic bioreactor treating animal manure; Wilkins et al. [39] found MCG archaea in a biological treatment reactor for beverage plant wastewater with abundance second only to methanogens. Pure cultures of MCG archaea have not been obtained yet, and therefore the physiological functional characteristics of the archaea have not been well understood. Nonetheless, some scholars have found that MCG archaea can metabolize \( \text{CH}_4 \) according to studies on environmental substrate enrichment and gene expression. Zhang [40] discovered substantial anaerobic oxidation of \( \text{CH}_4 \) in the gas hydrate zone of the Sea of Okhotsk and concluded that it was MCG archaea that metabolized \( \text{CH}_4 \). Butterfield, Etto, Becker and other scholars [41–43] also believed that MCG archaea could metabolize \( \text{CH}_4 \) anaerobically.

4.2. Bacterial groups in low-temperature biogas fermentation system

Clostridium (which can hydrolyze cellulose and hemicellulose) was the main bacterial groups in the low-temperature biogas system in this study. Seib et al. [44] conducted research on 10°C low-temperature biogas fermentation of municipal sewage, and found that Clostridium is the main dominant group. Bialek et al. [45] used cow farm wastewater as raw material to conduct low-temperature biogas fermentation at 10°C, and the results showed that Clostridium aminobutyricum was the bacteria with the highest abundance. Tian [15] conducted a 15°C low-temperature biogas fermentation experiment with pig manure as raw material. The study showed that Clostridium had the highest relative abundance, and found that hydrolysis and fermentation bacteria accounted for the vast majority of bacterial groups. Clostridium can become a dominant bacteria genus, which is closely related to the rich cellulose, hemicellulose and other macromolecular carbohydrates in the fermentation environment.

The BLAST comparative analysis of Clostridium in our low-temperature biogas fermentation system shows that Clostridium cellulovorans is the most dominant species in this genus of bacteria (the comparative similarity was 99%). Some scholars found that the optimal growth temperature of this bacteria was 37°C [45]. For example, Rui et al. [46] studied the microbial communities in 13 medium temperature pig manure biogas engineering systems, and the results showed that Clostridium was the most dominant bacterial group. However, a large number of Clostridium were found in the above low-temperature biogas fermentation systems, indicating that the bacteria of this genus have a strong ability to adapt to the environment. At the same time, some studies have shown that the bacterial group of low-temperature biogas fermentation system was similar to that of medium temperature biogas fermentation system, and the low-temperature fermentation conditions have no obvious effect on the bacterial group.
4.3. Dominant groups of methanogens in low-temperature biogas fermentation system

Among the top 9 archaea in terms of average relative abundance, 3 species belong to hydrogen trophic methanogens, with the total abundance of 6.95% (see table 1), which is second only to methyl trophic methanogens, indicating that hydrogen reducing carbon dioxide is the main way to generate CH4 in low-temperature biogas fermentation. Many researchers have studied the nutritional types of methanogens under low-temperature conditions from various perspectives, and the results are generally consistent with the results of this study. Yang et al [24] studied the anaerobic digestion of pig manure under 9 °C, and found that the relative abundance of hydrogen trophic methanogens Methanocorpusculum sinense was 4.15%–37.14%, which was the most dominant methanogen. Tian et al [25] studied the dominant methanogenic bacteria in anaerobic digestion system at 9 °C, 15 °C, 21 °C, 35 °C, 45 °C and 55 °C respectively, and found that almost all methane came from the hydrogen reducing carbon dioxide pathway at 9 °C and 15 °C. Zhang et al [47] studied the anaerobic digestion of municipal wastewater under low temperature (16.5 ± 2.0 °C), and found that the abundance of hydrogen trophic methanogens Methanobacterium was the highest. Under low temperature, CH4 in anaerobic digestion mainly comes from the metabolism of hydrogen trophic methanogens. On the one hand, it is related to the strong ability of hydrogen trophic methanogens to adapt to cold environment, for example, the growth temperature range of Methanocorpusculum is 3.6 °C–45 °C [48]; On the other hand, it is related to the lower energy required by the hydrogen trophic methanogenic pathway at low temperatures. Lettinga et al [49] studied the Gibbs free energy of the main biochemical reactions in the anaerobic digestion process at medium temperature 37 °C and low temperature 10 °C respectively, and found that the Gibbs free energy of the hydrogen trophic CH4 production pathway was −140.9 kJ at low temperature 10 °C, while the Gibbs free energy of −131.3 kJ was required at 37 °C. Lettinga et al showed from the thermodynamic point of view that the hydrogen trophic type is a more easily realized methanogenic pathway at low temperature than at medium temperature.

However, some scholars’ research shows different results from this study. Dong et al [50] collected the fermentation sludge from the rural household biogas digester located in Shangri La, a cold region on the Yunnan Plateau (the fermentation temperature of the low-temperature biogas digester was 12 °C, and the fermentation raw material was pig manure), and sequenced the microbial community in the sludge with high throughput. The results showed that the facultative trophic (hydrogen and acetic acid) methanogen Methanosarcina was the most dominant methanogen.

5. Conclusions

(1) In the malfunctioning low-temperature biogas fermentation system, the hydrolytic fermentation process, hydrogen and acetic acid production process, and methane production process were severely inhibited; denitrifying bacteria became the dominant bacterial group, and the system primarily produced N2.

(2) Biotic factors indicative of low-temperature biogas fermentation systems were identified. MCG archaea metabolized CH4, which leads to the loss of CH4 from biogas, while Pseudomonas inhibits the activity of hydrogenotrophic methanogens. Hence, we concluded that MCG archaea and Pseudomonas were important biotic factors indicative of poor operation of low-temperature biogas fermentation systems.

(3) This study studied the abnormal low-temperature biogas fermentation system, identified the biological factors that led to its abnormal operation, and provided a microbiological basis for the selection of low-temperature biogas fermentation inoculum, that is, the inoculum dominated by MCG archaea and Pseudomonas cannot be selected when selecting inoculum.

(4) In this study, under the fermentation temperature of 9 °C, although the operation of the low-temperature biogas fermentation system using 4 °C, domesticated inoculum is abnormal, the system has a significant effect on the increase of the gas production rate. How to maintain the normal operation of biogas fermentation based on the increase of the gas production rate is the next step of research to be concerned.

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**Data availability statement**

The data that support the findings of this study are available upon reasonable request from the authors.

**Declarations**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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