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Microbial community structure and metabolic pathways in temperature-controlled anaerobic biofilm processes for the treatment of municipal wastewater in Alpine regions

Mingzhe Guo, Jun Wang, Chunhui Fu, Junhao You, Yongchen Zong*

Tibet Agricultural and Animal Husbandry University, Nyingchi, Tibet 860000, China, emails: zongyongchen126@163.com (Y. Zong), guo_mingzhe126@163.com (M. Guo), 1574443725qq.com (J. Wang), 1354599589@qq.com (C. Fu), 991874823@qq.com (J. You)

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ABSTRACT

Microbial communities under Alpine regions show significant regional variation. As a main factor affecting microbial communities and metabolic processes, temperature was used as a single-factor control condition for 16S rRNA high-throughput sequencing analysis of microbial communities in anaerobic treatment systems in Alpine regions based on the Illumina MiSeq sequencing platform, and functional prediction was explored using the PICRUSt2 database. The results show that the biotic environment in Alpine regions can reduce microbial diversity. The microbial community composition is partially similar, and the microbial metabolic processes are slightly different. Low temperatures favor the methanogenesis module reaction, and for energy metabolism, 25°C is the optimal temperature working condition.

Keywords: Alpine regions; Anaerobic biofilm processes; Microbial community; Metabolic

1. Introduction

Municipal wastewater is a complex resource containing water, energy, and plant fertilizer to be exploited [1]. Traditional aerobic wastewater biological treatment processes allow municipal wastewater to be treated to standard, but the process requires an energy-intensive aeration and produces a residual sludge that requires costly further treatment and increases CO_2 emissions [2]. Anaerobic treatment is cost-effective for treating municipal wastewater with low energy consumption, reducing residual sludge generation, and enabling the capture and recycling of methane-based bioenergy [3]. Hence, anaerobic digestion is a sustainable municipal wastewater treatment technology that is in line with the concept of "carbon neutrality".

Anaerobic biological treatment is the conversion of organic matter into inorganic substances such as methane, nitrogen, sulfureted hydrogen, and ammonia by microorganisms in an anaerobic environment, and the process is accompanied by the release of energy, which allows for the conversion and reuse of energy in wastewater [4]. The process can be divided into three stages, namely the hydrolytic fermentation stage, the hydrogen/acetic acid production stage, and the methane production stage, each of which requires the participation of corresponding bacteria in the reaction and involves multiple strains of bacteria [5]. Anaerobic biofilm reactors have been widely studied and used in treating municipal wastewater. This reactor can balance the effects of hydraulic retention time and sludge retention time on the metabolic process of bacteria, maintaining high biomass and increasing load resistance [6]. A suitable temperature is a prerequisite for an efficient anaerobic process, and the acid-producing and methanogenic bacteria involved in this process are highly sensitive to changes in temperature [7]. Anaerobic reaction systems often show high methanogenic capacity in medium or high-temperature

* Corresponding author.

environments [8,9], while low temperatures can reduce anaerobic biological treatment capacity and also lead to changes in the properties of the wastewater [10,11]. However, when microorganisms are exposed to low temperatures for long periods, the microbial community may undergo cold domestication, whereby the microorganisms regulate their metabolic processes to increase the proportion of cold-tolerant species in the microbial community structure to adapt to the low temperatures [12,13]. Therefore, it is possible to achieve biodegradation at low temperatures, which has been confirmed in a number of recent studies. A study by Dolejs et al. [14] concluded that the biodegradation of most of the organic matter in the effluent can be achieved at 15°C. A study by Zhang et al. [15] showed that a temperature of 10°C can inhibit the anaerobic biological system to degrade organic matter. Currently, there are a series of studies on the anaerobic degradation process at low temperatures [16].

The average altitude of the Tibetan region is above 4,000 m, and as a typical Alpine region, the area is cold all year round, with an average annual temperature of 3°C–17°C and a daily diurnal temperature difference of over 10°C. At present, we see little research on anaerobic biological treatment of municipal wastewater in the unique biological environment of Alpine regions (low temperature, low pressure, strong UV). This study explored the anaerobic biofilm reactor in this region under the influence of the temperature by the single-factor controlled variable method. The study collected samples of the microbial community for 16S rRNA sequencing to explore the species composition of the bacteria involved and the changes in the community during temperature control in the anaerobic biotechnology used to treat municipal wastewater in the biological environment of the region. The study also used PICRUSt2 database to predict and analyze the metabolic pathways of microorganisms, identify the metabolic process, and derive the effective temperature during the operation, providing a sound theoretical basis for subsequent studies.

2. Test design

2.1. Test water

For the inlet water, the test used the underground sewage from the municipal sewage pipe of Tibet Agricultural and Animal Husbandry University (E94°21′46.75″, N29°39′57.23″), and the average inlet water quality index is shown in Table 1.

The test applies the 6B-3000A Water Quality (Multi-Parameter) Tester (Produced by Jiangsu Shengaohua Eco-Technology Co., Ltd., China) for the dilution assay of chemical oxygen demand, total nitrogen, total phosphorus and NH_3–N. And 5-d biochemical oxygen demand (BOD_5) was determined using a LB-805 Differential-Pressure BOD_5 Tester (Qingdao Lubo Jianye Environmental Protection Technology Co., Ltd., China).

Table 1
Intake water quality indicators

Chemical oxygen demand (mg/L)	490.60
5-d biochemical oxygen demand (mg/L)	160.52
Total phosphorus (mg/L)	3.15
Total nitrogen (mg/L)	121.29
NH_4^+–N (mg/L)	97.01
pH	8.3
Dissolved oxygen (mg/L)	0.15

2.2. Test set-up

The test set-up uses a laboratory-scale anaerobic biofilms reactor shown in Fig. S1. With a total volume of 7 L and an internal diameter of 10 cm, it was made of an acrylic sheet and wrapped with an external layer of insulation. It used polyurethane as a biofilm carrier with a 50% fill rate. The peristaltic pump controls both the bottom inlet and top outlet flow, while the top is equipped with an air vent. The test uses a high precision 1500W (PY-SM5, China) temperature controller to control the temperature.

2.3. Test method

During the start-up phase, the sludge culture was carried out using anhydrous aeration method with intermittent water intake for 50 d and sludge was inoculated after successful culture. The tests used polyurethane material as a biofilm carrier for the biofilm hangings. The average biofilm dry weight (BDW)was 406.05 mg/g, and the average biofilm volatile dry weight (BVDW) was 222.72 mg/g at the end of the hanging.

The test ran for 10 d in an anaerobic environment on a trial basis. The operational phase controlled the influent flow rate at 0.6 L/h, the hydraulic retention time at 11.67 h, the operating temperature at 30°C, and the dissolved oxygen at 0.08~0.12 mg/L. The official test took place after the instrumentation had stabilized in operation.

During the official test, the operating conditions, except for the temperature, are consistent with the operating conditions of the instrument during the trial run. The formal test has five temperature conditions, 15°C, 20°C, 25°C, 30°C, and 35°C. The biomass-rich parts of the filtrate biofilm samples were removed at the end of each working condition, a total of five samples, named temp_15, temp_20, temp_25, temp_30 and temp_35, were stored in a refrigerator at –20°C. At the end of the test, DNA was extracted uniformly and DNA integrity was checked by 1% agarose gel electrophoresis after testing the purity and concentration of DNA using a NanoDrop 2000 Ultra-Micro Spectrophotometer. The 16S rRNA in the V3-V4 region was amplified using an ABI GeneAmp® 9700 PCR instrument with universal primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). PCR amplification phase reaction system: 5×FastPfu Buffer 4 μL, 2.5 mM dNTPs 2 μL, Forward Primer (5 μM) 0.8 μL, Reverse Primer (5 μM) 0.8 μL, FastPfu Polymerase 0.4 μL, BSA 0.2 μL, Template DNA 10 ng. Template DNA 10 ng, supplemented with sterile ultrapure water (ddH_2O) to 20 μL. The reaction conditions were: pre-denaturation temperature 95°C (3 min), denaturation temperature 95°C (30 s), annealing temperature 55°C (30 s), and extension temperature 72°C (45 s). The extension was terminated at 72°C for 10 min after 27 cycles, and the Illumina MiSeq PE300 platform was used for high-throughput

sequencing. The sequencing data were spliced, quality-controlled, and de-spliced to obtain optimized sequences, which were OTU clustered to obtain OTU abundance tables for subsequent bioinformatics analysis. The sequencing for this study was completed by Majorbio Ltd., (Shanghai, China).

3. Results and discussion

The OTU sequence similarity of this sequencing result was 0.97, and the reference species taxonomic database was silva138/16s_bacteria with a classification confidence of 0.7. The five completed samples were sequenced to obtain a total of 301903 optimized sequences with 124701646 effective bases and an average length of 413. OTUs were filtered after OTU clustering and OTUs annotated to bacterial domains were retained. The results were drawn at the minimum sample temp-25 with a sequence number of 45963 and included 33 phyla, 81 orders, 164 families, 291 families, 542 genera, 822 species, and 1,206 OTUs, upon which the subsequent analysis was based.

3.1. Bacterial composition analysis

3.1.1. Alpha diversity analysis

The microbial community abundance index Sobs index (a) and the Shannon index (b), which combines the abundance and evenness of species in the community, are shown in Fig. S2, respectively. Both dilution curves showed a flat trend, and the coverage indexes in Table 2 were above 0.995. The sequencing results showed good coverage, and the sequenced samples were representative of the overall situation. Also combined with Table 2, in the plateau areas, the Chao1 index range is 1,002.88~1,093.71, and the Shannon index range is 4.77~5.01, both smaller compared to the Chao1 indexes range (1,042 to 1,790) and the Shannon indexes range (5.32~7.72) derived from the study by Ni et al. [17]. Combining the above values of BDW and BVDW, we can determine that the microbial diversity of wastewater treatment systems in alpine areas is less than in plain areas, a conclusion also reached by Fang et al. [18]. It can be seen that the microbial diversity also changes to a certain extent during the temperature change. That is, temperature control can affect the microbial abundance in the anaerobic reaction process, while there may be some regularity with temperature [19].

3.1.2. Bacterial composition analysis at the phylum level

After averaging and ranking the abundance of the bacterial phyla contained in the five samples, the distribution of bacterial phyla in anaerobic reaction systems in the biological environment of the Alpine region was derived (Fig. 1a). The screened bacterial phylum with a relative abundance (gene abundance as the proportion of the gene's abundance normalized by gene length to the total abundance of all genes normalized by gene length in the sample) greater than 2% was matched to five samples (Fig. 1b).

The bacterial phyla with relative abundance greater than 2% in Fig. 1a included Firmicutes (29.15%), Actinobacteria (21.26%), Proteobacteria (21.22%), Bacteroidetes (13.27%),

Table 2
Alpha diversity index table

Sample	Sobs	Shannon	Chao	Coverage
temp_35	922	4.884064	1,065.523	0.996106
temp_30	932	5.013376	1,050.194	0.995975
temp_25	875	4.92608	1,002.878	0.996258
temp_20	954	4.931202	1,093.709	0.995779
temp_15	915	4.769722	1,041.024	0.996127

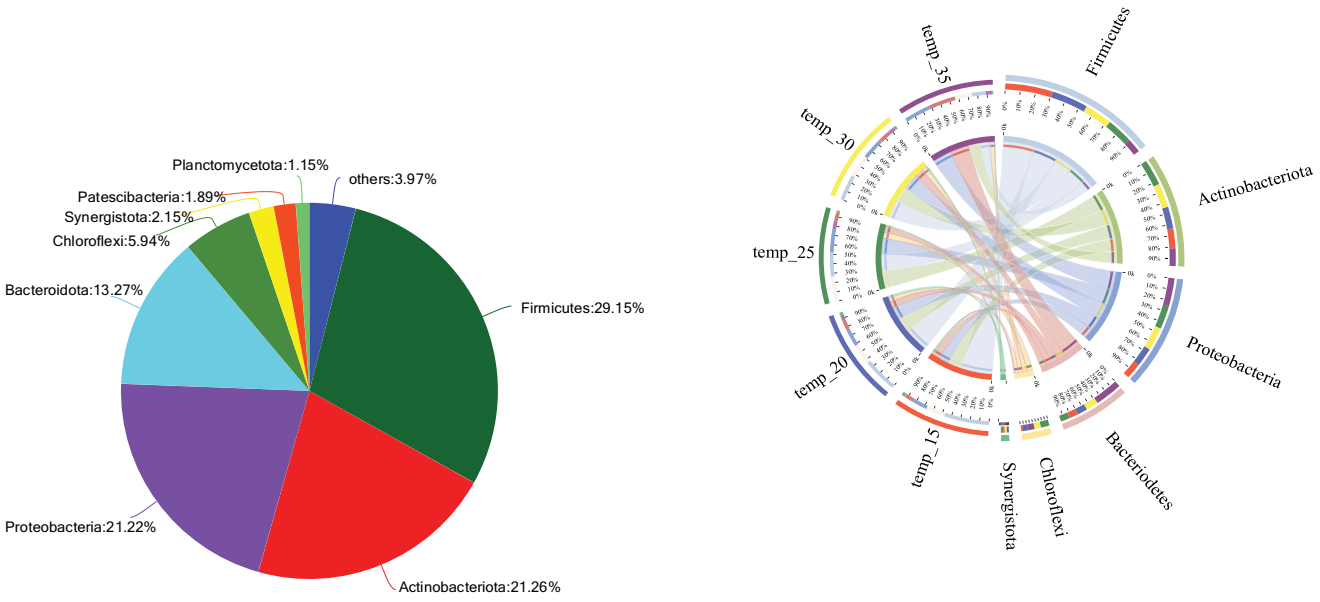

Fig. 1. Community analysis PiePlot on genus level. (a) Community analysis PiePlot on phylum level. (b).

Chloroflexi (5.94%), and Synergistota (2.15%). Among them, Firmicutes, Proteobacteria, Bacteroidetes, and Chloroflexi, as the main bacterial phylum in the anaerobic treatment process, had an absolute dominance in the relative abundance derived from the results of this experiment [20]. The comparison with the results of Houari et al. [21] on the structural composition of the microbial community derived in an anaerobic reactor can be concluded that there is a partial similarity between the two. Among them, Firmicutes, as the bacterial phylum with the highest proportion of relative abundance, is an important bacterial phylum in the anaerobic hydrolysis process, with an increase in relative abundance, indicating that anaerobic reaction systems in Alpine regions have a relatively strong capacity to metabolize a wide range of organic compounds [22]. Proteobacteria mainly contribute to the conversion of propionic, butyric, and valeric acids into acetic acid, which is essential for the production of methane [23]. Bacteroidetes, as a typical representative of fermentative microorganisms, mainly serve to degrade glycerol and polysaccharides and participate in the hydrolysis of macromolecular organic matter to VAF [24,25], and also play an important role in protein hydrolysis and the assimilation of semi-solid waste [26]. Notably, Actinobacteria is ranked as the second most abundant bacterial phylum in terms of relative abundance and is disproportionately overrepresented in terms of relative abundance. Previous studies have concluded that altitude has a small effect on Actinobacteria [27], and analysis of the causes of the substantial increase in the relative abundance of Actinobacteria in this test may be related to the biological sampling process, which requires further exploration in future studies.

In Fig. 1b, we can see that temperature has a certain effect on the level of the bacterial mesohyl, with the average relative abundance being more significantly affected by temperature in the ranking of Chloroflexi (2.03% to 9.79%), Firmicutes (14.95% to 48.92%) and Bacteroidetes (9.73% to 28.92%). The proportion of Chloroflexi peaks at 25°C and decreases either as the temperature rises or drops, suggesting that the ideal temperature for Chloroflexi in this reaction system is around 25°C. Studies have shown that an increase in temperature leads to a decrease in Chloroflexi abundance [28], and the results of this study suggest that a decrease in temperature also leads to a decrease in Chloroflexi abundance. It appears that the relative abundance of Firmicutes, famous for its tolerance of unfavorable conditions [29], is negatively correlated with temperature as it decreases, peaking at a temperature of 15°C. Notably, Bacteroidetes increased in abundance by approximately 123.69% as the temperature increased from 30°C to 35°C, which is considerably different from the research by Ferrari et al. [30] Bacteroidetes have the capacity for biofilm formation through the release of proteinaceous (EPS) [31], suggesting that the processes of biofilm formation differ in the Alpine region compared to the plains.

3.1.3. Bacterial composition analysis at the genus level

Fig. S3 shows the distribution of the top 50 genera in terms of relative abundance from five samples, with clustering at both the species level and the sample level using the form of average. The figure shows a clear pattern of differences in the relative abundance of bacterial genera between the different samples under the control of temperature, suggesting temperature is the main factor influencing the anaerobic biological response. Among the top fifty genera in terms of abundance, 40% are unmarked. Sample temp_15 and temp_30 show low overall abundance, and sample temp_20 shows the best species evenness. Among the various bacterial genera, *Proteiniclasticum* (0.74‰), *norank_f__AKYH767* (0.60‰), *Aestuariimicrobium* (0.44‰), *Romboutsia* (0.43‰), *Ottowia* (0.43‰), *Propioniciclava* (0.41‰), *Gallicola* (0.36‰), *Christensenellaceae_R-7_group* (0.36‰) are all bacterial genera with mean abundance greater than 0.30‰ of the mean total abundance of the sample. Among them, *norank_f__AKYH767* showed a positive trend in relation to temperature, and its abundance increased substantially compared to anaerobic microbial communities at lower altitudes [32], indicating that this genus is the dominant genus at higher altitudes. The abundance of *Proteiniclasticum* peaked at 15°C, followed by 20°C, suggesting that low temperatures cause an increase in protein consumption while aiding acetic acid production in the system [33]. *Gallicola* is a member of the Firmicutes phylum, which metabolizes amino acids and peptones in response to acetate and butyrate [34], and the temperature-dependent expression of abundance bears a strong resemblance to *Proteiniclasticum*. *Ottowia*, a genus of parthenogenic anaerobic bacteria, showed a high abundance in the anaerobic reactors in the Alpine region in this study. *Ottowia*, the dominant genus in Alpine regions, is capable of degrading refractory organic matter and performing nitrate reduction [35], and its relative abundance tends to increase during increasing temperatures.

3.2. KEGG pathway analysis at different levels

The PICRUSt2 database, currently the most accurate method for function prediction of microbial communities [36], was used for functional prediction analysis of the sequencing results. A total of 6837 KOs (KEGG Orthology), 352 Level 3 KEGG pathways, and 42 Level 2 KEGG pathways were detected in the analysis according to KEGG (Kyoto Encyclopedia of Genes and Genomes).

3.2.1. KEGG pathway analysis at level 2

After calculating the relative abundance of the 42 KEGG pathways of level 2 obtained in this test, they were grouped and sorted with the 6 KEGG pathways of level 1, and the distribution of each level 2 contained under level 1 was shown in Fig. 2.

The average distribution of the six level 1 KEGG pathways in Fig. 2 is metabolism (77.38%), genetic information processing (6.92%), environmental information processing (5.70%), cellular processes (4.49%), human diseases (3.60%), and organismal systems (1.91%). The three samples with the highest variance were carbohydrate metabolism, membrane transport, and amino acid metabolism. It is clear from the figure that the relative abundance of carbohydrate metabolism decreases with increasing temperature, and it can be concluded that there is a negative correlation with temperature. There was no clear positive or negative correlation between amino acid metabolism and temperature, with relative abundance peaking at 25°C. The relative

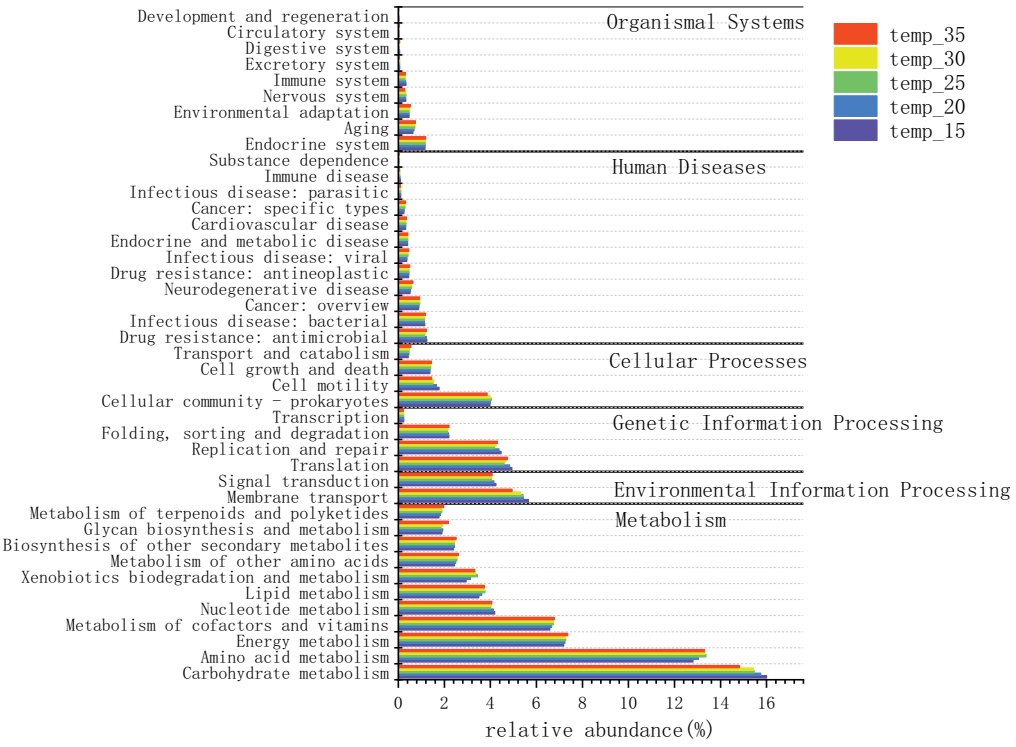

Fig. 2. Distribution of level 2 under level 1.

abundance of the two metabolism is significantly higher in Alpine regions when combined with the available studies.

Although the relative abundance of environmental information processing is smaller than that of genetic information processing, environmental information processing contains only two level 2 KEGG pathways, the membrane transport and the signal transduction. The high relative abundance of both species and the high signal transduction indicate that microbial systems in alpine areas are more sensitive to environmental signals than those in plain areas. Microbial systems modify the metabolic behavior of microbial systems by transmitting environmental signals to the metabolic network to adapt to the environment [37].

3.2.2. Different level 3 KEGG pathway analysis

Carbohydrate metabolism, energy metabolism, amino acid metabolism as the level 2 KEGG pathway with the highest relative abundance in metabolism need to be further investigated. The test yielded a total of 352 level 3 KEGG pathways. By calculating the relative abundance in each sample, the top 50 KEGG pathways of level 3 in carbohydrate metabolism, energy metabolism, and amino acid metabolism were ranked according to the relative abundance. Then the dispersion [Inline Equation 1]) during the temperature rise was calculated (Fig. 3).

In Fig. 3, starch and sucrose metabolism, valine, leucine, and isoleucine degradation, fructose and mannose metabolism, tryptophan metabolism showed the more apparent degree of dispersion in descending order. The relative abundance of starch and sucrose metabolism decreases at increasing temperatures, suggesting that low temperatures promote the conversion of some of the sugar carbon sources and facilitate the conversion to small molecule organic matter. Also, oxidative phosphorylation as an energy metabolic process was the highest in relative abundance in the overall metabolism in the test results, indicating that energy conversion was the signature metabolic process in this test. The relative abundance of this metabolic pathway, which is involved in the degradation of phosphorus-containing pollutants in wastewater, will stop increasing when the temperature is above 25°C. It differs slightly from the conclusion reached by Lin et al. [38] at low altitudes that the relative abundance of this metabolic pathway continues to increase over increasing temperatures. This phenomenon coincided with the trend in abundance of some of the above-mentioned genera with increasing temperature.

3.3. Energy metabolism

Energy metabolism is by far the most noteworthy type of biological metabolic activities in anaerobic reaction systems, and level 2's energy metabolism accounts for approximately 7.27% of the overall. The relative abundance of level 2 energy metabolism increases with increasing temperature, indicating that high temperatures contribute to energy conversion in anaerobic systems [39]. The four typical energy metabolism processes, level 3 of oxidative phosphorylation (map00190), methane metabolism (map00680), sulfur metabolism (map00920), and nitrogen metabolism (map00910) accounted for 26.64%, 16.13%, 10.37%, and 9.20% of energy metabolism, respectively, and each of these four typical energy metabolism processes will be analyzed in the following sections.

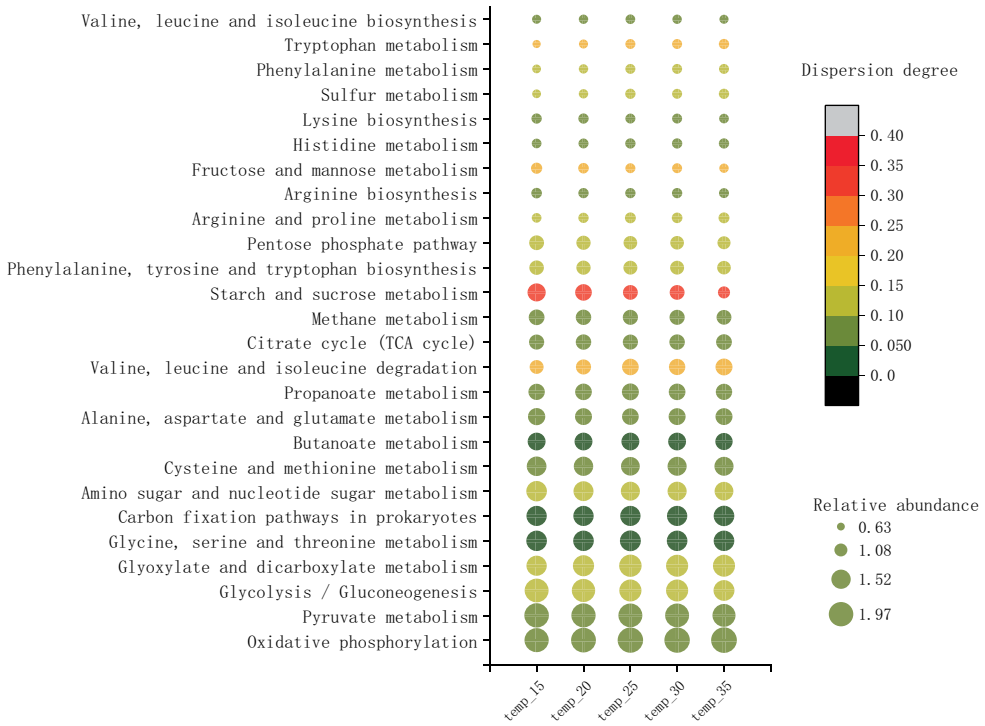

Fig. 3. Top 50 Level 3 KEGG pathways belonging to carbohydrate, amino acid and energy metabolism in terms of relative abundance.

3.3.1. *Oxidative phosphorylation*

After extracting all the KOs in map00190 (oxidative phosphorylation), the relative abundance percentage of KOs exceeding 1% was filtered out after its summation and sorting. Fig. S4 shows the distribution of the relative abundance of these KOs at each of the five temperature working conditions. Fig. S4 shows that except for K00336 (nuoG), K02110 (ATPFOC), and K03885 (ndh), the relative abundance ratios of almost all the other KOs show an upward trend with increasing temperature. A one-way ANOVA was performed using SPSS on KO and five temperature samples. Meeting the consistency of variance and a significance level of p = 0.05, it was concluded that KO was highly correlated with temperature working conditions. The test further analyzed the correlation between the two using Pearson's correlation coefficient (r) and came up with r = 0.323, which showed a positive correlation, but the strength of the correlation was low. Therefore, the trend of this metabolic pathway changing with temperature is one of the reasons for the increased energy conversion during temperature increases.

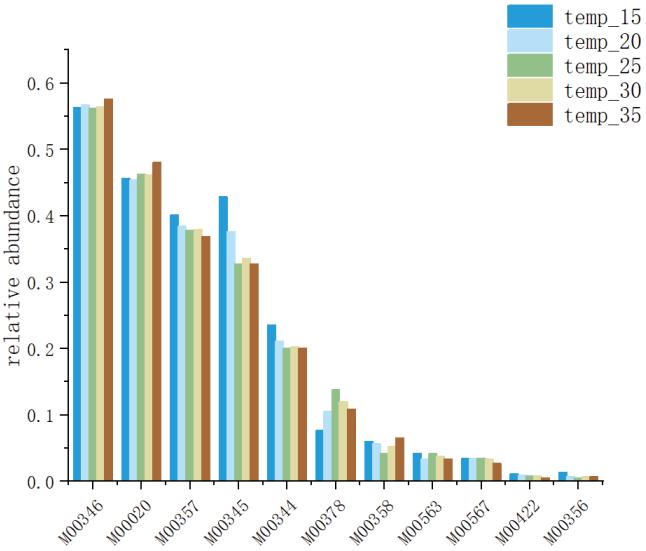

Fig. 4. Composition of the different modules in methane metabolism.

3.3.2. *Methane metabolism*

The relative abundance of methane metabolism (map00680) decreases with increasing temperature. This KEGG pathway includes 14 modules, and Fig. 4 shows the distribution of the modules included in this test. After analyzing the four methanogenic modules (M00356, M00357, M00563, M00567) of the defined methane metabolism (map00680) with the test results, M00357 (acetate => methane) is the main methanogenic module in the anaerobic reaction system in the region. The relative abundance of M00357 (acetate => methane) decreases with increasing temperature, followed by M00563 (methylamine/dimethylamine/trimethylamine => methane) and M00567 (CO2 => methane), and lastly M00356 (methanol => methane). In M00357 (acetate => methane), the two stages of the reaction with significant relative abundance were the conversion of acetate to acetylphosphate in the presence of acetate kinase, followed by the conversion to acetyl-CoA in the presence of phosphate acetyltransferase. Both enzymes involved show a

decreasing trend with increasing temperature, and Gunnigle et al. [40] also concluded that low temperatures can promote acetoclastic methanogenesis.

3.3.3. Nitrogen metabolism

The KOs involved in nitrogen metabolism obtained in this test were mapped in the nitrogen metabolism (map00910) diagram according to their relative abundance at five temperature levels and expressed in the form of the enzyme encoded by each KO in the diagram, as shown in Fig. 5. The relative abundance of enzymes at each of the five temperature levels is shown in descending order from left to right in each box containing the enzyme number. It is evident that dissimilatory nitrate reduction shows a high abundance in the reduction of nitrate, in particular, the process is more capable of reducing nitrite nitrogen to ammonia nitrogen and almost unaffected by changes in temperature conditions. The denitrification process is affected significantly by changes in temperature, but the holistic pattern is unclear. It is worth noting that the increase in temperature facilitates the conversion of NO to N_2. Also, the microbial community in this anaerobic system is not predicted to form a complete anaerobic ammonia oxidation process. Also, in the bacterial genera analyzed above, the anaerobic ammonia-oxidizing bacteria genus so far confirmed was not identified [41]. Whether the activated sludge used in treating municipal wastewater by biological methods in Alpine regions contains anaerobic ammonia-oxidizing bacteria needs further study.

3.3.4. Sulfur metabolism

Each box marked with the enzyme number in Fig. 6 has been divided into five small pieces. From left to right, the color of each plot represents the relative abundance of the enzyme under each of the five working conditions, namely temp_15, temp_20, temp_25, temp_30 and temp_35. Fig. 6 shows that the assimilatory sulfate reduction, dissimilatory sulfate reduction and oxidation, and the whole S metabolic process of the SOX system are involved in this anaerobic reaction system. Assimilative sulfur metabolism is the assimilation of sulfate into organic compounds and the resulting sulfide is a component of microbial cells, a process which, as an energy-consuming process, is predominant in this reduction of sulfate. The present anaerobic reaction system showed strong conversion of sulfate to APS in the presence of the bifunctional enzyme CysN/CysC and of PAPS

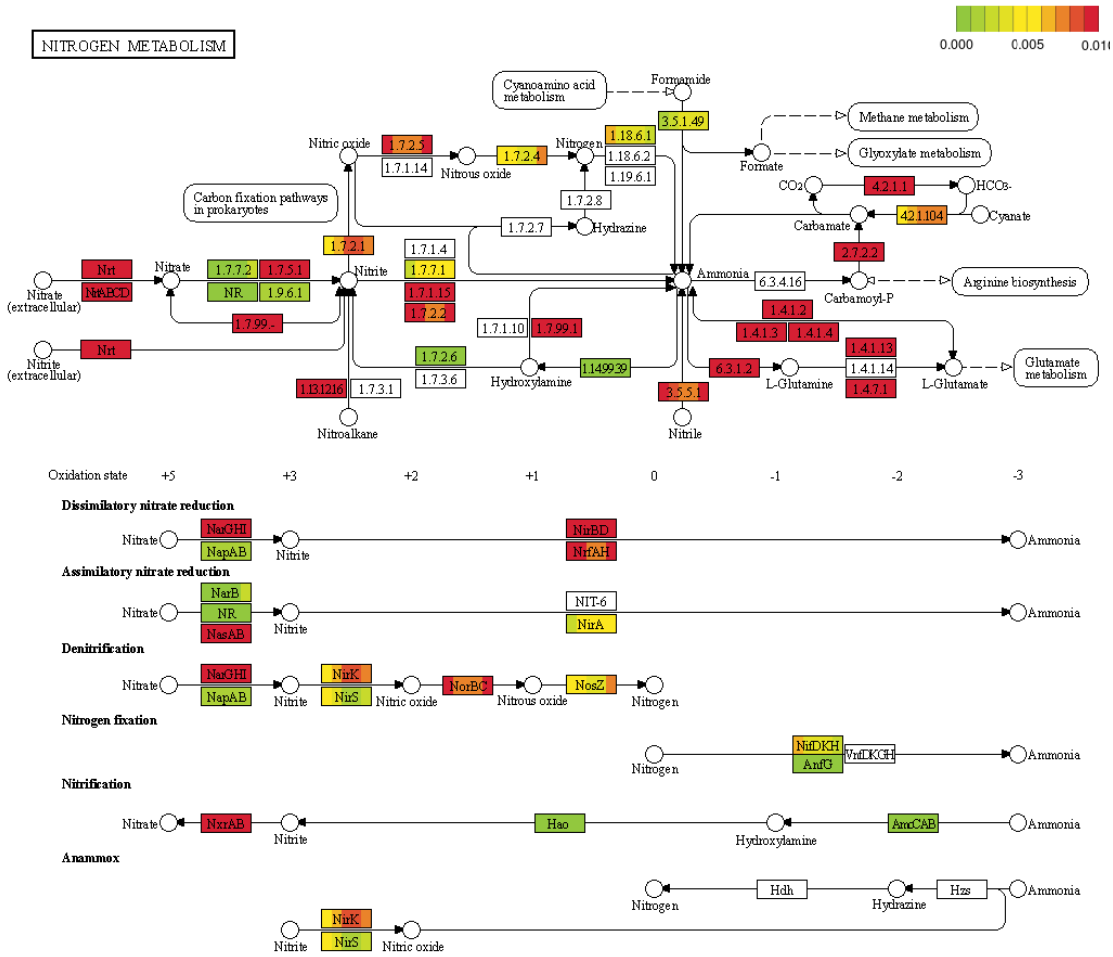

Fig. 5. Nitrogen metabolism (map00910) network diagram.

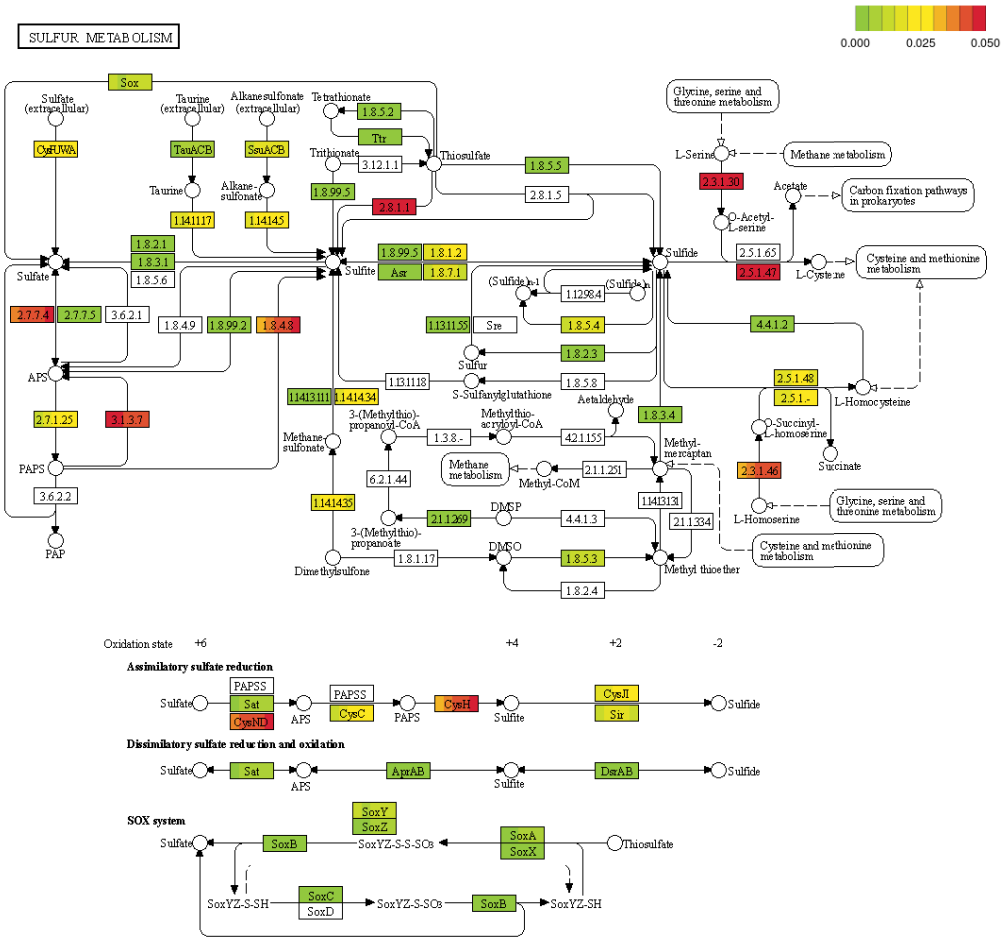

Fig. 6. Sulfur metabolism (map00920) network diagram.

to Sulfite in the presence of phosphoadenosine phosphosulfate reductase. The figure also shows that the process is facilitated by the increase in temperature, suggesting that the increase in temperature helps the microorganisms to reproduce [42]. The relative abundance of enzymes involved in dissimilatory sulfate reduction and oxidation as a productive process is low and does not vary significantly with temperature, suggesting that temperature does not have a strong influence on the growth of sulfate-reducing microorganisms [43]. The SOX system as a complete sulfur oxidation system showed the same insignificant variation with temperature in the results of this test.

4. Conclusions and outlook

The Alpine region has restrained microbial diversity, but the main species composition of the anaerobic reaction system is similar to that of the plains. Yet the trend in the relative abundance changes of certain genera of bacteria with increasing temperature is different from that of the plains. Further research is required to address the substantial increase in Actinobacteria abundance in this test.

Energy metabolism is the signature metabolic process, with 25°C being the optimal temperature working condition. In this test, M00357 (acetate => methane) served as the main methanogenic module in the region's anaerobic reaction system, and its module's reaction preferred low temperatures, while the increase in temperature facilitates the conversion of NO to N_2 and the growth and reproduction of cells.

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Author's statement

All authors have read, understood, and have complied as applicable with the statement on "Ethical Responsibilities of Authors" as found in the instructions for authors and are aware that with minor exceptions, no changes can be made to authorship once the paper is submitted.

Data availability

Due to the confidentiality of the experimental data and the experimental research being in the basic mechanism research stage, the experimental data will not be disclosed for the time being. The data set generated and/or analyzed during the current research period cannot be publicly obtained but can be obtained from the corresponding author according to reasonable requirements obtained there. The data in this article can be used after obtaining the consent of

all authors. The email address for obtaining data: guo_mingzhe126@163.com.

Author contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Mingzhe Guo, Jun Wang, Yongchen Zong, Chunhui Fu, Junhao You. The first draft of the manuscript was written by Mingzhe Guo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict interests

The authors declare no competing interests.

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Supporting information

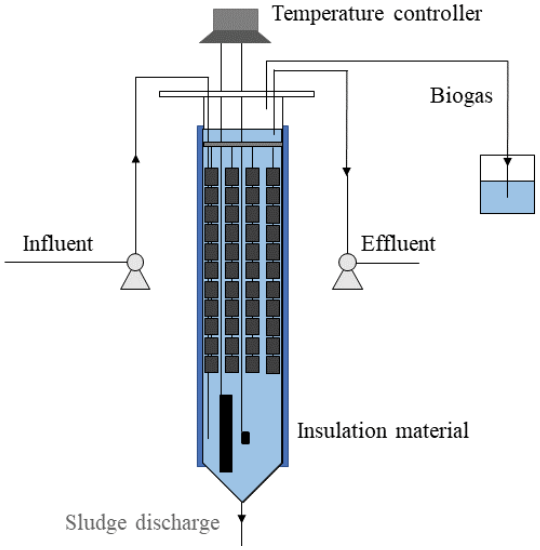

Fig. S1. Anaerobic biofilm reactor.

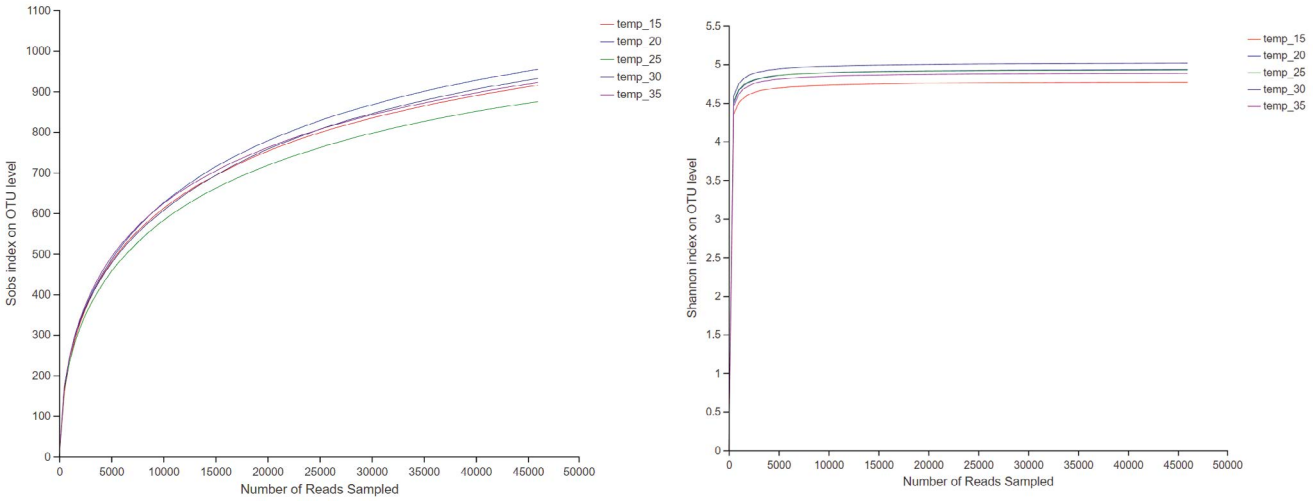

Fig. S2. Shannon (a) and rarefaction curve chart (b).

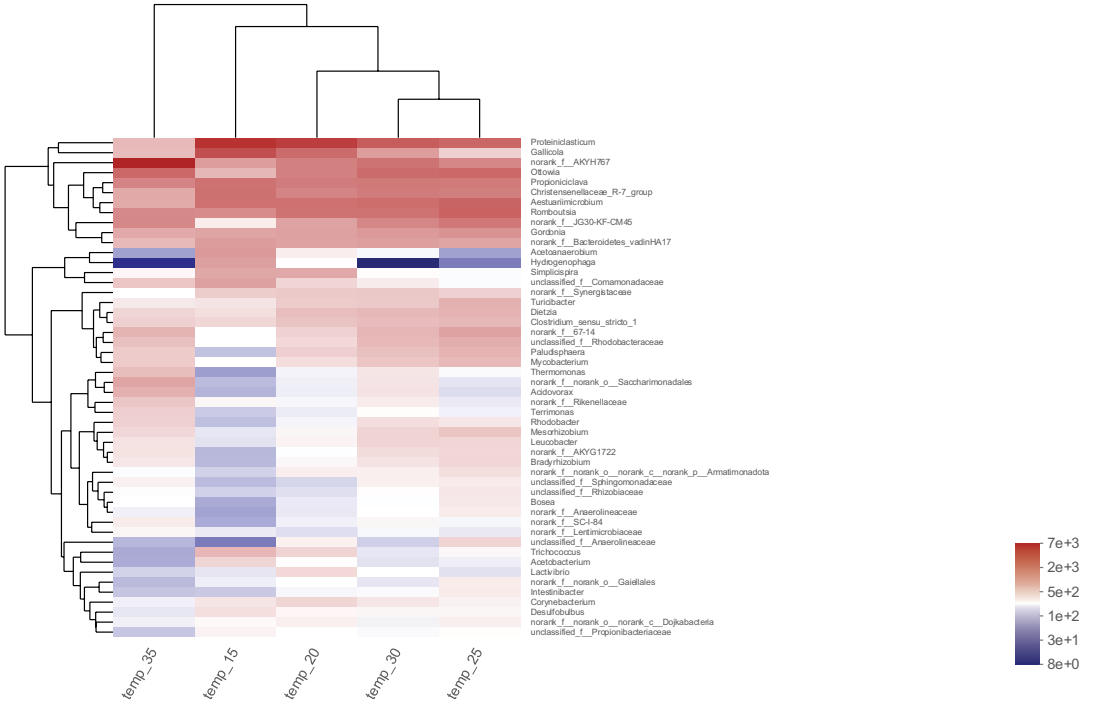

Fig. S3. Heat map of the distribution of the top 50 genera in abundance in the 5 samples.

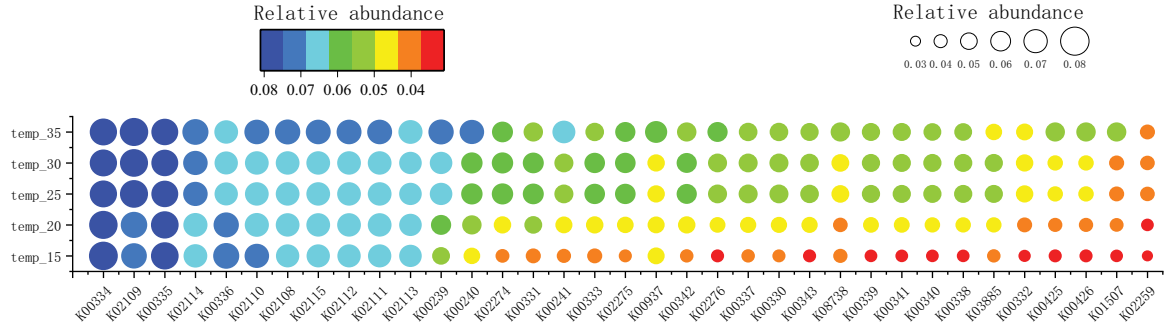

Fig. S4. Sample distribution of relative abundance of KO in map00190.