ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Effect of Temperature on Anaerobic Fermentation of Poplar Ethanol Wastewater: Performance and Microbial Communities

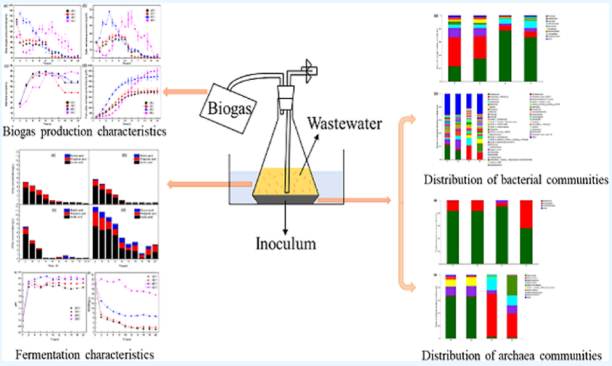
Xiaofan Yan, Pingping Deng, Tingting Ding, Zuoyu Zhang, Xingong Li, and Zhiping Wu*

Cite This: *ACS Omega* 2023, 8, 5486−5496

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Temperature plays an important role in anaerobic digestion (AD), and different substrates have different optimum temperatures in AD. However, the effect of temperature on the performance of AD when cellulosic ethanol wastewater was used as a substrate was rarely reported. Therefore, the digestion characteristics of cellulosic ethanol wastewater at 25, 35, 45, and 55 °C were investigated, and the microbial communities of the sludge sample were analyzed after fermentation. The results showed that the cumulative methane production was the highest at 55 °C, 906.40 ± 50.67 mL/g VS, which was 81.06, 72.42, and 13.33% higher than that at 25, 35, and 45 °C, respectively. The content of methane was 68.13, 49.26, 70.46, and 85.84% at the terminal period of fermentation at temperatures of 25, 35, 45, and 55 °C, respectively. The testing of volatile fatty acids (VFAs) indicated that the accumulation of VFAs did not occur when the fermentation was carried out at 25, 35, and 45 °C; however, the VFA content at 55 °C was much larger than that in the three groups (25, 35, and 45 °C), and the ratio of propionic acid to acetic acid was larger than 1.4 at the late stage of fermentation, so it inhibited the fermentation. The diversity of the microbial community indicated that the floral structure and metabolic pathway of fermentation were alike at 25 and 35 °C. *Firmicutes* and *Proteobacteria* were the main flora covering the 25−55 °C-based phylum or below it. The relative abundance of *Methanosaeta* was the highest when fermentation temperatures were 25 and 35 °C; however, its relative abundance decreased sharply and the relative abundance of *Methanosarcina* increased substantially when the temperature increased from 35 to 45 °C, which indicated that *Methanosarcina* can exist in higher temperatures. At the same time, hydrogenotrophic methanogens such as *Methanoculleus* and *Methanothermobacter* were dominant when fermentation temperatures were 45 and 55 °C, which indicated that the metabolic pathway changed from acetoclastic methanogenesis to hydrogenotrophic methanogenesis.



1. INTRODUCTION

Fossil fuel is not only non-renewable but also causes environmental pollution and greenhouse effect owing to its massive consumption, which have brought great challenges to the survival of human beings. Therefore, it is necessary to search for a low-cost, environmentally friendly, clean energy.[1] Ethanol fuel is cleaner and renewable compared to conventional fossil fuels, so it has been extensively studied and applied to practical life.[2] It neither "competes with people for grains" nor "competes with grain for fields" when ethanol fuel is produced by lignocellulose as the raw material, so many countries have devoted great efforts to developing it. However, a large amount of wastewater is generated during the production of cellulosic ethanol, and it has been reported that at least 20 tons of wastewater is generated when 1 ton of ethanol is produced.[3] This wastewater has the characteristics of highly suspended solid content, high chemical oxygen demand (COD), and a low pH, which may cause serious water pollution if directly discharged.[4] It has become a major problem in limiting the production of cellulosic ethanol.

Anaerobic digestion (AD) is a good way to reduce organic pollution and produce biogas for energy recovery,[5,6] which has been widely used to treat wastewater sludge, municipal solid waste, livestock wastewater, and food wastewater.[7] Therefore, it is a favored option to produce biogas from cellulosic ethanol wastewater through AD.[8,9] Many parameters such as the pH, temperature, and C/N ratio can affect AD. Of these parameters, the temperature has an important effect on hydrolysis and methane production rates, so it plays a crucial role in AD.[10] According to the optimal growth temperature range of microorganisms, it can generally be divided into ambient temperature (10−25 °C), mesophilic temperature

Received: October 18, 2022
Accepted: January 16, 2023
Published: January 31, 2023

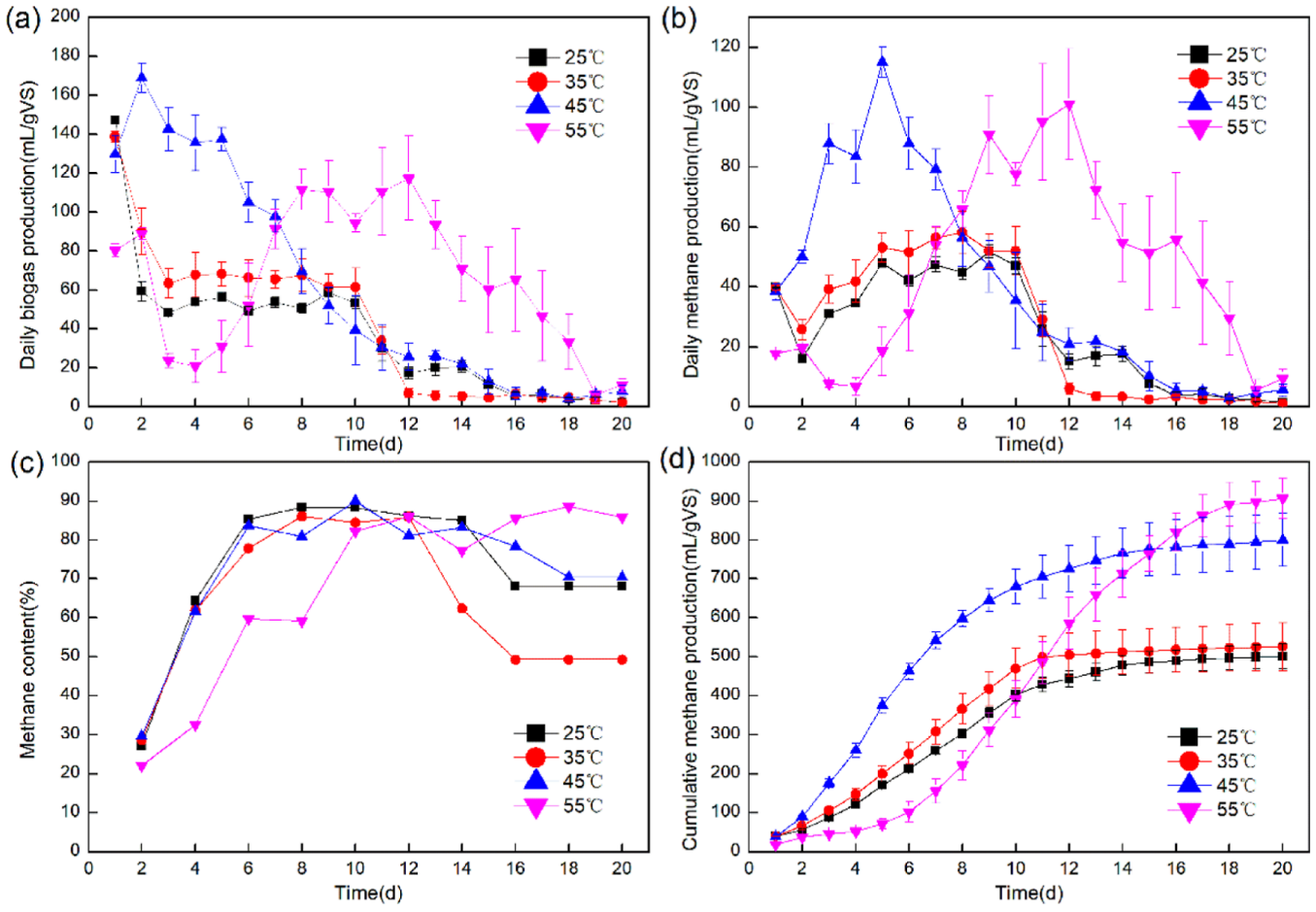


Figure 1. Effects of different temperatures on (a) daily biogas production, (b) daily methane production, (c) methane content, and (d) cumulative methane production.

(35−37 °C), and thermophilic temperature (55−60 °C).[11] Many studies have shown that temperature has a significant effect on the performance of AD. For example, Deng et al.[11] used swine manure as feedstock for AD at 15, 25, and 35 °C, and their maximum methane yields were 0.036, 0.226, and 0.237 $L{\cdot}g^{-1}$ VS, respectively. Kafle and Kim[12] found that the biogas yield was significantly higher in thermophilic conditions (55 °C) than in mesophilic conditions (36.5 °C) when a mixture of apple and swine manure was used as the substrate for AD at 55 and 36.5 °C. Some studies focus on the effect of different temperatures of psychrophilic, mesophilic, and thermophilic conditions. Tian et al.[13] explored the effect of temperature on the anaerobic fermentation of pig manure at 9, 15, 21, 35, 45, and 55 °C. The results showed that the biogas production was different, of which the highest biogas production occurred at 45 and 55 °C. The effect of temperatures on the biogas production performance has attracted the interest of many researchers. However, the effect of temperature on the fermentation performance of ethanol wastewater was rarely reported.

On the other hand, with the development of molecular biology techniques, many researchers have tried to reveal the relationship between microbial communities and anaerobic fermentation performance at different temperatures. High-throughput sequencing technology, also known as second-generation sequencing technology, can analyze a large number of microbial sequences in a shorter analytical time, which can favor understanding the composition of microorganisms and their interactions.[5] It has become an advanced method for studying microbial composition. Many studies have shown that temperature plays an important role in the growth and metabolism of microorganisms as well as the interaction between microbial communities,[14] and the microbial communities of anaerobic fermentation at different temperatures are also different. Generally, the bacterial communities observed at different temperatures are mainly *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, but the methanogen communities will be different. Khan et al.[15] found that the predominant archaea are *Methanosaetaceae*, which can only utilize acetic acid when molasses wastewater and straw ethanol wastewater are used for combined fermentation at 35−37 °C. Pap et al.[16] and Tian et al.[17] also found that the hydrogenotrophic methanogenesis increased and the acetoclastic methanogenesis decreased when the temperature increased from 35 to 55 °C. However, Röske et al.[18] found that *Methanosaetaceae* played an important role in AD when CSTR was employed to ferment industrial bioethanol wastewater at 55 °C. Moreover, Tian et al.[13] also found that the composition of the microbial community at 15−35 °C was similar when pig manure was used as the raw material to ferment at 9, 15, 35, 45, and 55 °C, but the number of bacteria decreased and the metabolic pathways also changed from hydrogenotrophic to acetoclastic methanogenesis when the temperature was increased to 55 °C. Therefore, many studies have demonstrated that temperature has an effect on the community composition of microorganisms during anaerobic fermentation, but the metabolic pathways of

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

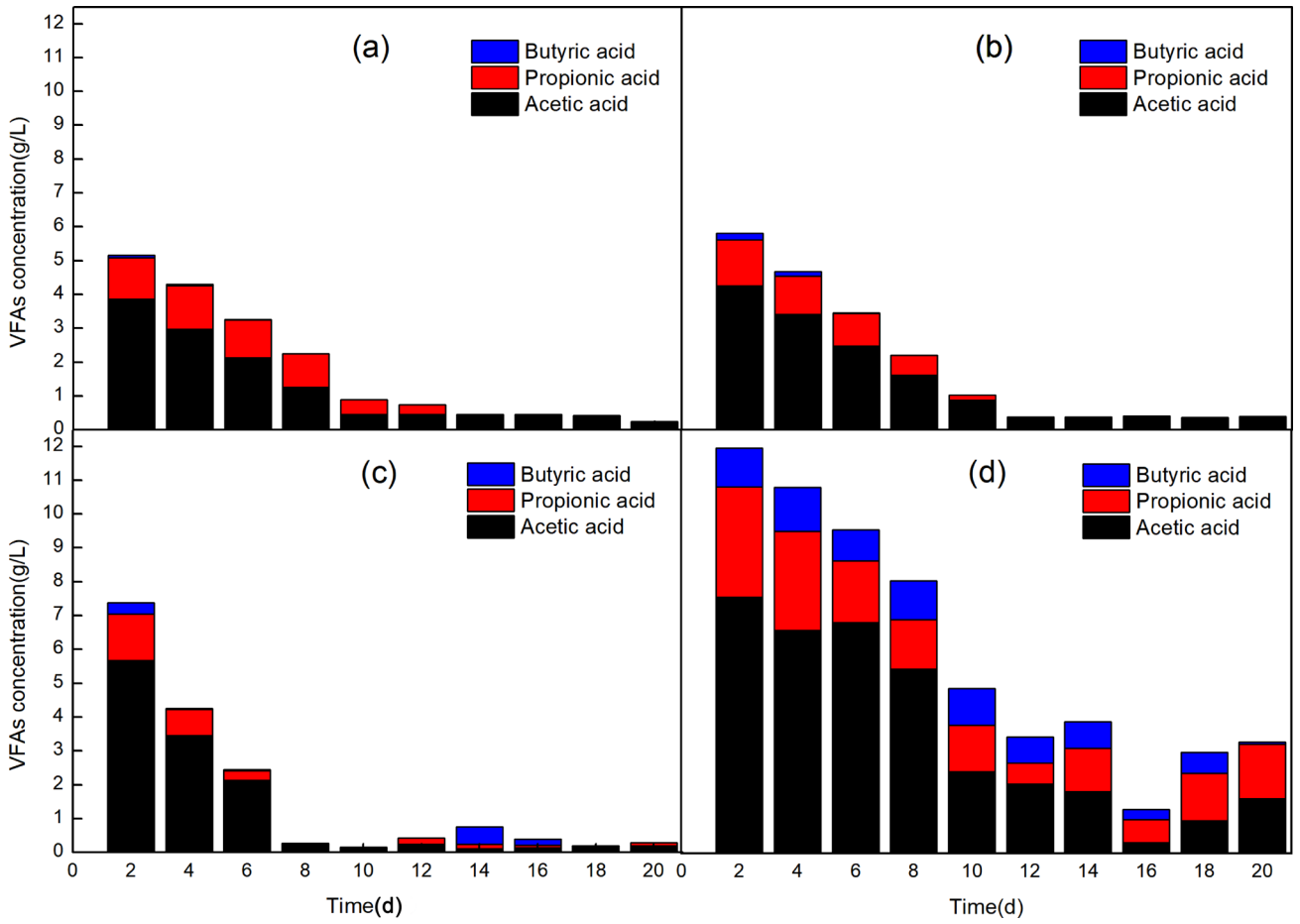


Figure 2. Variation of VFAs concentration with time at different temperatures: (a) 25; (b) 35; (c) 45; (d) 55 °C.

methanogenesis are different under different operating conditions and raw materials. Most of the research works have focused on the exploration of the effect of fermentation temperature on the larger VS of substances such as kitchen waste,[19] pig manure,[13] straw,[14,20,21] sewage sludge,[22,23] and so forth, and few studies have reported the effect of temperature on the fermentation properties and microbial community when low VS of wastewater such as poplar ethanol wastewater was treated by AD.

The objective of this study was to compare the fermentation performance of poplar ethanol wastewater (smaller VS compared to other substrates) at different temperatures. The variation of amounts and compositions of volatile fatty acids (VFAs) was measured during the fermentation process, and the changes in microbial community structure and metabolic pathways at different temperatures were analyzed by high-throughput sequencing technology. This study provides the theoretical and practical bases for the efficient treatment of bioethanol wastewater.

2. RESULTS AND DISCUSSION

2.1. Effect of Temperature on Biogas Production Characteristics of AD. The biogas production characteristics of AD of poplar ethanol wastewater at different temperatures are shown in Figure 1. It can be seen from Figure 1a that the trend of biogas changes at 25 and 35 °C was similar, and both reached the biogas production peak on the 1st day. This can be explained by the higher amount of easily digestible organic compounds supplied initially to the system by the substrate.[24] But the superior performance of this substrate only remained for the 1st day, and then biogas production began to decline. Biogas production kept stable in 3−10 days because the metabolic activity of methanogens was stable at this time. At 45 °C, the peak of biogas production was reached on the 2nd day. Although the biogas production was stable on the 3−5th day, it generally showed a downward trend. The reason was that the methanogens at 45 °C quickly adapted to the environment, so the rate of methane production was quicker than the rate of hydrolysis and acidification, which was also in line with the trend of a sharp decrease in the concentration of VFAs (Figure 2c). The rapid decline in biogas production at 55 °C on the 3rd day, it can be attributed that the microorganisms were still in the adaptation period subjected to high concentrations of VFAs in thermophilic temperatures,[25] which make the fermentation long start-up time, this was consistent with Lin et al.[26] After the 5th day, the microorganisms adapted to the high concentration of VFAs (Figure 2d), and the biogas production began to increase and reached a peak on the 8th and 12th days.

As can be seen from Figure 1a,b that the variation trends of daily biogas production and daily methane production were different, and these differences were mainly determined by the metabolic activity of methanogens. As shown in Figure 1c, the methane content at all temperatures was the lowest on the 2nd day, indicating that the metabolic activity of methanogens was poor at this phase. Methane content increased as anaerobic fermentation proceeded at all temperatures (Figure 1c).

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

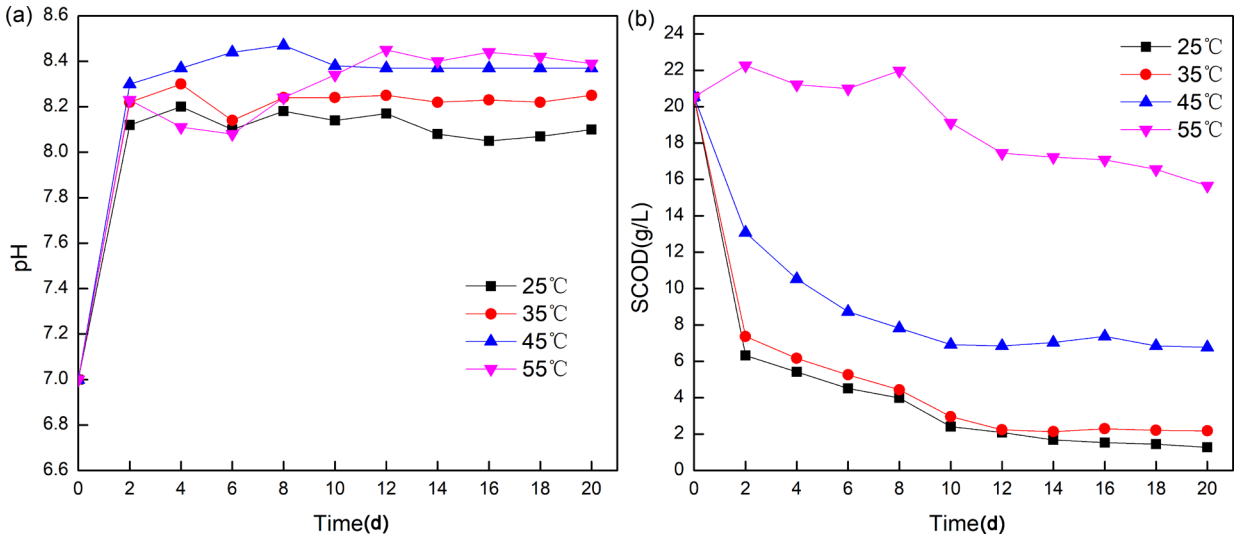


Figure 3. Effects of different temperatures on (a) pH and (b) SCOD.

Although the methane content at 25, 35 and 45 °C was similar in the rising stage, the metabolic rate of methanogens at 45 °C was higher than that at 25 and 35 °C at this phase (Figure 1b). The methane content at 25, 35, and 45 °C remained above 78% from the 6th to the 12th day, after which the content decreased. The reason may be that the VFAs reduced, which weakened the activity of methanogens. While the methane content kept an upward trend at 55 °C, indicating that thermophilic temperature significantly promoted the activity of methanogens.[27] From the effect of temperature on cumulative methane production in Figure 1d, it can be concluded that temperature and biogas production were generally positively correlated, and the higher the temperature, the higher the cumulative methane production. This was inconsistent with the previous report that 45 °C was the transition zone in the anaerobic fermentation process and its gas production should be lower than 35 and 55 °C.[28] It can be seen from the Figure 1d that the cumulative methane yields at 25 and 35 °C were almost the same and that those at 45 and 55 °C were not much different. The cumulative methane production was the highest at 55 °C, which was 81.06, 72.42, and 13.33% higher than that at 25, 35, and 45 °C, respectively.

2.2. Effect of Temperature on Fermentation Characteristics of AD Process. *2.2.1. Variation of VFA Concentration.* The concentrations of VFAs are usually used as the essential parameters to indicate the stability of the AD process. VFAs mainly include acetic acid, propionic acid, and butyric acid.[29] The variation of VFAs during the whole AD process at different temperatures is shown in Figure 2. The concentrations of VFAs reached their maximum value on the 2nd day, and the concentrations of VFAs increased along with temperature on the 2nd day, indicating that the higher the temperature, the higher the rate of hydrolysis and acidification.[30] With the progress of anaerobic fermentation, the concentrations of VFAs in each experimental group gradually decreased. The acetic acid content generally accounted for more than 50% of VFAs in the whole AD at 25, 35, and 45 °C, indicating that acetic acid was the main fermentation substrate. Therefore, the mechanism of methane formation was acetoclastic methanogenesis at 25, 35, and 45 °C, which was consistent with the large proportion of acetoclastic methanogens found in the microbial community analysis (Figure 5). Acetic acid content was higher in VFAs at 55 °C on the 2−14th days, but propionic acid reached more than 50% of VFAs after day 16.

During the AD, the variation trend of VFAs at 25 and 35 °C was similar. They all decreased uniformly from 2 to 10 days, which was consistent with the variation trend of gas production at 25 and 35 °C in Figure 1a, and then decreased to about 1 g/L on the 12th day. The VFAs at 45 °C decreased rapidly from the 2nd to the 8th day, indicating that the methane production rate was much higher than the hydrolysis and acidification rates. This was consistent with a fast rate of methane generation in 2−8 days (Figure 1d). Then methane production gradually decreased with the decrease of the VFAs. The maximum concentration of VFAs reached 11.98 g/L on the 2nd day at 55 °C; it may be that the high temperatures promoted the degradation of organic matter, resulting in higher concentrations of VFAs. On the 4th to 8th day, VFAs decreased slowly. At this phase, the methanogens slowly adapt to the high concentration of VFAs, resulting in the increase in biogas production. On the 16th−20th day, the VFAs contents were 1.30, 2.99, and 3.29 g/L, respectively, but the gas production showed a downward trend. The reason was that the ratio of propionic acid to acetic acid was larger than 1.4 at this phase, and it was generally believed that the anaerobic fermentation process was unstable when the ratio of propionic acid to acetic acid was larger than 1.4.[31]

It can be seen that the concentration of propionic acid in thermophilic temperatures was higher than that in lower temperatures, which was consistent with the result of Wang et al.[32] who revealed mesophilic temperatures were more beneficial for the degradation of propionic acid. Butyric acid was degraded as soon as it was produced, indicating that butyric acid was more easily degraded than propionic acid. The energy needed to convert butyric acid into acetic acid was lower than that of propionic acid (eqs 1 and 2).[33] From the variation of VFAs at each temperature, it was indicated that the decrease of VFAs was consistent with the trend of cumulative methane production (Figure 1d). The higher the temperature, the greater the reduction of VFAs and the higher the cumulative methane production.

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

Table 1. Alpha Diversity Index of Bacteria and Archaea in Samples at Different Temperatures

samples		ACE	Chao	Shannon	Simpson
bacterial	25°C	960.18 ± 4.51	964.28 ± 5.75	4.52 ± 0.01	0.06 ± 0.01
	35°C	938.39 ± 22.45	953.40 ± 29.00	4.36 ± 0.51	0.07 ± 0.05
	45°C	782.30 ± 40.40	797.47 ± 44.29	4.08 ± 0.23	0.07 ± 0.02
	55°C	840.44 ± 37.69	868.55 ± 23.03	4.35 ± 0.64	0.07 ± 0.06
archaeal	25°C	21.63 ± 3.91	21.44 ± 4.03	1.25 ± 0.10	0.47 ± 0.05
	35°C	18.47 ± 1.20	18.17 ± 1.76	1.18 ± 0.15	0.46 ± 0.06
	45°C	18.35 ± 11.05	13.78 ± 3.98	1.18 ± 0.35	0.47 ± 0.17
	55°C	28.77 ± 15.86	24.58 ± 9.82	1.43 ± 0.03	0.33 ± 0.03

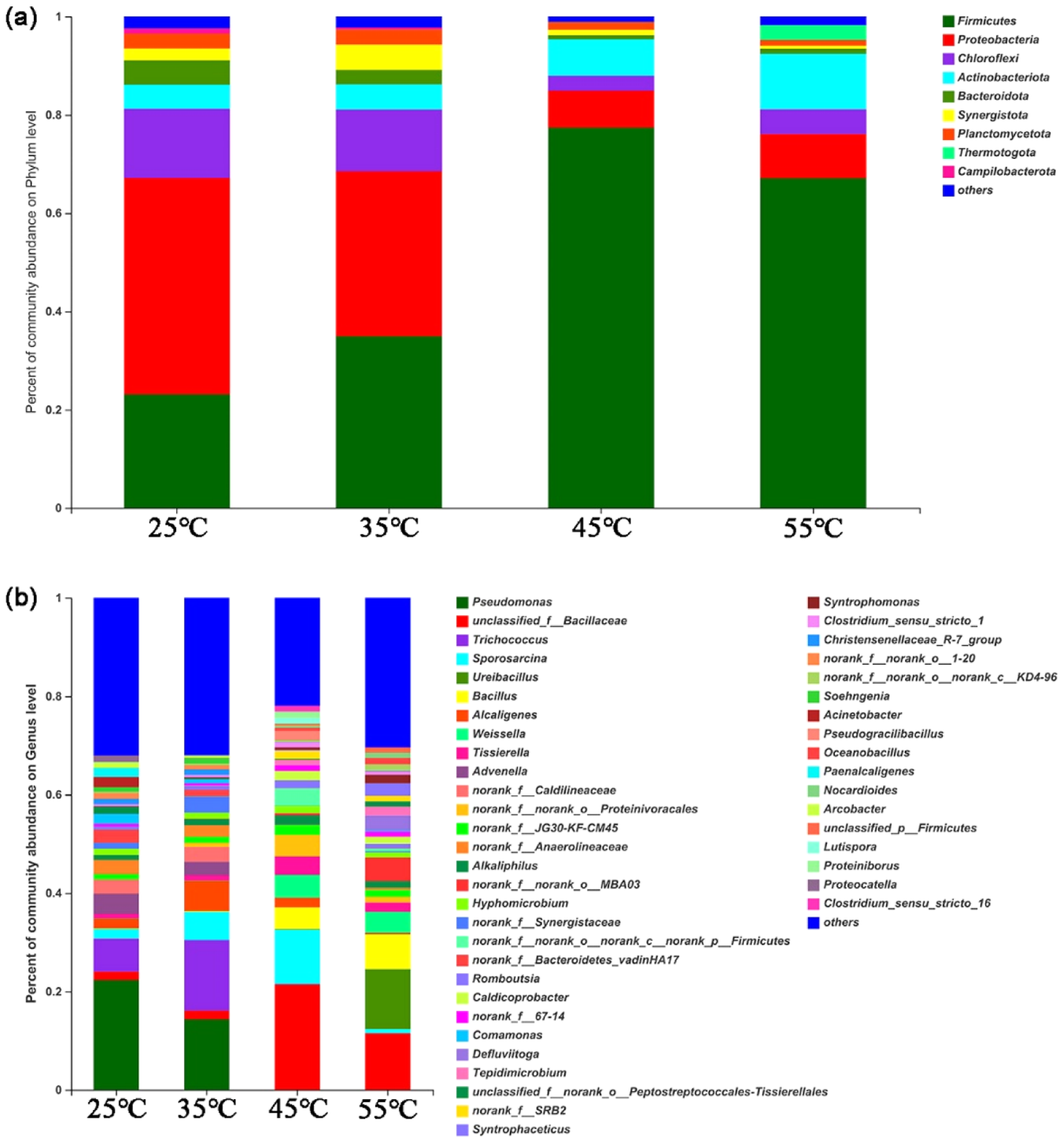


Figure 4. Distribution of bacterial communities at the phylum level (a) and genera level (b) in sludge samples at different temperatures.

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

$$CH_3CH_2CH_2COOH + 2H_2O = 2CH_3COOH + 2H_2$$
$$\Delta G = 48.4\ kJ/mol \quad (1)$$

$$CH_3CH_2COOH + 2H_2O = CH_3COOH + 3H_2 + CO_2$$
$$\Delta G = 76.1\ kJ/mol \quad (2)$$

2.2.2. Variation of pH and SCOD. Anaerobic fermentation of biogas is the result of the common activity of many different kinds of microorganisms, each of which has its own suitable pH. Different bacterial species interact with each other in AD, causing fluctuations in VFA, which in turn lead to the variation of pH.[34] Figure 3a showed the variation of pH with time at different temperatures. At the beginning of AD, the pH value of each temperature rose from 7.0 to 8.10−8.40. The trend of pH at 25 and 35 °C was similar; both increased on the 4th day and gradually stabilized after the 6th day. The pH of 45 °C was in the rising stage from the 2nd to the 8th day. This was attributed to the higher biogas production (Figure 1a,b), which caused the rapid decomposition of VFAs. The trend of pH at 55 °C was also consistent with the change of biogas production (Figure 1). Biogas production was low and VFAs accumulated in 2−6 days, which caused pH decreasing, and pH increased along with gas production increased. The pH range (7.00−8.50) provided a suitable living environment for methanogens.[35]

The variety of SCOD concentrations reflects the efficiency of hydrolysis and acidification during AD. The fermentation temperature of AD can significantly affect the efficiency of enzyme activity and hydrolysis rate.[36] The variation of SCOD with time at different temperatures is shown in Figure 3b. The SCOD concentration at 25, 35, and 45 °C decreased sharply on the 2nd day, followed by a slow decline, and finally stabilized. The SCOD concentration in the AD at 55 °C increased from 20.54 g/L at the initial to 22.27 g/L after 2 days of treatment, and the concentration remained almost stable during the 2nd to 8th day. The increase in SCOD concentration can be ascribed to the fast hydrolysis of easily biodegradable organics in wastewater. On the 9th day, the SCOD concentration began to decline, which corresponded to peak biogas production on the 9th day (Figure 1a). This indicated that the activity of methanogens was enhanced. At the same time, it can be seen from Figure 3b that the variation trend of SCOD concentration and VFAs (Figure 2) was generally consistent, which was consistent with the finding by Magdalena et al.[37] that VFAs represented around 60% of the SCOD content in effluent, whereas the rest (40%) were soluble compounds, which were not converted into methane. Studies have shown that a thermophilic temperature can increase the activity of extracellular enzymes and enhance the hydrolysis rate compared to a mesophilic condition.[38] Therefore, the concentration of soluble substances will be higher at thermophilic temperatures, which will lead to an increase of SCOD in the effluent. The higher the temperature, the higher the SCOD. This indicated that the temperature promoted the degradation of organic compounds in the poplar ethanol wastewater, which was consistent with the fact that Zhang et al.,[39] which found that the content of SCOD in thermophilic temperature was higher than that in mesophilic fermentation using food waste as substrate. Although the SCOD at 45 and 55 °C was higher than that at 25 and 35 °C, the methane content in the biogas was similar to that at 25 and 35 °C, or even higher, indicating that the methanogenic activity was not inhibited.

2.3. Effect of Temperature on Microbial Communities in AD. The bacterial and archaeal 16SrRNA gene sequences of fermentation sludge were analyzed to reveal the microbial community composition and possible pathways at different temperatures.

2.3.1. Analysis of Microbial Community Diversity. The diversity and function of microbial communities at different temperatures may be interrelated. As a key factor affecting AD, the temperature will directly affect the diversity of microorganisms. Therefore, the richness and diversity of bacteria and archaea in sludge at different temperatures were investigated, as shown in Table 1.

ACE and Chao indexes reflect the microbial richness in the sample, and the higher their values, the higher richness of the microbial communities. Shannon and Simpson represent the diversity of microbial communities.[40] The larger the Shannon value, the higher the diversity in the sample, while the opposite is for Simpson. According to the results, the bacterial ACE index and Chao index showed a downward trend from 25 to 45 °C. They rose slightly at 55 °C but were still lower than that at 25 and 35 °C, and Shannon showed the same trend. It indicated that the richness and diversity of the bacterial community decreased from 25 to 45 °C, and it increased a little from 45 to 55 °C. In the archaeal communities, the ACE, Chao, and Shannon indexes at 35 and 45 °C were lower than those at 25 and 55 °C. The highest value at 55 °C indicated that the abundance and diversity of archaea increased at 55 °C. In general, the richness of bacterial communities gradually decreased along with temperature. The archaeal communities differed little at 25, 35, and 45 °C but obviously increased at 55 °C, which were consistent with the highest cumulative methane production at 55 °C (Figure 1d).

2.3.2. Composition of Bacterial Communities. The diversity of bacterial communities at the phylum level under different temperatures is shown in Figure 4a. *Firmicutes* and *Proteobacteria* were the dominant phyla in the AD system at different temperatures, accounting for more than 60% of the total abundances, followed by *Chloroflexi* and *Actinobacterota*. The relative abundances of *Firmicutes* were the highest at 45 °C (75.48%) and 55 °C (62.25%), which were much higher than those at 25 °C (23.07%) and 35 °C (34.95%). *Firmicutes* are reported to be able to tolerate unfavorable environments and produce methanogenic precursors.[41] The relative abundance of *Proteobacteria* showed a decreasing trend along with temperature, and the highest content was 44.12% at 25 °C. *Chloroflexi* also showed a downward trend along with temperature, while *Actinobacterota* was the opposite. It was worth noting that *Thermotogota* only appeared at 55 °C, accounting for 3.02%. Meanwhile, a number of other phyla presented in the AD system might play important roles, although the proportion was relatively low, such as *Bacteroidota*, *Synergistota*, *Planctomycetota,* and so on.

Phylum *Firmicutes*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria,* and *Thermotogata* contain a large number of hydrolytic bacteria as well as acidogenic and fermentative bacteria with different functions. Previous studies have reported that *Firmicutes* are the dominant bacteria in the AD system.[13,20,42] Bacteria belonging to this phylum are fermentative members for the degradation of organic substrates and play an important role in the acetogenic metabolism, with a final product of acetate.[42] *Proteobacteria* are considered a class of bacteria that

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

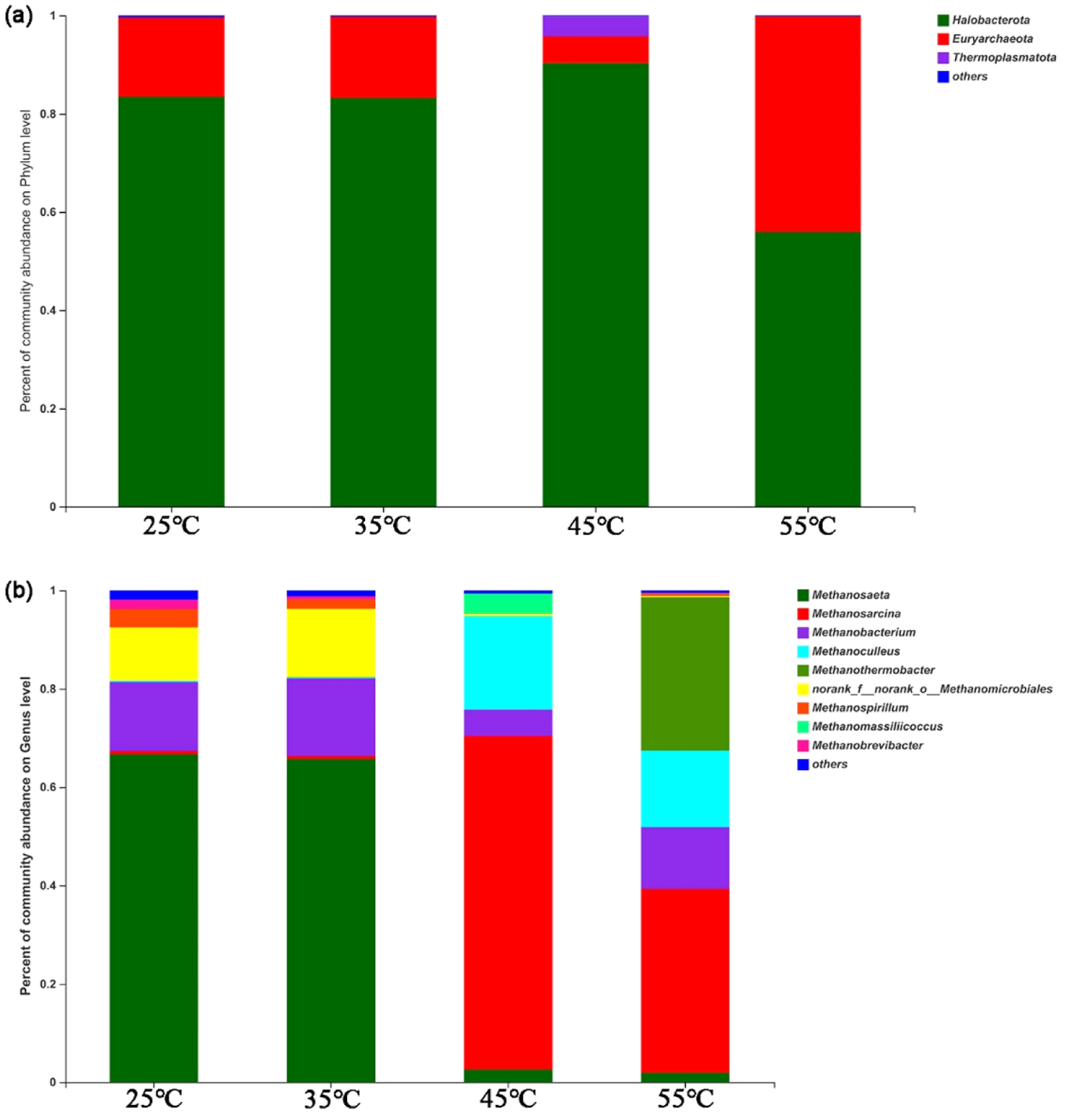


Figure 5. Distribution of archaeal communities at phylum level (a) and genus level (b) in samples at different temperatures.

can degrade glucose, propionic acid, butyric acid, and acetic acid.[43] *Chloroflexi* plays an important role in AD and can degrade both monosaccharides and polysaccharides, as well as generate acetic acid.[34] Some of the *Actinobacteria* contribute to VFAs and propionate production along with hydrolysis.[44] *Thermotogata*, which is commonly found in thermophilic temperature reactors, is capable of degrading acetic acid.[45] The total abundance of these main hydrolytic acid-producing bacteria at 45 and 55 °C was higher than that at 25 and 35 °C, which may be the reason for the higher concentration of VFAs.

Figure 4b shows the distribution of the sample microorganisms at the genus level. It can be seen from Figure 4 that the communities were similar in structure at 25 and 35 °C but showed great differences at 45 and 55 °C. *Pseudomonas*, the predominant genus in the phylum *Proteobacteria*, and *Trichococcus*, the predominant genus in the phylum *Firmicutes*, dominated at 25 and 35 °C because both of those bacteria were mesophilic. *Unclassified _f_ Bacillaceae*, the relative abundance at 45 °C (21.45%) was higher than that at 25 °C (1.74%), 35 °C (1.80%), and 55 °C (11.49%). *Bacillus* is a kind of bacteria that can degrade cellulose.[14] But it only appeared at 45 and 55 °C, which meant that cellulose and

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

other substances in the fermentation liquid could be effectively degraded. *Ureibacillus* is a thermophilic bacterium with the highest content at 55 °C, reaching 12.22%. *Ureibacillus* was reported that can effectively enhance the content of soluble organic matter in thermophilic digestion.[46] In summary, Variation of temperature can remarkably affect the structure and function of microbial communities.

2.3.3. Composition of Archaeal Communities. The distributions of archaeal at the phylum level are shown in Figure 5a. *Halobacterota* and *Euryarchaeota* were the dominant groups in the archaeal phyla. Archaeal communities were similar at 25 and 35 °C, and the same conclusions can be drawn from Figure 5b. But obvious differences of archaeal communities existed between 45 °C (55 °C) and 25 °C (35 °C).

The distribution of archaeal communities at the genus level is shown in Figure 5b. It can be seen from Figure 5b that *Methanosaeta* was the dominant genus at 25 and 35 °C, and its relative abundance reached 66.67 and 65.72%, but decreased substantially to 2.64 and 1.94% at 45 and 55 °C, respectively. The abundance of the genus *Methanosarcina* increased from 25 °C (0.07%) to 45 °C (67.76%), which is in agreement with a previous study (Shin et al. 2019).[10] *Methanobacterium* was present in four samples with abundances of 14, 15.79, 5.38, and 12.57%, respectively. *Norank_f_norank_o_Methanomicrobiales* mainly existed at 25 and 35 °C, and their abundance was less than 1% at both 45 and 55 °C, while *Methanoculleus* was the opposite. *Methanomassiliicoccus* was the exclusive genus at 45 °C (4.09%), while *Methanothermobacter* was the exclusive genus at 55 °C, accounting for 31.14% of the total samples.

Methanosaeta can only utilize acetic acid as a substrate for methanogenesis.[47] *Methanosarcina*, a generalist known to have a high metabolic versatility and able to use acetate, hydrogen, formate, secondary alcohols, and methyl compounds as energy sources.[31] *Methanobacterium*, *Methanoculleus*, and *Methanothermobacter* are all considered to be hydrogenotrophic methanogens, which can produce methane from H_2/CO_2.[48] *Methanoculleus* and *Methanothermobacter* can withstand high temperatures and are often found in various high-temperature reactors, which was consistent with the results of this study.

Based on the above study, it showed that the main metabolic mechanism of methane formed at 25 and 35 °C was acetoclastic methanogenesis. While the relative abundance of *Methanosarcina* and the hydrogenotrophic *Methanoculleus* was increased at 45 °C, it indicated that the metabolic pathway changed from acetoclastic methanogenesis to the hydrogenotrophic methanogenesis. The high relative abundance of hydrogen-producers' bacteria and the accumulation of acetic acid content in the system confirmed that biogas was produced via the hydrogenotrophic pathway at 55 °C. The dominance of acetoclastic methanogenesis and hydrogenotrophic methanogenesis species depends upon the substrate used in AD. When considering thermodynamic stability, the hydrogenotrophic methanogenesis pathway is more promising than the acetoclastic methanogenesis pathway (eqs 3 and 4).[44] In summary, with the increased of temperature, the metabolic pathway of methanogens gradually changed from acetoclastic to hydrogenotrophic methanogenesis, indicating that thermophilic temperature effectively promoted the interspecific electron transfer during anaerobic fermentation. It may also be a reason for the high production of methane in thermophilic temperatures.

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \Delta G = -31.60\ \text{kJ} \tag{3}$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G = -135.00\ \text{kJ} \tag{4}$$

In this study, anaerobic batch experiments were performed on cellulosic ethanol wastewater at 25, 35, 45, and 55 °C. The results showed that with the increase of temperature, the production of biogas and methane increased, and the methanogenic pathway also changed from acetoclastic to hydrogenotrophic methanogenesis. However, SCOD exhibited a negative correlation with temperature, attributed to thermophilic temperatures promoting the dissolution of organic matter. Therefore, it can be inferred that temperature had a great influence on the fermentation performance of cellulosic ethanol wastewater.

3. CONCLUSIONS

Poplar ethanol wastewater can be used for biogas anaerobic fermentation at different temperatures, of which the biogas production was the highest at 55 °C, which was 81.06, 72.42, and 13.33% higher than that of 25, 35, and 45 °C, respectively. The analysis of VFAs and SCOD indicated that the hydrolysis rate was faster along with the temperature. Analysis of the sludge samples after fermentation showed that the main bacterial communities at the four temperatures were *Proteobacteria* and *Firmicutes*, but the microbial community structures were different. *Proteobacteria* was the predominant bacterial community at 25 and 35 °C, while *Firmicutes* dominated at 45 and 55 °C. In the archaeal community, as the temperature increased, the microbial flora began to transform from *Methanosaeta* to *Methanosarcina* and even to hydrogenotrophic methanogens such as *Methanoculleus* and *Methanothermobacter*. This change indicated that the metabolic pathway of methanogens began to shift from the acetoclastic to the hydrogenotrophic methanogenesis.

In summary, temperature is a key factor affecting anaerobic fermentation. This study can provide theoretical and practical bases for the efficient treatment of bioethanol wastewater, but the evolution of various microbes at different temperatures is not explored in this study and can be done in future studies.

4. MATERIALS AND METHODS

4.1. Material and Inoculum. Poplar ethanol wastewater was the residual wastewater obtained by distillation of fermentation liquor, which was derived from poplar saccharification and fermentation in our laboratory. The wastewater was stored at 4 °C until used. The sludge (inoculum) was taken from a wastewater treatment plant in Heze City, Shandong Province. The inoculum was added to the reactor and acclimated at 25, 35, 45, and 55 °C for 20 days, respectively. The characteristics of poplar ethanol wastewater and inoculum are shown in Table 2.

4.2. Experimental Design. Four batch AD experiments (each including three reactors) were conducted in a 1 L Erlenmeyer flask with 0.6 L working volume at 25, 35, 45, and 55 °C. The pH of the wastewater was adjusted to 7 with sodium hydroxide before adding it to the flasks. Each bottle was inoculated with 360 g sludge (inoculum) and 180 mL wastewater, as a ratio of 7.9 (based on VS). Nitrogen was used to blow off for 5 min to construct an anaerobic environment, and then a rubber pad was used to seal the system. Then flasks were placed in water baths at 25, 35, 45, and 55 °C for AD.

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

Table 2. Characteristics of Poplar Ethanol Wastewater and Inoculum[a]

	poplar ethanol wastewater	inoculum
***TS (%)	4.04 ± 0.01	14.20 ± 0.16
*VS (%)	1.77 ± 0.11	7.00 ± 0.11
***pH	5.79 ± 0.10	
COD (g/L)	27.39	
C/N	21.92	
acetic acid (g/L)	3.70	
propionic acid (g/L)	1.04	
butyric acid (g/L)	0	

[a]The date represents the means ± SD, $n = 3$; *, $P < 0.05$; ***, $P < 0.001$.

Each experiment was conducted in triplicate. Biogas and digestive samples were periodically collected from the corresponding reactors.

4.3. Physicochemical Analysis. TS and VS were determined by standard methods (APHA).[49] Total organic carbon and total nitrogen of poplar ethanol wastewater were determined by standard methods (HJ501-2009 and HJ636-2012). Biogas production and methane content were detected by the drainage method and gas chromatography (Agilent 6850, USA), respectively. Digestive samples were centrifuged at 8000 rpm for 10 min, and the supernatant was filtered with a 0.45 μm membrane filter to remove suspended solids for pH, SCOD, and VFAs analysis. The pH was measured by a pH meter (Lemag pHS-3E, Shanghai INESA Scientific Instrument Co., Ltd.). A digestion apparatus (Beijing Lianhua Yongxing Technology Co., Ltd.) and a high-performance liquid chromatograph (Shimadzu LC-20A, Japan) were used to measure the SCOD and VFAs.

4.4. Microbial Community Analysis. The total genomic DNA of microbial communities in sludge after AD was extracted using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The extracted genomic DNA was detected by 1% agarose gel electrophoresis. 515FmodF (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806RmodR(5′-GGACTACNVGGGTWTCTAAT-3′) were used as primers for polymerase chain reaction in the V4 region of 16SrRNA. PCR was performed as described by Cao.[50] Then, high-throughput sequencing was performed on the Illumina Miseq platform (Illumina, San Diego, CA, USA). The structure and distribution of microbial communities in each group were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com).

Original fastq files were treated with the Trimmomatic software package and FLASH software, and low-quality sequence operational taxonomic units (OTUs) were filtered, 97% similarity of OTUs was clustered by UPARSE (version 7.1 http://drive5.com/uparse). The Silva16SrRNA database (version 128 http://www.arb-silva.de) with a confidence threshold of 0.7 was used for taxonomic analysis. The data analyzed were the mean ± standard deviation of triplicate measurements.

AUTHOR INFORMATION

Corresponding Author

Zhiping Wu − *College of Materials Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan 410004, China;* orcid.org/0000-0001-8022-4837; Email: wuzhiping02@163.com

Authors

Xiaofan Yan − *College of Materials Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan 410004, China;* orcid.org/0000-0002-1099-1309

Pingping Deng − *College of Materials Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan 410004, China*

Tingting Ding − *College of Materials Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan 410004, China*

Zuoyu Zhang − *College of Materials Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan 410004, China*

Xingong Li − *College of Materials Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan 410004, China;* orcid.org/0000-0001-6546-8999

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c06721

Author Contributions

X.F.Y.: conceptualization, methodology, investigation, data curation, original draft preparation, and review and editing. T.T.D.: support for formal analysis. Z.Y.Z.: resources and support for formal analysis. X.G.L. and Z.P.W.: conceptualization, supervision, project administration, reviewing results and manuscript, and funding acquisition.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported by the National Key Research and Development Program of China (2019YFB1503805), the Science and Technology Innovation Program of Hunan Province(2021RC4062), and the University Science and Technology Innovation Fund (2022CX02069).

ABBREVIATIONS

AD, anaerobic digestion
PCR, polymerase chain reaction
SCOD, soluble chemical oxygen demand
TS, total solids
TOC, total organic carbon
TN, total nitrogen
VS, volatile solids
VFAs, volatile fatty acid

REFERENCES

(1) Kumari, D.; Conceptualisation, Y. J.; Singh, R. A Study on Green Pretreatment of Rice Straw using Petha Wastewater and Mausami Waste Assisted with Microwave for Production of Ethanol and Methane. *Energy Convers. Manag.* **2020**, *10*, 100067.

(2) Wang, K.; Zhang, J.; Mao, Z. Research Advances in Bioethanol Wastewater Treatment Technologies. *Food Sci. Biotechnol.* **2018**, *37*, 7.

(3) Shan, L.; Yu, Y.; Zhu, Z.; Zhao, W.; Wang, H.; Ambuchi, J. J.; Feng, Y. Microbial community analysis in a combined anaerobic and aerobic digestion system for treatment of cellulosic ethanol production wastewater. *Environ. Sci. Pollut. Res.* **2015**, *22*, 17789−17798.

(4) Wang, X. L. Z. G.; Li, X.; Gong, Z.; Wang, X.; Liang, H.; Gao, D. Co-metabolic substrates enhanced biological nitrogen removal from

cellulosic enthanol biorefinery wastewater using aerobic granular sludge. *Environ. Technol.* **2020**, *41*, 389−399.
(5) Kim, M.; Kim, D.; Yun, Y. Effect of operation temperature on anaerobic digestion of food waste: Performance and microbial analysis. *Fuel* **2017**, *209*, 598−605.
(6) Yang, Y.; Wang, J.; Zhou, Y. Enhanced Anaerobic Digestion of Swine Manure by the Addition of Zero-Valent Iron. *Energy Fuels* **2019**, *33*, 12441−12449.
(7) Park, J.; Kang, H.; Park, K.; Park, H. Direct interspecies electron transfer via conductive materials: A perspective for anaerobic digestion applications. *Bioresour. Technol.* **2018**, *254*, 300−311.
(8) Shan, L.; Zhang, Z.; Yu, Y.; Ambuchi, J. J.; Feng, Y. Performance of CSTR−EGSB−SBR system for treating sulfate-rich cellulosic ethanol wastewater and microbial community analysis. *Environ. Sci. Pollut. Res.* **2017**, *24*, 14387−14395.
(9) Han, Z.; Chen, F.; Zhong, C.; Zhou, J.; Wu, X.; Yong, X.; Zhou, H.; Jiang, M.; Jia, H.; Wei, P. Effects of different carriers on biogas production and microbial community structure during anaerobic digestion of cassava ethanol wastewater. *Environ. Technol.* **2017**, *38*, 2253−2262.
(10) Shin, J.; Jang, H. M.; Shin, S. G.; Kim, Y. M. Thermophilic anaerobic digestion: Effect of start-up strategies on performance and microbial community. *Sci. Total Environ.* **2019**, *687*, 87−95.
(11) Deng, L.; Chen, C.; Zheng, D.; Yang, H.; Liu, Y.; Chen, Z. Effect of temperature on continuous dry fermentation of swine manure. *J. Environ. Manage.* **2016**, *177*, 247−252.
(12) Kafle, G. K.; Kim, S. H. Anaerobic treatment of apple waste with swine manure for biogas production: Batch and continuous operation. *Appl. Energy* **2013**, *103*, 61−72.
(13) Tian, G.; Yang, B.; Dong, M.; Zhu, R.; Yin, F.; Zhao, X.; Wang, Y.; Xiao, W.; Wang, Q.; Zhang, W.; et al. The effect of temperature on the microbial communities of peak biogas production in batch biogas reactors. *Renewable Energy* **2018**, *123*, 15−25.
(14) Wang, S.; Ma, F.; Ma, W.; Wang, P.; Zhao, G.; Lu, X. Influence of Temperature on Biogas Production Efficiency and Microbial Community in a Two-Phase Anaerobic Digestion System. *Water* **2019**, *11*, 133.
(15) Khan, S.; Lu, F.; Jiang, Q.; Jiang, C.; Kashif, M.; Shen, P. Assessment of Multiple Anaerobic Co-Digestions and Related Microbial Community of Molasses with Rice-Alcohol Wastewater. *Energies* **2020**, *13*, 4866.
(16) Pap, B.; Györkei, Á.; Boboescu, I. Z.; Nagy, I. K.; Bíró, T.; Kondorosi, É.; Maróti, G. Temperature-dependent transformation of biogas-producing microbial communities points to the increased importance of hydrogenotrophic methanogenesis under thermophilic operation. *Bioresour. Technol.* **2015**, *177*, 375−380.
(17) Tian, Z.; Zhang, Y.; Li, Y.; Chi, Y.; Yang, M. Rapid establishment of thermophilic anaerobic microbial community during the one-step startup of thermophilic anaerobic digestion from a mesophilic digester. *Water Res.* **2015**, *69*, 9−19.
(18) Röske, I.; Sabra, W.; Nacke, H.; Daniel, R.; Zeng, A.; Antranikian, G.; Sahm, K. Microbial community composition and dynamics in high-temperature biogas reactors using industrial bioethanol waste as substrate. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 9095.
(19) Jiang, J.; Li, L.; Cui, M.; Zhang, F.; Liu, Y.; Liu, Y.; Long, J.; Guo, Y. Anaerobic digestion of kitchen waste: The effects of source, concentration, and temperature. *Biochem. Eng. J.* **2018**, *135*, 91−97.
(20) Liu, C.; Wachemo, A. C.; Tong, H.; Shi, S.; Zhang, L.; Yuan, H.; Li, X. Biogas production and microbial community properties during anaerobic digestion of corn stover at different temperatures. *Bioresour. Technol.* **2018**, *261*, 93−103.
(21) Zhang, W.; Wang, Z.; Guo, H.; Li, L.; Zhang, M.; Zhang, W.; Sun, X.; Sun, S.; Kou, C.; Zhao, W. Biochemical Process and Microbial Evolution in the Conversion of Corn Straw Combined with Coal to Biogas. *ACS Omega* **2022**, *7*, 31138−31148.
(22) Ho, D. P.; Jensen, P. D.; Batstone, D. J. Methanosarcinaceae and Acetate-Oxidizing Pathways Dominate in High-Rate Thermophilic Anaerobic Digestion of Waste-Activated Sludge. *Appl. Environ. Microbiol.* **2013**, *79*, 6491−6500.
(23) Hu, Y.; Wang, F.; Lv, G.; Chi, Y. Enhancing the Biogas Production of Sludge Anaerobic Digestion by a Combination of Zero-Valent Iron Foil and Persulfate. *Energy Fuels* **2019**, *33*, 7436−7442.
(24) Basinas, P.; Rusín, J.; Chamrádová, K. Assessment of high-solid mesophilic and thermophilic anaerobic digestion of mechanically-separated municipal solid waste. *Environ. Res.* **2021**, *192*, 110202.
(25) Yin, Q.; Gu, M.; Wu, G. Inhibition mitigation of methanogenesis processes by conductive materials: A critical review. *Bioresour. Technol.* **2020**, *317*, 123977.
(26) Lin, R.; Cheng, J.; Ding, L.; Murphy, J. D. Improved efficiency of anaerobic digestion through direct interspecies electron transfer at mesophilic and thermophilic temperature ranges. *Chem. Eng. J.* **2018**, *350*, 681−691.
(27) Du, N.; Li, M.; Zhang, Q.; Ulsido, M. D.; Xu, R.; Huang, W. Study on the biogas potential of anaerobic digestion of coffee husks wastes in Ethiopia. *Waste Manage. Res.* **2021**, *39*, 291−301.
(28) Mohd, N. S. Investigation of the Performance and Kinetics of Anaerobic Digestion at 45°C. *J. Water Resour. Protect.* **2015**, *7*, 59796.
(29) Meng, X.; Cao, Q.; Sun, Y.; Huang, S.; Liu, X.; Li, D. 16S rRNA genes- and metagenome-based confirmation of syntrophic butyrate-oxidizing methanogenesis enriched in high butyrate loading. *Bioresour. Technol.* **2022**, *345*, 126483.
(30) Cho, H. U.; Kim, Y. M.; Choi, Y.; Kim, H. G.; Park, J. M. Influence of temperature on volatile fatty acid production and microbial community structure during anaerobic fermentation of microalgae. *Bioresour. Technol.* **2015**, *191*, 475−480.
(31) Franke-Whittle, I. H.; Walter, A.; Ebner, C.; Insam, H. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. *Waste Manag.* **2014**, *34*, 2080−2089.
(32) Wang, Z.; Ma, T.; Xing, L. Process performance and microbial interaction in two-stage continuously stirred tank reactors for sludge anaerobic digestion operated at different temperatures. *Biochem. Eng. J.* **2020**, *161*, 107682.
(33) Saady, N. M. C. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *Int. J. Hydrogen Energy* **2013**, *38*, 13172−13191.
(34) Xu, R.; Zhang, K.; Liu, P.; Khan, A.; Xiong, J.; Tian, F.; Li, X. A critical review on the interaction of substrate nutrient balance and microbial community structure and function in anaerobic co-digestion. *Bioresour. Technol.* **2018**, *247*, 1119−1127.
(35) Deng, P.; Yan, X.; Liu, R.; Ding, T.; Chen, J.; Wu, Z. Effect of cow dung on anaerobic digestion characteristics of poplar fuel ethanol wastewater. *Biomass Convers. Biorefin.* **2022**, 1 DOI: 10.1007/s13399-022-02549-3.
(36) Nie, E.; He, P.; Zhang, H.; Hao, L.; Shao, L.; Lü, F. How does temperature regulate anaerobic digestion? *Renewable Sustainable Energy Rev.* **2021**, *150*, 111453.
(37) Magdalena, J. A.; Greses, S.; González-Fernández, C. Valorisation of bioethanol production residues through anaerobic digestion: Methane production and microbial communities. *Sci. Total Environ.* **2021**, *772*, 144954.
(38) Yan, S.; Miyanaga, K.; Xing, X.; Tanji, Y. Succession of bacterial community and enzymatic activities of activated sludge by heat-treatment for reduction of excess sludge. *Biochem. Eng. J.* **2008**, *39*, 598−603.
(39) Zhang, L.; Loh, K.; Sarvanantharajah, S.; Tong, Y. W.; Wang, C. H.; Dai, Y. Mesophilic and thermophilic anaerobic digestion of soybean curd residue for methane production: Characterizing bacterial and methanogen communities and their correlations with organic loading rate and operating temperature. *Bioresour. Technol.* **2019**, *288*, 121597.
(40) Yang, F.; Li, W.; Liu, C.; Wang, M.; Li, Q.; Sun, Y. Impact of total carbon/sulfate on methane production and sulfate removal from co-digestion of sulfate-containing wastewater and corn stalk. *J. Environ. Manage.* **2019**, *243*, 411−418.

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496

(41) Ng, K. K.; Shi, X.; Ong, S. L.; Ng, H. Y. Pyrosequencing reveals microbial community profile in anaerobic bio-entrapped membrane reactor for pharmaceutical wastewater treatment. *Bioresour. Technol.* **2016**, *200*, 1076−1079.
(42) Xu, R.; Yang, Z.; Zheng, Y.; Liu, J.; Xiong, W.; Zhang, Y.; Lu, Y.; Xue, W.; Fan, C. Organic loading rate and hydraulic retention time shape distinct ecological networks of anaerobic digestion related microbiome. *Bioresour. Technol.* **2018**, *262*, 184−193.
(43) Zhao, S.; Chen, W.; Luo, W.; Fang, H.; Lv, H.; Liu, R.; Niu, Q. Anaerobic co-digestion of chicken manure and cardboard waste: Focusing on methane production, microbial community analysis and energy evaluation. *Bioresour. Technol.* **2021**, *321*, 124429.
(44) D'Silva, T. C.; Isha, A.; Chandra, R.; Vijay, V. K.; Subbarao, P. M. V.; Kumar, R.; Chaudhary, V. P.; Singh, H.; Khan, A. A.; Tyagi, V. K.; et al. Enhancing methane production in anaerobic digestion through hydrogen assisted pathways—A state-of-the-art review. *Renew. Sustain. Energy Rev.* **2021**, *151*, 111536.
(45) Nobu, M. K.; Narihiro, T.; Mei, R.; Kamagata, Y.; Lee, P. K. H.; Lee, P.; McInerney, M. J.; Liu, W. Catabolism and interactions of uncultured organisms shaped by eco-thermodynamics in methanogenic bioprocesses. *Microbiome* **2020**, *8*, 111.
(46) Jang, H. M.; Park, S. K.; Ha, J. H.; Park, J. M. Microbial community structure in a thermophilic aerobic digester used as a sludge pretreatment process for the mesophilic anaerobic digestion and the enhancement of methane production. *Bioresour. Technol.* **2013**, *145*, 80−89.
(47) Lee, J.; Hwang, S. Single and combined inhibition of Methanosaeta concilii by ammonia, sodium ion and hydrogen sulfide. *Bioresour. Technol.* **2019**, *281*, 401−411.
(48) Gao, B.; Wang, Y.; Huang, L.; Liu, S. Study on the performance of HNO3-modified biochar for enhanced medium temperature anaerobic digestion of food waste. *Waste Manag.* **2021**, *135*, 338−346.
(49) Walter, W. G. APHA Standard Methods for the Examination of Water and Wastewater. *Freshw. Sci.* **1998**, *56*, 387.
(50) Cao, Q.; Liu, X.; Ran, Y.; Li, Z.; Li, D. Methane oxidation coupled to denitrification under microaerobic and hypoxic conditions in leach bed bioreactors. *Sci. Total Environ.* **2019**, *649*, 1−11.

https://doi.org/10.1021/acsomega.2c06721
ACS Omega 2023, 8, 5486−5496