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Research article

Process stability and microbial adaptation during temperature shift from thermophilic to mesophilic temperature in anaerobic digestion

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Abstract: Thermophilic anaerobic digestion often achieves higher biogas yields than mesophilic conditions, but its high energy demand limits long-term sustainability. As a result, many thermophilic digesters may transition toward mesophilic conditions. This study examined the effects of stepwise temperature shift from thermophilic to mesophilic ranges on biogas production and microbial community dynamics, while identifying factors that support or inhibit methane generation. At 55 °C and 50 °C, methane yields reached 342 and 311 mL CH_4/g COD, respectively, with a predominance of hydrogenotrophic *Methanobacterium*, alongside abundant *Coprothermobacter, Defluviitoga, Acetomicrobium, Candidatus Bipolaricaulis, Brachyspira*, and *Dictyoglomus*, suggesting potential syntrophic interactions between these microorganisms. At 45 °C and 40 °C, the methane yield declined to 234 and 219 mL CH_4/g COD but recovered to 289 and 251 mL CH_4/g COD at 35 °C and 30 °C, respectively. In the mesophilic range, frequent accumulation of acetate and butyrate coincided with a methanogenic shift from hydrogenotrophic *Methanobacterium* to acetoclastic *Methanothrix*, indicating a pathway transition. These results reveal that lowering the digestion temperature from thermophilic to mesophilic conditions enables recovery of acetogenic activity and shifts methanogenesis from hydrogenotrophic to acetoclastic pathways, with implications for optimizing biogas production during long-term operation.

Keywords: anaerobic digestion; microbial community; mesophilic; temperature decrease; thermophilic

1. Introduction

Global sustainable development has been encountering two major issues: the escalating energy crisis and ongoing environmental degradation. Anaerobic digestion (AD) offers a promising solution by converting diverse residual biomass sources, such as sewage sludge, anaerobic sludge, agricultural residues, and food waste, into renewable bioenergy and biofertilizer, thereby addressing both issues simultaneously. AD is considered one of the most efficient technologies, where diverse microorganisms degrade organic matter through sequential metabolic processes [1,2]. The AD process yields several products, most notably biogas, which consists primarily of methane (50%–70%) and carbon dioxide (30%–50%), along with minor impurities such as hydrogen sulfide, ammonia, and moisture [2–4]. Biogas production relies on the synergistic activity of microbial communities involved in four key stages of AD: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [5,6].

During hydrolysis, complex organic matter is broken down into simpler, soluble compounds [1,7]. In acidogenesis, these compounds are converted into volatile fatty acids (mainly acetate, butyrate, and propionate), along with hydrogen and carbon dioxide [7]. During acetogenesis, intermediate products are further transformed into acetate, hydrogen, and carbon dioxide [7,8]. Finally, in methanogenesis, methane is produced mainly from acetate or from hydrogen and carbon dioxide via acetoclastic and hydrogenotrophic pathways [9,10]. In the acetoclastic pathway, acetate is directly converted to methane, a process mainly carried out by members of the genus *Methanothrix* and *Methanosarcina*. *Methanothrix* often dominates under low acetate concentrations (100–150 mg COD/L) because of its higher substrate affinity compared to *Methanosarcina* [9,10]. In contrast, *Methanosarcina* becomes more competitive at higher acetate concentrations due to its greater substrate uptake capacity and tolerance to fluctuating conditions [10]. On the other hand, the hydrogenotrophic pathway relies on hydrogen and carbon dioxide to generate methane, and the majority of methanogens belong to this group, particularly those within the orders Methanomicrobiales, Methanobacteriales, Methanococcales, Methanopyrales, and Methanocellales [11,12].

The AD system is commonly operated within three temperature ranges: psychrophilic (<20 °C) [13], mesophilic (20–43 °C, with an optimum of 35–37 °C) [14], and thermophilic (50–60 °C) [15]. Previous studies have explored the influence of temperature on biogas production from anaerobic sludge. Mirmasoumi et al. [16] reported that methane productivity was substantially higher under thermophilic conditions (0.64 m^3 CH_4/m^3·day at 55 °C) compared with mesophilic conditions (0.246 m^3 CH_4/m^3·day at 37 °C). Similarly, Kasiński [17] observed higher methane yields at thermophilic temperatures (0.56–0.70 L CH_4/g VS) than at mesophilic temperatures (0.25–0.32 L CH_4/g VS). These findings suggest that thermophilic operation generally enhances biogas productivity. However, maintaining thermophilic conditions in full-scale Wastewater Treatment Plants (WWTPs) requires substantial energy input, thereby increasing operational costs.

To address these limitations, previous studies explored the effect of transitioning from thermophilic to mesophilic conditions to discover potential microbial adaptations that may lead to the enhancement of methane production. Zhang et al. [18] conducted a stepwise temperature reduction from 55 to 35 °C at a rate of 1 °C per day and observed a substantial decline in performance within the transition zone. However, the study did not provide a detailed discussion of biogas production or

microbial community dynamics specifically within the 40–45 °C range during the transition to 35 °C. A few studies have examined the effects of stepwise temperature decreases on biogas production and microbial community dynamics. Some studies applied large temperature shifts of 12–20 °C per step, which caused a sharp decline in the abundance of key microbial groups such as *Proteobacteria* [19], *Ruminococcaceae*, and *Methanobacterium* [20]. Other studies employed smaller stepwise shifts (3–7 °C), such as from 55 to 45 °C, which resulted in a mild reduction in biogas production [21,22].

Nonetheless, the impacts of prolonged temperature transitions from thermophilic to mesophilic conditions, particularly with extended incubation at each temperature step, on biogas production and microbial community dynamics remain insufficiently understood. While previous studies, including our earlier work [21,22], have examined stepwise temperature reductions and associated microbial succession, these investigations primarily focused on shorter transition periods with bigger temperature intervals, which may prevent microbial communities from adapting to the changing environment. In contrast, the present study extends the incubation period to two months at each temperature, allowing the anaerobic microbial community to undergo deeper acclimation and stabilization at each thermal condition. This approach enables a more detailed evaluation of sustained biogas production performance, potential inhibitory factors, and long-term shifts in microbial community structure and interactions that are not readily captured in shorter-term experiments. Therefore, this study aims to provide new insights into the resilience, recovery, and strain dominance of methanogenic communities during prolonged thermophilic-to-mesophilic transitions, offering a clearer mechanistic understanding of temperature-driven microbial adaptation in anaerobic digestion systems.

2. Materials and methods

2.1. Inoculum and substrates

In the current study, digested sewage sludge was utilized as the source of microorganisms. The digested sludge was obtained from the Eastern Wastewater Treatment Plant in Ube City, Yamaguchi Prefecture, Japan, with its characteristics summarized in Table 1. For the experiments, 250 mL of digested sludge was transferred into 500 mL vials. To ensure anaerobic conditions, nitrogen gas was purged into each vial before sealing with aluminum caps and butyl rubber stoppers. To ensure a simultaneous fermentation and to avoid the death phase for methanogens, a fed-batch approach was employed. A glucose-based substrate (20 mL) was supplemented to the vials every 15–20 days. This feeding interval was selected based on the observation that biogas production consistently ceased within 15–20 days in each batch cycle, consistent with our previous studies [21,23,24]. The substrate was also supplemented with several nutrients to support the growth of microbial communities, as detailed in Table 2, where solutions A, B, and C represent the inorganic nutrient salts. Specifically, solution A contained 350 g/L $(NH_4)_2HPO_4$; solution B comprised 75 g/L KCl, 85 g/L NH_4Cl, 42 g/L $FeCl_3 \cdot 6H_2O$, 81 g/L $MgCl_2 \cdot 6H_2O$, 25 g/L $MgSO_4 \cdot 7H_2O$, and 1.8 g/L $CoCl_2 \cdot 6H_2O$; and solution C contained 150 g/L $CaCl_2 \cdot 2H_2O$ [25,26].

Table 1. Characteristics of digested sewage sludge used in the current study.

Parameter	Concentration	Units
pH	7.89	-
SS	11,000	mg/L
VSS	8000	mg/L
COD	1760	mg/L
NH_4^+	1167.6	mg/L
Acetic acid	290.34	mg/L

Table 2. Composition of glucose-based substrate.

Composition	Concentration	Units
Glucose	15	g/L
Yeast extract	0.1	g/L
$NaHCO_3$	5	g/L
K_2HPO_4	5	g/L
Solution A	2.0	ml/L
Solution B	10	ml/L
Solution C	1.0	ml/L

2.2. Experimental setup

In the present study, the incubation temperature was gradually reduced from thermophilic to mesophilic conditions. The vials were first incubated at 55 °C as the optimal temperature for thermophilic anaerobic digestion for two months. The temperature was then lowered to 50 °C, followed by 45 °C, and subsequently to 40 °C, with an interval of 2 months incubation in each temperature. Incubation from 55 to 40 °C was performed using a water bath incubator (BT 300; Yamato Scientific Co., Ltd., Tokyo, Japan). Afterward, the temperature was further decreased to 35 °C and finally to 30 °C, each for two months, using an SLI-700 EYELA incubator (RIKAKIKAI Co., Ltd., Tokyo, Japan).

2.3. Data collection and analysis

Biogas production was quantified using a glass syringe, while gas composition was analyzed by gas chromatography (GC) with a GC-8APT/TCD system (Shimadzu Corp., Kyoto, Japan), targeting H_2, CH_4, and CO_2. The injector, column, and detector temperatures were set to 50, 60, and 50 °C, respectively. Volatile fatty acids (VFAs) were determined using high-performance liquid chromatography (HPLC) (LC-20AD, Shimadzu Corp., Kyoto, Japan) with a SH1011 column. VFAs, defined as fatty acids with six or fewer carbon atoms, are known to inhibit methanogenic activity [27]. A 5 mM H_2SO_4 solution was used as the mobile phase for the HPLC, with the column maintained at 50 °C and a flow rate of 0.6 mL/min. In addition, pH, chemical oxygen demand (COD), suspended solids (SS), volatile suspended solids (VSS), and ammonia were measured following the Standard Methods for the Examination of Water and Wastewater [28]. Ammonia is essential for microbial growth; however, excessive ammonium ions are known to inhibit methanogenic activity, similar to VFAs [29].

2.4. DNA extraction and microbial analysis

A total of 1.5 mL sludge samples were taken from various temperature conditions for DNA extraction and stored at -22 °C [22]. DNA was extracted following the standard protocol of the NucleoSpin® Soil Manual using the NucleoSpin® kit, and DNA concentration was determined with the Qubit® dsDNA Assay Kit on a Qubit® 4.0 Fluorometer (Life Technologies, CA, USA). PCR amplification for NGS was conducted in two phases. In the first PCR, the V4 region of the 16S rRNA gene was amplified using primers 515F/806GC and the KAPA HiFi HotStart ReadyMix (TaKaRa Bio Inc., Shiga, Japan). Amplicons were purified with the NucleoSpin® Gel and PCR Clean-Up kit to remove residual primers. The second PCR was performed with Illumina sequencing adapters using the Nextera XT V2 Index Kit, followed by purification with AMPure XP beads, 10 mM Tris-HCl (pH 8.5), and 80% ethanol. High-throughput next-generation sequencing (NGS) was carried out on the Illumina iSeq 100 platform at the Department of Environmental Engineering, Yamaguchi University, Japan. Taxonomic classification was performed using the Dragen Metagenomics Pipeline with the Extended Kraken database as the reference. Microbial diversity was assessed to evaluate changes in methanogen populations during anaerobic digestion under shifted temperature conditions, using the Shannon diversity index, Simpson's index, and evenness index. Correlation analysis was conducted with Pearson's correlation in RStudio (version 2024.12.0 Build 467), and heatmap visualization was performed with MeV (version 4.9.0).

3. Results

3.1. Changes in biogas production following the change in temperature conditions

Under the stepwise temperature decrease from the thermophilic range, changes in biogas yield, daily biogas production, and biogas composition are shown in Figure 1(a)–(c). As seen in Figure 1(a), at 55 °C, biogas production reached 342 mL CH_4/g COD and 273 mL CO_2/g COD. When the temperature was lowered to 50 °C, methane and carbon dioxide production decreased slightly to 311 mL CH_4/g COD and 256 mL CO_2/g COD. In this condition, the methane-to-carbon dioxide ratio remained stable in the range of 1.20–1.25, indicating uninhibited methanogenesis with dominance of the acetoclastic pathway [23,30]. This condition was also supported by the stable daily methane production of 13.49–14.40 mL CH_4/day, followed by methane content within the range 40%–60%, despite a transient drop to 16% at 55 °C. At both thermophilic temperatures, elevated acetic acid concentrations were detected, particularly during the early phase of incubation at 50 °C, where the acetic acid production reached 800 mg HAc/L (Figure 2(a)). This accumulation suggests an intense activity of acetogenic bacteria under thermophilic conditions.

When the temperature shifted to 45 °C, acetic acid production declined markedly. Correspondingly, methane and carbon dioxide production decreased to 234 mL CH_4/g COD and 216 mL CO_2/g COD, representing a 25% reduction compared to 50 °C. The methane-to-carbon dioxide ratio also declined to 1.08, indicating weaker methanogenic activity relative to the thermophilic condition. At this temperature, the methane content ranged from 15% to 55%, with average daily production tumbling to 10.93 mL CH_4/day. From day 150 until the end of incubation at 45 °C, acetic acid was no longer detected, suggesting diminished acetogenesis, which in turn limits the acetoclastic methanogenesis that further contributes to the reduction in methane production. The decline in biogas production continued to the level of 219 mL CH_4/g COD and 178 mL CO_2/g COD when the temperature was further lowered to 40 °C. Despite the declining biogas yield, the methane-to-carbon dioxide ratio increased significantly to 1.23, indicating incremental methane production followed by

the higher CO_2 conversion through hydrogenotrophic methanogenesis at 40 °C, compared to 45 °C.

Interestingly, the acetic acid concentration experienced a significant spike, reaching 1,000 mg HAc/L, following the shift to 40 °C, then consumed and maintained below 400 mg HAc/L throughout the incubation period. This substantial change in acetic acid production marked a potential recovery by acetogenic bacteria as they adapted to thermotolerant conditions. Methane production showed signs of recovery upon further shift to 35 °C, reaching 289 mL CH_4/g COD. Carbon dioxide production also rose sharply to 253 mL CO_2/g COD, resulting in a methane-to-carbon dioxide ratio of 1.14. Interestingly, after the temperature was lowered, butyric acid concentration spiked to 600 mg HBu/L. However, it was no longer detected after day 250, suggesting that it was rapidly consumed and converted to acetic acid through acetogenic bacteria.

Despite a transient spike at 35 °C, a further shift to 30 °C caused biogas production to decline markedly to 251 mL CH_4/g COD and 233 mL CO_2/g COD. At this temperature, both acetic and butyric acids were frequently detected, in contrast to other conditions. These results suggest the temperature shift to 30 °C may favor a wide array of microbial communities involved in hydrolysis and acidogenesis while potentially inhibiting methanogenesis. Despite fluctuations in biogas production (Figure 2(b)), the pH remained stable within the range of 6.8–7.6, which is considered optimal for anaerobic digestion. In addition, although the ammonium concentrations reached 951 mg NH_4^+ N/L (Figure 2(c)), no apparent inhibitory effects on biogas production were observed under the present conditions.

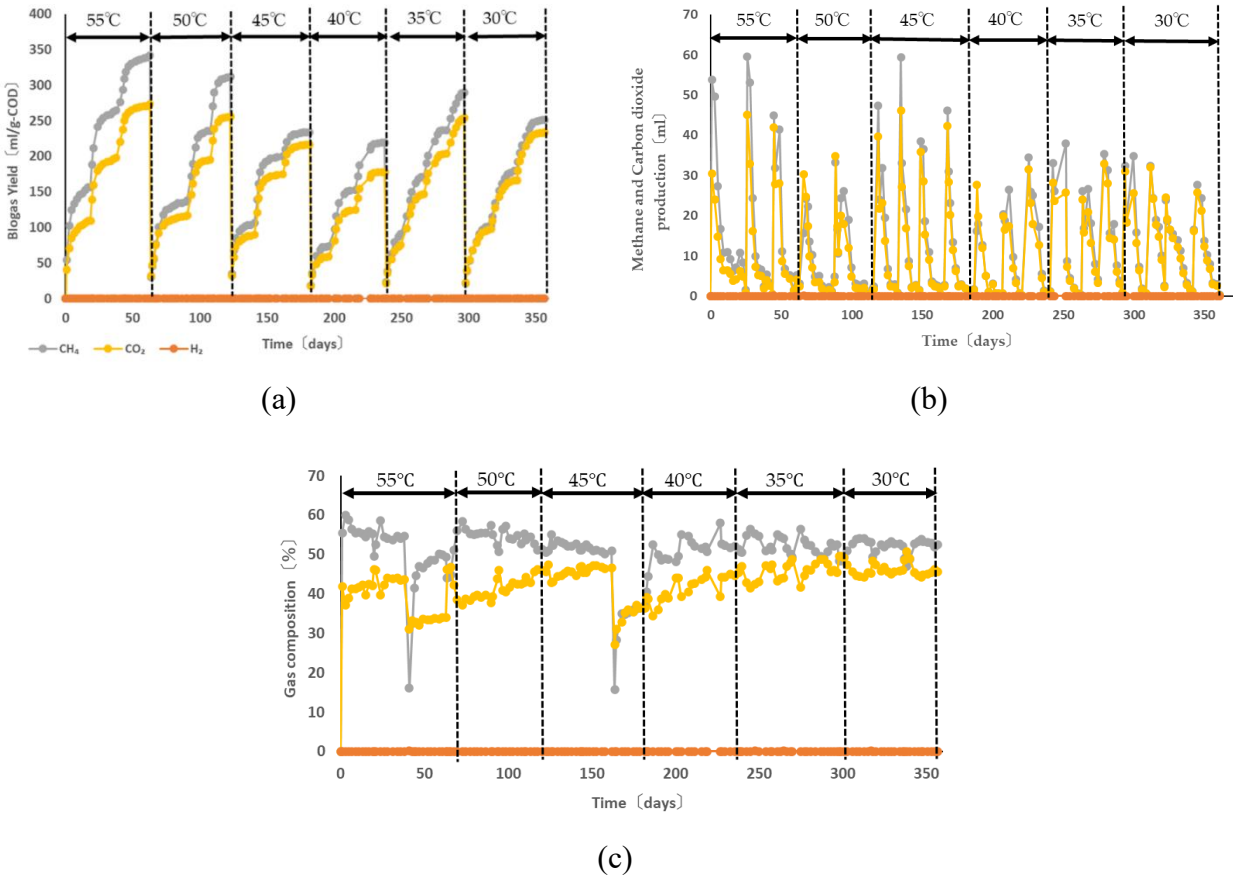


Figure 1. Profile of biogas production yield (a), daily production (b), and biogas composition (c) at each temperature during the digestion process.

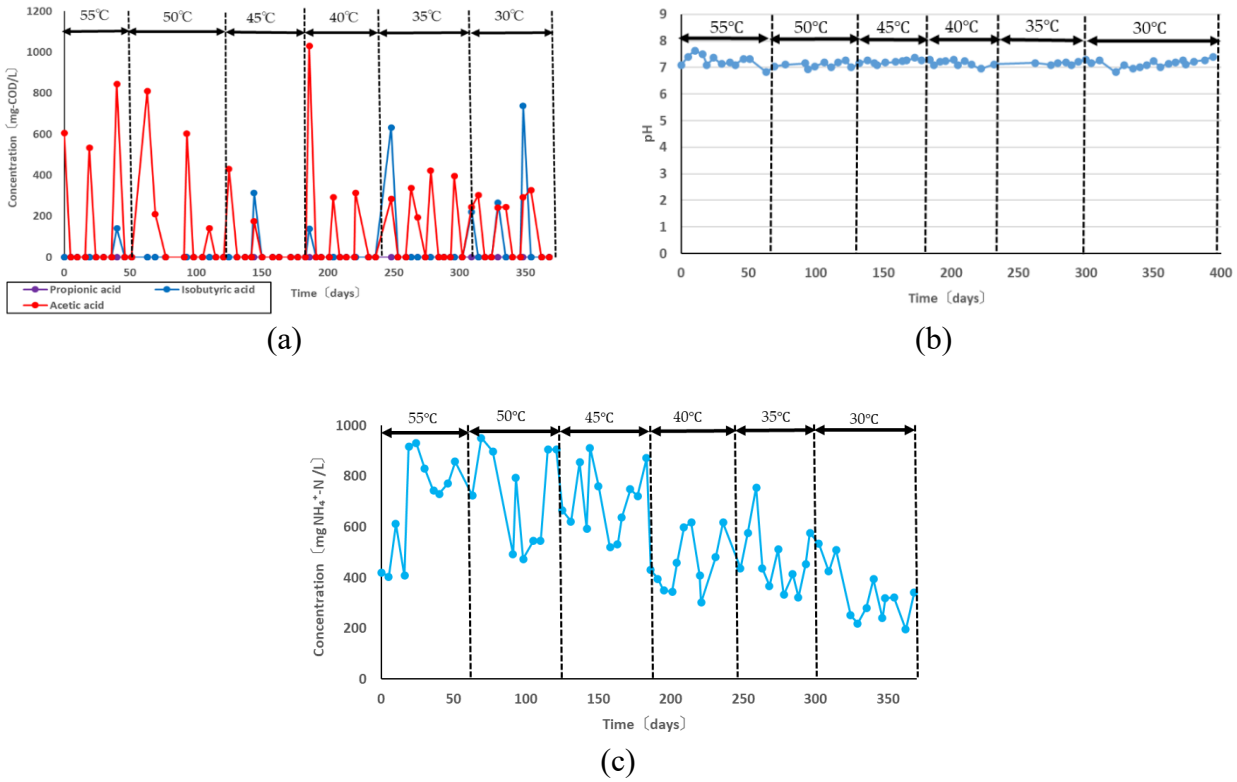


Figure 2. Level of VFAs (a), pH (b), and ammonia nitrogen (c) concentration in each temperature condition.

3.2. Diversity analysis of microorganisms

Alpha diversity analysis was conducted to evaluate changes in microbial abundance and diversity across temperature conditions during the transition from thermophilic to mesophilic ranges. Diversity was assessed using Shannon's, Evenness, and Simpson's indices. These indices have been widely applied in previous studies to analyze microbial community abundance under varying temperatures, providing insights into community responses to temperature shifts [31–33]. As shown in Figure 3, Shannon's diversity index gradually declined as the temperature decreased to 40 °C, whereas Simpson's index exhibited a transient increase at 45 °C, despite both being measures of diversity and abundance. This discrepancy likely arises because the two indices emphasize different aspects of diversity: the Shannon index is more sensitive to species richness and the presence of rare taxa, while the Simpson index is more strongly influenced by the abundance of dominant species [34]. Consequently, microbial communities with the same species count, but differing distributions, can yield distinct Shannon and Simpson index values.

The evenness index also showed a temporary increase at 45 °C, suggesting that microbial species were more evenly distributed under this condition. Combined with the elevated Simpson's index at the same temperature, this indicates that community dominance was reduced, with no single species exerting strong dominance over the others. In this condition, the methane production started to decline while the VFA was detected in low abundance. The elevated microbial evenness may lead to the utilization of VFAs and other substrates by a broader range of microorganisms for diverse metabolic pathways rather than being directed primarily toward methane production.

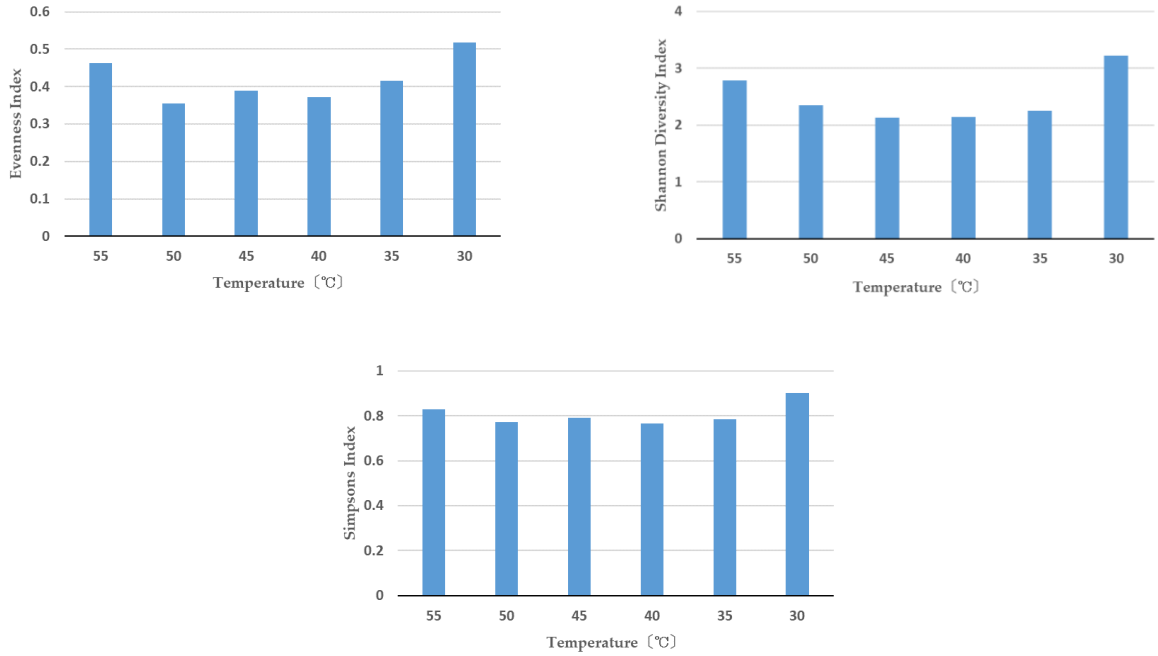


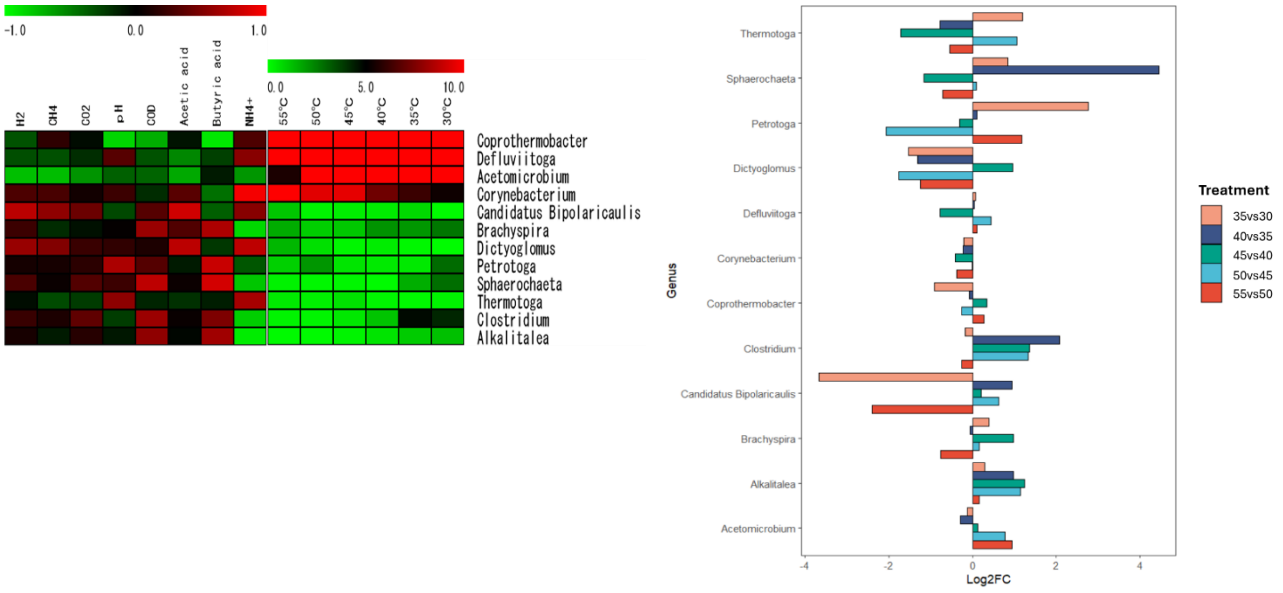
Figure 3. Alpha diversity analysis of microbial communities under different temperature conditions.

3.3. Microbial community analysis in each temperature shift during incubation

To examine the microbial community structure under downshifted temperature conditions and its interrelationship with key fermentation parameters, both a relative abundance heatmap and a correlation plot were generated. The heatmap of hydrolytic and acidogenic taxa relative abundance is presented in Figure 4(a). Among the hydrolytic and acidogenic bacteria identified in this study, *Coprothermobacter* (21%–43%) and *Defluviitoga* (15%–26%) consistently exhibited high relative abundance across all temperature conditions. *Coprothermobacter* is known to metabolize sugars and proteins, producing acetate, hydrogen, and CO_2 as end products [35], and its activity is closely linked to supporting hydrogenotrophic methanogens in providing available hydrogen and CO_2 for methane production [36,37]. Similarly, *Defluviitoga* ferments a wide range of carbohydrates to produce acetate, ethanol, hydrogen, and CO_2, and has been frequently isolated from thermophilic biogas reactors [36,37]. Another dominant genus, *Acetomicrobium*, which metabolizes VFAs to produce acetate, hydrogen, and CO_2, also maintained a relatively high abundance (10%–19%) across all temperature conditions, although its prevalence was lower under thermophilic conditions. These strains exhibited relatively stable abundances compared to other communities, highlighting their resilience to temperature fluctuations (Figure 4(b)).

However, despite the domination of *Coprothermobacter*, *Defluviitoga*, and *Acetomicrobium* in all temperature conditions, they exhibited low correlation to the concentration of methane, hydrogen, acetic acid, and butyric acid. This finding suggests that these microorganisms are not strictly dependent on the availability of such compounds, and their presence did not exert a direct influence on the production of key metabolites, particularly methane and hydrogen. In contrast to the dominant strains, the acidogenic *Candidatus Bipolaricaulis* and the hydrolytic genera *Brachyspira* and *Dictyoglomus* showed stronger correlations with the production of methane, hydrogen, and acetic acid, highlighting their significant role in the fermentation process. However, despite the high correlations, their relative

abundances fluctuated and tended to decline with decreasing temperature, indicating a greater sensitivity to thermal shifts.

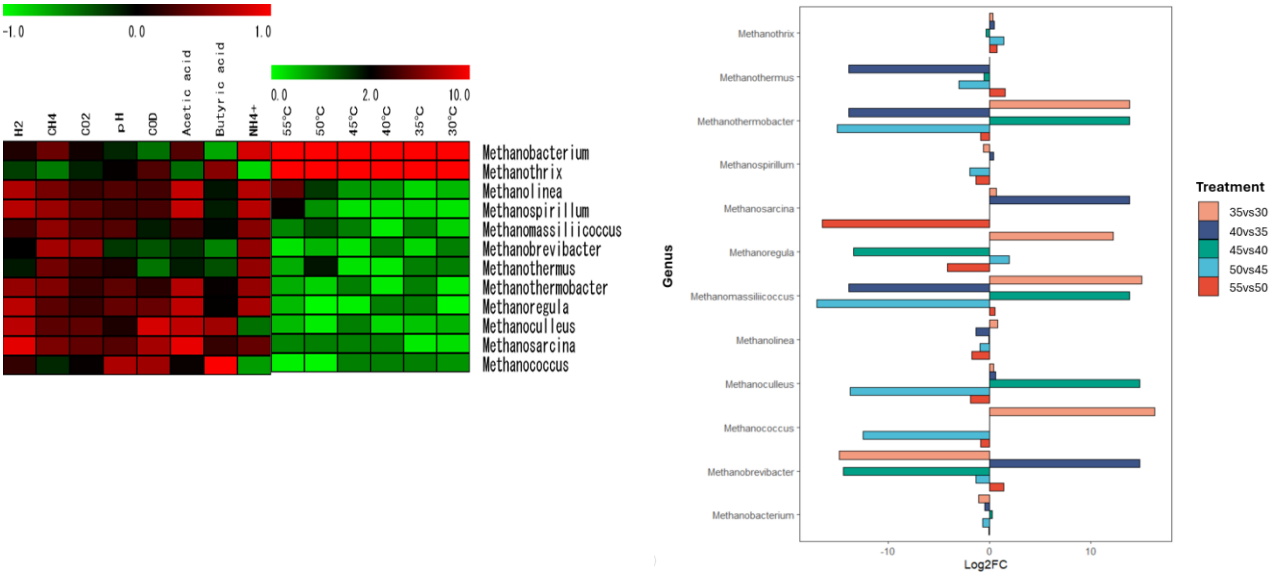


(a) (b)

Figure 4. Heatmap representing the relative abundance of hydrolytic, acidogenic, and acetogenic bacteria (right side) and the correlation plot between microbial abundance to several fermentation parameters (left side) (a). Log_2 fold-change was performed to clearly illustrate the change of microbial abundance in each temperature condition (b).

Among the methanogenic archaea identified in this study, *Methanobacterium* accounted for the highest relative abundance, comprising 73%–76% of the total methanogens under thermophilic conditions (Figure 5(a)). However, its abundance declined sharply when the temperature shifted to the thermotolerant range, falling below 60%, and ultimately decreased to only 19% at 30 °C. In contrast, the acetoclastic *Methanothrix* exhibited relatively low abundance at thermophilic temperatures (55–50 °C), comprising only 11%–19% of the total methanogens. However, its abundance increased markedly under thermotolerant and mesophilic conditions, surpassing *Methanobacterium*. At 35 and 30 °C, *Methanothrix* dominated the methanogenic community, accounting for 57%–73% of the total methanogens. Interestingly, when linked to methane production yield, the shift in dominance from *Methanobacterium* to *Methanothrix* was followed by a reduced methane output. Both the yield and production rate were higher when *Methanobacterium* dominated the methanogenic community compared to periods of *Methanothrix* prevalence. Correlation analysis further confirmed that *Methanobacterium* exhibited a stronger positive association with methane production than *Methanothrix*.

These findings suggest that hydrogenotrophic methanogenesis contributes more effectively and significantly to biogas production than acetoclastic methanogenesis, even under mesophilic conditions. However, other methanogenic pathways, such as methylotrophic routes, were not analyzed in detail due to the low and highly variable abundance of the corresponding taxa, but these microorganisms may still play indirect or supporting roles in the anaerobic digestion process.



(a) (b)

Figure 5. Heatmap showing the relative abundance of methanogens (right side) alongside the correlation plot between methanogen abundance and selected fermentation parameters (left side) (a). Log_2 fold-change analysis illustrating microbial abundance across temperature conditions (b).

4. Discussion

Temperature is a critical factor influencing microbial communities [38]. Deviations beyond the optimal range can significantly impact metabolic activity and growth, as microorganisms are susceptible to environmental stress, especially thermal changes [38,39]. Previous studies have shown that methanogenic activity is reduced under decreasing temperatures [21,22]. However, these studies applied relatively large temperature shifts (3–7 °C intervals), which often resulted in suppressed biogas production due to thermal shock, particularly when transitioning into the thermotolerant range (40–45 °C). Unlike previous studies that examined abrupt temperature shifts, this study systematically extended the transition from thermophilic to lower mesophilic conditions to assess the potential for biogas production recovery and to investigate microbial community dynamics during the shift.

The present study found that methane production performed optimally under thermophilic conditions compared to when transitioned to the mesophilic range. Reducing the temperature from 55 to 50 °C led to a decline in methane production (342 to 311 mL CH_4/g COD), which coincided with a lower minimum threshold of acetic acid concentration. In this condition, the hydrogenotrophic *Methanobacterium* dominated the methanogen community, while the acetoclastic *Methanothrix* grew at a slow pace. This aligns with previous studies reporting that thermophilic anaerobic digestion favors hydrogenotrophic methanogenesis, especially *Methanobacterium*, largely because elevated temperatures, followed by high free ammonia concentrations, and increased levels of VFA promote this pathway over acetoclastic methanogenesis [24,40,41].

However, as the temperature decreased further to 45 and 40 °C, as shown in Figure 5(b), a pronounced decline in key methanogenic populations was observed, particularly when the temperature was shifted from 50 to 45 °C, which likely contributed to the reduction in methane production. The dominance of hydrogenotrophic methanogens shifted toward acetoclastic methanogens, marked by the

decline of *Methanobacterium, Methanoculleus*, *Methanothermobacter, Methanospirilium,* and *Methanothermus* abundance, while the acetoclastic *Methanothrix* exhibited an increase in abundance. This finding was accompanied by a decline in biogas production to below 250 mL CH_4/g COD. This observation is consistent with previous studies reporting transient decreases in methane production within the temperature range of 40–45 °C [21,42]. Previous studies have suggested that this temperature range may exceed the upper growth threshold of mesophilic microorganisms while still being insufficient to support the stable growth of thermophilic strains [31,43]. At 35 °C, the methane production managed to recover to 289 mL CH_4/g COD with acetoclastic *Methanothrix* as the leading methanogens, followed by a slight depletion to 251 mL CH_4/g COD at 30 °C.

In addition to temperature, ammonia concentration may also play an important role in regulating methanogenesis. In this study, relatively high ammonia levels (approximately 400–1000 mg NH_4^+/L) were observed during incubation at 55–45 °C, a temperature range in which hydrogenotrophic methanogens were predominant. As the temperature was further reduced to mesophilic conditions, ammonia concentrations declined to below 700 mg NH_4^+/L. This decrease was accompanied by a reduction in the abundance of hydrogenotrophic methanogens and a concurrent increase in acetoclastic methanogens. This shift likely reflects differences in microbial tolerance toward temperature, with acetoclastic methanogens exhibiting greater resilience to temperature transitions compared with hydrogenotrophic methanogens and ammonia-producing microorganisms. Previous studies have shown that hydrogenotrophic methanogens are generally more sensitive to temperature changes and fluctuations than acetoclastic methanogens [44,45]. In addition, decreases in temperature may also inhibit ammonia production by slowing microbial reaction rates; however, this effect has not been extensively evaluated or investigated in terms of the AD process [46,47].

The decrease in ammonia concentration favored the growth of acetoclastic methanogens, as supported by the observed negative Pearson correlation. Previous studies have reported the sensitivity of acetoclastic methanogens to ammonia stress. Their thin, filamentous morphology provides a larger surface area than the rod-shaped hydrogenotrophic methanogens, facilitating faster diffusion of undissociated NH_3 into the cell, making them more susceptible to free ammonia nitrogen [47–50]. In contrast, the presence of multiple energy-converting hydrogenases in hydrogenotrophic methanogens, such as the Eha/Ehb and Ech complexes, enhances their resistance to ammonia stress [51]. *Methanothermobacter,* equipped with these complexes, showed remarkable adaptation under ammonia stress compared to *Methanothrix*, which lacks these hydrogenase enzymes [51]. Methanogens with such multiple energy-converting systems are more energy-efficient, enabling them to regulate proton balance and replenish H^+ under elevated ammonia levels [51,52].

Compared with the previous study, which experienced a recovery of methane production through hydrogenotrophic methanogenesis [24], the current study discovered that the recovery of methane production on temperature variations was weaker when acetoclastic methanogenesis dominated. However, this finding should be interpreted with caution, as the statistical correlation does not imply functional insignificance or infer inherent inefficiency of *Methanothrix*-dominated systems. There are a number of factors that may influence methane production. The support of hydrolytic, acidogenic, and acetogenic bacteria also played an important role in methane production by sustaining methanogen growth. *Coprothermobacter*, *Defluviitoga*, and *Acetomicrobium* were prevalent across all conditions. Although their correlation with fermentation-related parameters was low in the present study, previous research has highlighted their potential syntrophic associations with both hydrogenotrophic and acetoclastic methanogens by providing available acetate, hydrogen, and CO_2 [23,53–55]. This suggests that their role may be indirect, facilitating methanogenesis through substrate supply rather than direct linkage to fermentation performance.

On the other hand, *Candidatus Bipolaricaulis*, *Brachyspira*, and *Dictyoglomus* exhibited strong correlations with methane, hydrogen, and acetic acid production, underscoring their pivotal role in driving the fermentation process. *Candidatus Bipolaricaulis* is involved in the acidogenesis process and related to hydrogenotrophic methanogenesis, as it establishes syntrophic associations with hydrogenotrophic methanogens [24,56]. Through the conversion of acetate, propionate, and other organic acids into CO_2 and H_2, these microorganisms render the hydrogenotrophic pathway thermodynamically feasible [27,57]. Previous studies have further emphasized the syntrophic interactions between syntrophic acetate-oxidizing bacteria (SAOB), such as *Candidatus bipolaricaulis*, and hydrogenotrophic methanogens when attached to granular activated carbon (GAC) [58]. Similarly, *Dictyoglomus* contributes significantly to anaerobic digestion by hydrolyzing complex polysaccharides such as cellulose, leading to the production of acetate and hydrogen [59,60]. This efficient fermentation process accelerates biomass degradation and enhances overall biogas yield. Moreover, the substantial hydrogen output from *Dictyoglomus* supports hydrogenotrophic methanogens, which utilize CO_2 and H_2 to produce methane [59,60]. Interestingly, both *Candidatus Bipolaricaulis* and *Dictyoglomus* decreased in abundance following the decrease in temperature, which at the same time may lead to a shift in pathway from hydrogenotrophic to acetoclastic methanogenesis.

This finding suggests that temperature shifts may drive a transition in the dominant methanogenesis pathway, as reflected by changes in methanogen abundance and their syntrophic interactions with associated microbial communities. However, a key limitation of this study is the use of glucose as a biodegradable substrate, which does not fully represent the complexity of real anaerobic digestion feedstocks. Glucose was selected to provide a soluble carbon source, especially for the sewage sludge microbiome as an inoculum source, providing a clearer interpretation of microbial responses and methanogenic shifts under decreasing temperature while minimizing variability associated with hydrolysis and substrate heterogeneity. However, in full-scale digesters treating complex substrates, microbial pathways are influenced by multi-step degradation processes, including hydrolysis and acidogenesis, leading to more diverse VFA profiles and potentially different syntrophic interactions. Consequently, the VFA dynamics and dominance of methanogenic pathways observed in this study may differ from those in real systems, where substrate composition, particulate matter, and inhibitory compounds play significant roles. Therefore, the findings should be interpreted as mechanistic insights derived from a simplified model system rather than direct predictors of full-scale digester performance. Future research employing real and heterogeneous feedstocks is necessary to validate the observed microbial responses and methanogenic trends and to improve the engineering relevance and scalability of the results.

5. Conclusions

The current study demonstrates that stepwise reduction from thermophilic to mesophilic conditions strongly affects methane yield, VFA accumulation, and microbial community dynamics. At 55 and 50 °C, methane production peaked, dominated by hydrogenotrophic *Methanobacterium* in association with syntrophic partners including *Coprothermobacter*, *Defluviitoga*, *Acetomicrobium*, *Candidatus Bipolaricaulis*, *Brachyspira*, and *Dictyoglomus*. A decline in methane yield was observed at 45 and 40 °C, accompanied by acetic acid accumulation and reduced methanogenic activity, as reflected by a marked decrease in the abundance of most methanogens. Further temperature reduction to 35 and 30 °C resulted in a recovery of methane yield, characterized by consistent detection of acetate and butyrate and the dominance of the acetoclastic methanogen *Methanothrix*. This pattern indicates enhanced acetoclastic methanogenic activity under mesophilic conditions. Based on taxonomic

observations, these results suggest a shift in pathway dominance from hydrogenotrophic to acetoclastic methanogenesis, driven by the recovery of acetogenic activity at lower, mesophilic temperatures. The results suggest that gradual temperature reduction may conceptually help maintain methane production while lowering heating demand. This interpretation is based on batch-scale experiments and should not be directly extrapolated to full-scale systems. Further studies under continuous and full-scale conditions are needed to confirm its effects on process stability and energy efficiency.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

Yung-Tse Hung and Tsuyoshi Imai are the Guest Editors of special issue "Current advances in wastewater treatment" for AIMS Environmental Science. Yung-Tse Hung and Tsuyoshi Imai were not involved in the editorial review and the decision to publish this article.

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