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Biogas Efficiency and Microbial Community Characteristics in Thermophilic Anaerobic Digestion of Kitchen Waste

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Abstract

Anaerobic digestion of kitchen waste (KW) for biogas production has significant promise both economically and environmentally. However, low biogas production and fluctuating methane concentration remain key challenges that need to be overcome to make this a viable solution. This study implements a two-stage evaluation framework to link temperature-dependent microbial characteristics with process optimization. First, high-throughput sequencing was performed across a wide temperature gradient (25 °C, 37 °C, 45 °C, 50 °C, 55 °C, and 60 °C) to characterize the baseline microbial community screening and succession trajectories of methanogenic archaea. Based on the identified thermophilic transition threshold, five batch anaerobic reactors were subsequently configured under the target thermophilic condition (50 ± 1 °C) with substrate-to-inoculum (S/I) ratios calculated on a volatile solids (VS) basis (1:0, 1.5:1, 1:1, 1:1.5, and 1:2) to evaluate the biomethane potential, organic removal rates, and volatile fatty acid (VFA) conversion. The results showed that an optimal S/I ratio of 1:1 was crucial for maximizing methane production performance, achieving the highest cumulative biogas yield of 1494.07 mL/g VS and methane yield of 746.28 mL/g VS. Notably, *Methanoculleus* and *Candidatus Methanoplasma* were identified as the core functional methanogens enriched at high-temperature stages. Both genera are obligate hydrogenotrophic methanogens that consume H_2 and CO_2 as substrates, and their enrichment plays a vital syntrophic role in alleviating intermediate acid accumulation and maintaining steady methanogenesis. This study provides clear theoretical insights and experimental data supporting the efficient thermophilic utilization of KW via precise microbial load balancing.

Keywords: kitchen waste; biogas slurry; methane; inoculation ratio; methanogenic archaea

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1. Introduction

Currently, with the continuous advancement of industrialization and urbanization, the output of kitchen waste (KW) in China has increased sharply, which has brought enormous pressure to its disposal and resource utilization [1]. The relevant statistics show that KW has accounted for 40% to 60% of the total urban domestic waste in China in recent years. Due to the high moisture content of KW, it increases the difficulty and cost of pretreatment for resource utilization technologies. Two conventional disposal methods, incineration and

landfill, could interfere with the natural biogeochemical cycle, disrupting the balance of carbon, nitrogen and other material cycles in the ecosystem, and lead to the emission of large amounts of greenhouse gases (GHGs), thus posing a serious threat to the ecological environment and human health [2].

In the modern era, the concepts of Sustainable Development Goals (SDGs) and circular bioeconomy have been increasingly emphasized, and the resource utilization and harmless disposal of KW have become important components of promoting ecological civilization construction and achieving carbon neutrality goals [1]. Consequently, it is imperative to adopt appropriate reprocessing technologies to solve the existing problems of KW disposal, thereby realizing the resource recycling of KW, reducing environmental pollution, and ultimately meeting the requirements of a sustainable circular bioeconomy [2]. Using biogenic carbon instead of fossil carbon is the basis for the renewable fuels industry. Compared with other resource treatment technologies, anaerobic digestion (AD) can not only effectively convert KW into green energy, but also has a low life-cycle cost related to economic sustainability [3,4].

Temperature is one of the primary operating parameters that influence the methane production of AD [5,6]. Thermophilic anaerobic digestion (TAD) is associated with promoted methane productivity and improved solids removal efficiency. This is due to the state of archaeal communities on an intercellular and intracellular basis, both of which are influenced by temperature. This temperature impact has an important role in accelerating the conversion process and in determining the rate of hydrolysis, further increasing the rate of methanogenesis of the system [7,8]. For example, methanogens show higher growth rates under thermophilic conditions, which may induce efficient methane production [9]. An increase in hydrogenotrophic methanogens at 55 °C compared to 35 °C [10] may result in alternative methanogenic pathways in anaerobic digestion. Furthermore, compared to mesophilic conditions, thermophilic conditions can promote the solubilization of organic polymers to volatile fatty acids (VFAs), reduce hydraulic retention time and shorten the total duration of AD. However, this may also increase the probability of VFA accumulation and make the system prone to instability [11]. For these reasons, increasing the anaerobic digestion temperature can not only promote the formation of methane in the system, but also shorten anaerobic digestion time, consequently improving gas production efficiency.

Previous studies have demonstrated that the functional stability and methane production performance of anaerobic digestion systems are closely associated with the composition and activity of methanogenic archaeal communities [12,13]. Organic matter rich in microorganisms such as animal manure, wastewater treatment sludge, or biogas slurry is well characterized and widely used in anaerobic treatment. Among these forms of organic matter, biogas slurry is commonly used as an inoculum for anaerobic digestion because of its favorable buffering capacity and abundant anaerobic microbial communities, which can facilitate reactor start-up and process stability [14]. Additionally, the methane production from AD is largely governed by the microbial community in the inoculum, including the type, abundance, and activity of the flora [15]. The substrate-to-inoculum (S/I) ratio has been considered a major factor in ensuring the economic sustainability of large digesters [16]. The S/I ratio varies depending on the waste composition and digestion conditions. Due to the biogas slurry characteristics, the concentration of total ammonia nitrogen and free ammonia contained in it will also affect the digestion process [17]. Accordingly, the S/I ratio is an important operational parameter affecting the stability and performance of batch anaerobic digestion systems. By adjusting the inoculation amount to regulate microbe-mediated AD, methanogenic microorganisms are maximized and supplied with corresponding amounts of nutrients, thereby shortening the lag time of methane production, and achieving the resource utilization of kitchen waste [18].

Previous studies have extensively investigated the anaerobic digestion (AD) of kitchen waste (KW), particularly the influence of operational temperature on digestion performance. However, microbial community structures across a broad temperature gradient and the responses of thermophilic systems to operational parameters, such as substrate-to-inoculum (S/I) ratios, have often been studied separately. Understanding the interactions between these factors is important because methanogenic microorganisms exhibit distinct temperature preferences. These preferences strongly influence system stability and methane production efficiency.

To address these limitations, this study adopted a two-stage experimental design. First, high-throughput sequencing was used to characterize baseline microbial community variations across six temperature conditions (25 °C, 37 °C, 45 °C, 50 °C, 55 °C, and 60 °C). A critical microbial transition was identified at the thermophilic threshold of 50 °C. Based on this result, 50 °C was selected as the core operational condition for further investigation. Different S/I ratios were then applied to systematically evaluate biomethane production potential, volatile fatty acid (VFA) conversion, and variations in ammonia nitrogen. Rather than directly linking microbial community shifts to methane production performance, the temperature screening stage was intended to identify a thermophilic condition that favored the enrichment of methanogenic microorganisms. The selected temperature (50 °C) was subsequently used as the fixed operating condition for S/I ratio optimization. It also provides practical experimental evidence for improving the thermophilic anaerobic digestion of kitchen waste.

2. Materials and Methods

2.1. Microbial Community Analysis

To characterize the microbial community succession across the designated temperature gradient (25–60 °C), total genomic DNA was extracted from the liquid samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using universal primers (F: 5′-GYGCASCAGKCGMGAAW-3′, R: 5′-GGACTACVSGGGTATCTAAT-3′) and paired-end sequenced on an Illumina platform by Annoroad Gene Technology Co., Ltd. (Beijing, China). Bioinformatic pipeline processing was executed via the BMKCloud platform (www.biocloud.net, accessed on 7 March 2024). Raw paired-end reads were quality-filtered using Trimmomatic (v0.33) and primer-trimmed via Cutadapt (v1.9.1). High-quality sequences were merged using USEARCH (v10) and chimera-filtered through UCHIME (v8.1) to generate clean tags. Operational Taxonomic Units (OTUs) were clustered at a 97% similarity threshold using USEARCH (v10.0), with rare OTUs below an abundance threshold of 0.005% filtered out. Taxonomic annotation was performed using the Naive Bayes classifier via the classify-sklearn plugin in QIIME2 (v2020.6) against the SILVA database (Release 138) with a confidence threshold of 0.7. Alpha diversity (Chao1, ACE, Shannon, Simpson) and beta diversity (PCoA, NMDS, UPGMA) metrics were computed, followed by functional potential and phenotypic trait predictions using PICRUSt2 and BugBase.

2.2. Kitchen Waste and Inoculum

The KW was collected from the canteen of Beijing Union University. Impurities such as bones, plastics, disposable spoons, and paper towels were removed from the KW samples, and the remaining KW was crushed to a particle size of 2 mm or less. After high-temperature degreasing and three-phase separation, the solid content was adjusted to less than 10%, and the liquid KW was placed in a refrigerator at 4 °C for use [19].

The additive used was a biogas slurry taken from the normally operating KW fermenter of Dong village Comprehensive Treatment Plant of the Beijing Environmental

Sanitation Group. After collection, the impurities in the biogas slurry were filtered out, and the slurry placed at 4 °C for further use. The properties of the KW and the biogas slurry are shown in Table 1.

Table 1. Properties of kitchen waste and inoculum.

Item	Kitchen Waste	Inoculum
TS (%)	21.5 ± 2.2	2.4 ± 0.02
VS (%)	19.9 ± 3.6	1.2 ± 0.5
pH	4.2 ± 0.12	8.1 ± 0.04
TAN (mg/L)	106.7 ± 11.6	935.8 ± 4.2
FAN (mg/L)	0 ± 0.01	261.16 ± 0.01
TC (%)	45.1 ± 0.01	26.3 ± 0.01
TN (%)	3.3 ± 0.05	4.4 ± 0.01
C/N ratio	13.6 ± 0.22	6.0 ± 0.01

TS: total solids, VS: volatile solids, TAN: total ammonia nitrogen, FAN: free ammonia nitrogen, TC: total carbon concentration, TN: total nitrogen concentration.

2.3. Batch Experimental Setup

These batch experiments were carried out in 1000 mL glass reactors with a working volume of 600 mL, sealed with rubber bungs and connected to gas collection bottles. The reactors were equipped with an automatic stirring device to ensure good mass transfer, and stirring speed was fixed at 80 rpm. The temperature was kept at 50 ± 1 °C. Five different VS ratios of S/I (1:0, 1.5:1, 1:1, 1:1.5, 1:2) were introduced into different AD systems to determine the best-performing ratio. The 16-day experimental duration was designed to evaluate the overall methane production performance and the complete anaerobic digestion process rather than rapid kinetic dynamics alone. Furthermore, the operational duration was determined according to standard Biochemical Methane Potential (BMP) testing principles, in which batch digestion is typically continued until daily gas production becomes negligible. In addition, an inoculum-only blank reactor containing biogas slurry without substrate was operated in parallel throughout the experiment to evaluate endogenous biogas production from the inoculum.

2.4. Analytical Procedures

Before being fed into the digester, the substrate and inoculum were chemically analyzed for pH, total solids (TS), volatile solids (VS), total carbon concentration (TC), total nitrogen concentration (TN), and total ammonia nitrogen (TAN). The pH was measured using a multi-parameter analyzer (Rex Electric Chemical, Shanghai, China) equipped with a pH electrode (PHS-3C). The TS and VS concentration levels were calculated according to the standard method [20]. Measurements were made using an elemental analyzer (Vario MACRO cube, Elementar, Langenselbold, Germany) to determine the amounts of TC and TN, and the C/N ratio. TAN was measured using a Lianhua kit (Lianhua Technology, Beijing, China) and FAN was calculated according to Equation (1) [21]. VFAs were measured using a high-performance liquid chromatograph (HPLC, LC-20A, Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (7.8 mm i. d. × 300 mm) and a diode array detector. The mobile phase was prepared by dissolving 15 mmol H_2SO_4 in dd H_2O solution. The flow rate was 0.6 mL/min, and the temperature was set at 40 °C [22]. The water displacement method was used to measure the amount of biogas produced, and then the methane content in the biogas was determined by a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) [23]. Calculating the amount of biogas and methane produced per gram of substrate in the AD system yielded the volume, expressed as milliliters per gram (mL/g).

$$[\mathrm{FAN}] = [\mathrm{TAN}] \times (1 + \frac{10^{-\mathrm{pH}}}{10^{-(0.09018+\frac{2729.92}{\mathrm{T(k)}})}})^{-1} \quad (1)$$

where FAN is the concentration of free ammonia (mg/L), TAN is the total ammonia nitrogen concentration (mg/L), and T is the thermodynamic temperature (K).

3. Results and Discussion

3.1. Microbial Community Structure

Figures 1 and 2 show the distribution of archaeal communities at the phylum and genus levels, respectively, under different temperature conditions, revealing distinct archaeal succession patterns. *Halobacterota* and *Thermoplasmatota* were the dominant archaeal phyla across all experimental stages. The relative abundance of *Halobacterota* increased continuously with rising temperature and reached a maximum value of 26.77% at T5 (60 °C), representing a 71.25% increase compared with T1 (37 °C). It should be noted that *Halobacterota* is a phylum-level archaeal taxonomic group containing diverse archaeal lineages, and its enrichment should not be interpreted as evidence of enrichment of the halophilic genus *Halobacterium*. In addition, the relative abundance of P. acidophilus increased with temperature and reached its highest abundance (7.92%) at T5.

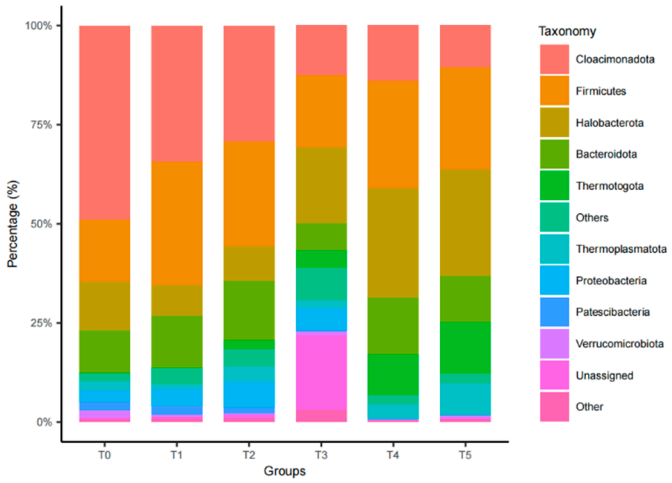

Figure 1. The relative abundance of microorganisms on phylum level at each temperature.

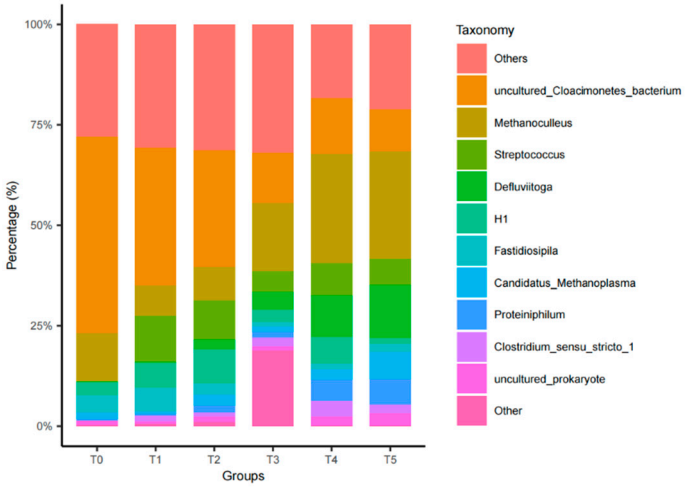

Figure 2. The relative abundance of microorganisms at the genus level at each temperature stage.

At the genus level, *Methanoculleus* and *Candidatus Methanoplasma* were identified as the dominant archaeal genera. *Methanoculleus* is widely recognized as a hydrogenotrophic methanogen in anaerobic digestion systems and is commonly associated with hydrogen-dependent methane production [24]. Previous studies have shown that hydrogenotrophic methanogens play an important role in maintaining syntrophic metabolism by consuming hydrogen and facilitating volatile fatty acid oxidation, thereby contributing to process stability during anaerobic digestion [25]. In the present study, the relative abundance of *Methanoculleus* increased with temperature and reached its highest level under thermophilic conditions. This trend may indicate that hydrogenotrophic methanogens were favored at elevated temperatures. However, because the present analysis was based solely on 16S rRNA gene sequencing, these observations should be regarded as potential ecological associations rather than direct evidence of methanogenic activity or pathway shifts.

Notably, *Methanoculleus* possesses strong ammonia tolerance, which can be partially attributed to the absence of ammonium transporters and methylammonium permeases, supporting its adaptability to ammonium-rich or ammonia-stressed environments. Relevant experiments have shown that, when TAN concentration exceeds 1000 mg/L, *Methanoculleus* becomes the dominant genus [26]. TAN concentration in this experiment is above 1000 mg/L during the high-temperature stages, consistent with the high relative abundance of *Methanoculleus*. This indicates that methanogenesis under high-temperature conditions was mainly driven by the hydrogenotrophic pathway. The increased relative abundance of *Methanoculleus* under high-temperature conditions suggests that this genus may be associated with thermophilic methanogenic processes. However, as this observation is based solely on relative abundance data, further quantitative microbial analyses are required to determine its specific contribution to methane production.

Candidatus Methanoplasma is a genus of methyl-hydrogen and hydrogenotrophic trophic methanogens. The increase in its relative abundance of *Candidatus Methanoplasma* may be associated with hydrogen release induced by environmental acidification during the reaction process [27]. Specifically, the syntrophic degradation of accumulated VFAs, such as propionate and butyrate, strictly relies on and generates H_2, which in turn provides abundant electron donors that strongly stimulate the growth of hydrogenotrophic methanogens. During the anaerobic digestion process, the excessive accumulation of specific volatile fatty acids (VFAs), particularly propionic acid, can disrupt the kinetic balance between acidogenesis and methanogenesis, exerting a significant inhibitory effect on the growth and activity of methanogens [28].

Overall, the experimental results showed that the relative abundances of hydrogenotrophic methanogens *Methaanoculleus* and *Candidatus Methanoplasma* increased with the accumulation of ammonia and VFA during the continuous operation, and the temperature rose, making them the primary dominant strains.

It should be noted that the increased relative abundances of *Methanoculleus* and *Candidatus Methanoplasma* under elevated temperature conditions mainly reflected microbial community succession across temperature gradients. High-throughput sequencing primarily represents relative abundance rather than absolute biomass. Therefore, the enrichment observed at T4 (55 °C) and T5 (60 °C) should be interpreted cautiously. In the present study, methane production performance was primarily evaluated under the target thermophilic operational condition (50 °C). Microbial community analyses at different temperatures were mainly used to characterize the enrichment trajectory of methanogenic microorganisms. The observed microbial shifts may be associated with methane production performance under thermophilic conditions. However, further studies involving multi-temperature methane production experiments and functional analyses are still required to establish direct mechanistic relationships.

3.2. Biogas and Methane Production

The calculated daily and cumulative biogas yields under different S/I ratios (1:0, 1.5:1, 1:1, 1:1.5, and 1:2) during the 16-day digestion period are shown in Figure 3a,b. The daily biogas yield of all groups reached its maximum on Day 1, with values of 100.08, 654.59, 929.14, 643.22, and 502.33 mL g^{-1} VS for S/I ratios of 1:0, 1.5:1, 1:1, 1:1.5, and 1:2, respectively. Thereafter, biogas production gradually declined in all treatments. In the 1:0 group, gas production ceased on Day 5 due to the absence of inoculum microorganisms. Biogas production continued until Days 14–16 in the inoculated treatments, depending on the S/I ratio (Figure 3a).

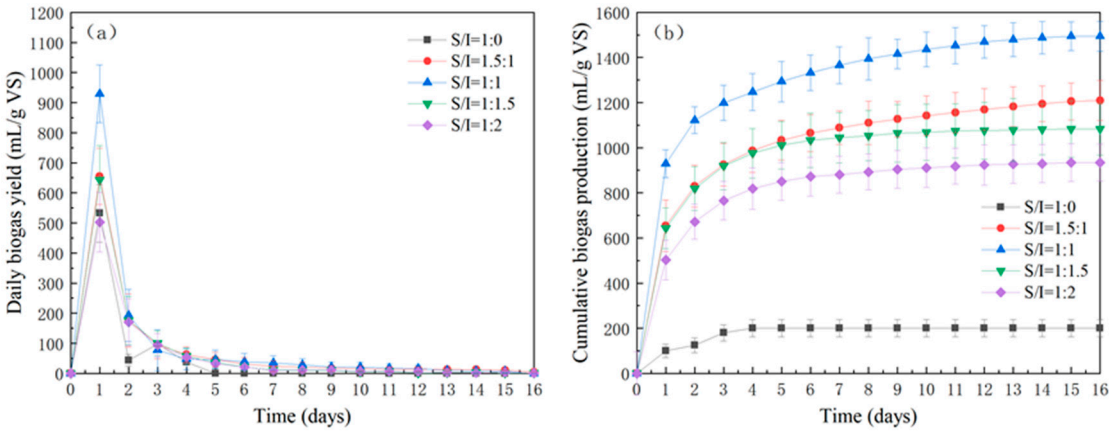

Figure 3. Cumulative biogas yield from different S/I ratios: (**a**) daily biogas yield; (**b**) cumulative biogas production.

An inoculum-only blank reactor operated in parallel produced no measurable biogas throughout the experiment. Therefore, the observed methane production was primarily attributed to the degradation of organic matter in the kitchen waste substrate, and blank correction had a negligible effect on the calculated methane yields.

Compared with the mono-digestion of kitchen waste or biogas slurry, co-digestion at appropriate S/I ratios resulted in substantially higher biogas yields, indicating that the microbial community introduced with the biogas slurry enhanced the degradation of organic matter and promoted methane production.

Although measurable biogas production persisted until Days 14–16 in several treatments, a longer production period should not be interpreted as evidence of superior digestion performance. Extended gas production may reflect slower degradation kinetics, delayed hydrolysis of complex organic matter, or temporary inhibitory effects. Therefore, digestion performance was evaluated primarily based on cumulative methane yield, methane concentration, and process stability rather than digestion duration alone.

Since methane production remained detectable after the initial rapid production phase, terminating the experiment at 72 h would have underestimated the cumulative methane yield and substrate degradation performance. Nevertheless, we acknowledge that the 24 h sampling interval used in this study may not fully capture the rapid metabolic transitions occurring during the early digestion stage. Future studies employing shorter sampling intervals (e.g., 2–4 h) during the first 72 h will be conducted to better characterize early-stage digestion dynamics.

Figure 3b shows the cumulative biogas yields with the different S/I ratios. Comparative maximum cumulative biogas yields of 200.20, 1209.65, 1494.07, 1083.11, and 934.67 mL g^{-1} VS were observed from the AD systems amended with S/I ratios of 1:0, 1.5:1, 1:1, 1:1.5 and 1:2 respectively. It is clear from the results that the AD system with a 1:1 ratio of S/I produced the highest biogas yield, indicating that this inoculation ratio

was more conducive to inducing methanogens to consume organic acids produced during digestion, thus increasing biogas production. However, too much or too little inoculation can result in unsatisfactory biogas production. In S/I 1:0 AD systems, there are rich substrates, and the methanogenic microorganisms are less active and therefore unable to utilize the VFAs produced during digestion. This resulted in excessive accumulation of VFAs. In contrast, excessive inoculum AD systems at 1:1.5 and 1:2 S/I resulted in insufficient degradable organic matter, hindering the biogas production process.

3.3. Methane Production and Content

The highest methane production was observed in reactors containing a 1:1 S/I ratio, with a cumulative methane yield of 746.28 mL g^{-1} VS (Figure 4a). The maximum cumulative methane yields in the 1.5:1, 1:1.5 and 1:2 S/I groups were 512.06, 521.08 and 483.01 mL g^{-1} VS, respectively. In contrast, no methane was detected throughout the entire experiment in the 1:0 S/I reactor, indicating that inoculum addition was essential for methane production under the tested conditions. Previous studies have suggested that reduced methane yields at non-optimal S/I ratios may be related to substrate inhibition or inoculum overloading [14]. In addition, increasing inoculum loading within an appropriate range can enhance methane production and shorten the digestion period, whereas excessively high inoculum proportions may reduce methane yields [29]. A similar trend was observed in the present study, with the highest methane yield obtained at an S/I ratio of 1:1. However, the underlying mechanisms could not be conclusively determined because VFA, alkalinity, ammonia concentration, and microbial activity were not monitored in this study.

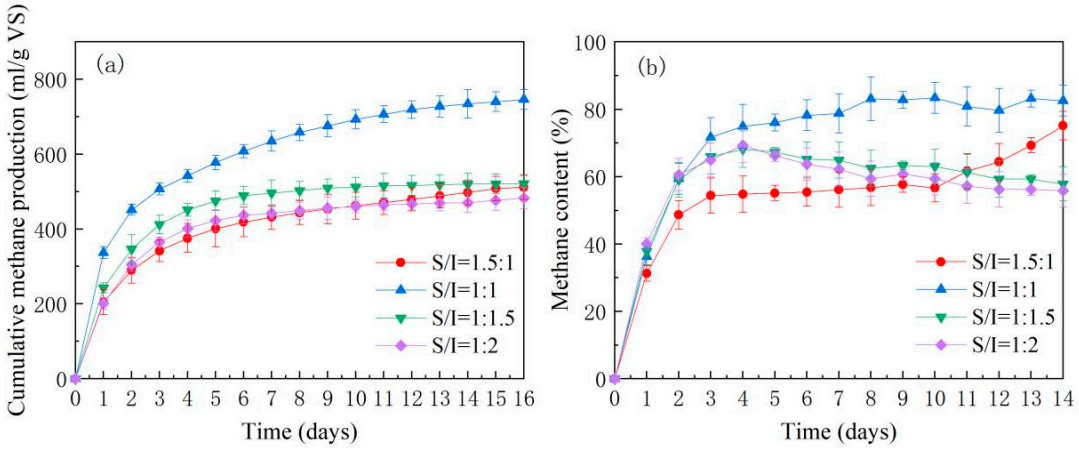

Figure 4. Cumulative methane production (**a**) and methane content (**b**) at different S/I ratios.

Figure 4b illustrates the variation in methane content with digestion time under different S/I ratios. Except for the 1.5:1 S/I ratio, methane content generally increased with increasing inoculum proportion, and the 1:1 S/I ratio exhibited the highest methane content (73.19%) and maintained values above 70% throughout most of the digestion period. The maximum methane contents of the 1:1.5 and 1:2 S/I ratio reactors were 72.14% and 71.35%, respectively, with no significant difference between them. Although methane content increased rapidly during the initial stage in these two reactors, both showed a gradual decline after Day 4, eventually decreasing to 59.36% and 55.83%, respectively. Since VFAs, TAN/FAN, and alkalinity were not monitored in this study, the exact mechanism responsible for this decline could not be conclusively determined. However, acidification is unlikely because the pH values remained within the suitable range for methanogenic activity throughout the digestion process (Figure 5). The observed differences may be related to variations in substrate availability and inoculum proportion under different

S/I ratios. In contrast, the 1.5:1 S/I ratio exhibited a prolonged lag phase before methane production increased substantially. Overall, within the tested range of S/I ratios, the 1:1 S/I ratio showed the most favorable methane production performance and methane content stability.

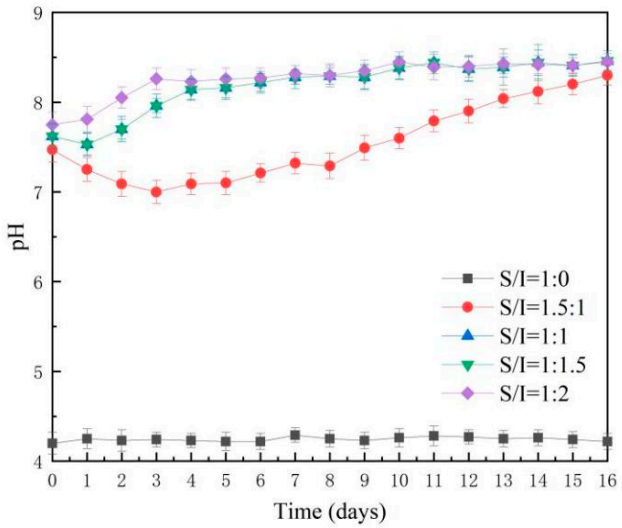

Figure 5. pH at different S/I ratios.

3.4. pH, TS, and VS Degradation Rates

The optimum pH range for methanogens is 6.8~7.2 [3], but pH within 6.5~9 has small impact on the AD process [30]. Perhaps, due to the different characteristics of inoculants, the pH of each experimental group was slightly higher than that described in the literature. Even under these conditions, the metabolic activity of methanogens remains stable and they can also utilize organic matter to produce methane [31]. Figure 5 demonstrates that KW was digested alone without significant changes in pH. Compared with other experimental groups, the pH value of the 1.5:1 S/I ratio quickly decreased (7.47~7) in the first three days due to VFA accumulation, while pH value gradually increased in 1:1 S/I because of methanogen consumption of VFAs on the 3rd day; 1:1.5 and 1:2 S/I completed the process within 1 day. The occurrence of abnormal pH values may be related to the inability of methanogens to obtain sufficient substrate for degradation in the AD system, so the pH value fluctuates within a small range [32]. The pH of the AD system containing 1:1 S/I varied between (7.62~8.46), which is the optimum pH for this digestion process, and it provides favorable conditions to methanogen archaea; thus, the highest biogas was produced at this ratio.

The removal rates of TS and VS directly reflect substrate biodegradation efficiency. As shown in Figure 6a,b, the degradation of organic matter in the AD system was evaluated based on TS and VS removal rates [33]. Excessively high substrate solids content may reduce slurry fluidity and mixing efficiency, thereby increasing energy demand during agitation.

The highest TS and VS removal rates were observed at an S/I ratio of 1.5:1. However, this condition exhibited a temporary pH decline during the initial digestion stage and a prolonged lag phase during methane production, suggesting transient process imbalance. In contrast, the reactor operated at an S/I ratio of 1:1 achieved TS and VS removal rates of 50.34% and 57.92%, respectively, while maintaining a more stable digestion process and producing the highest cumulative methane yield.

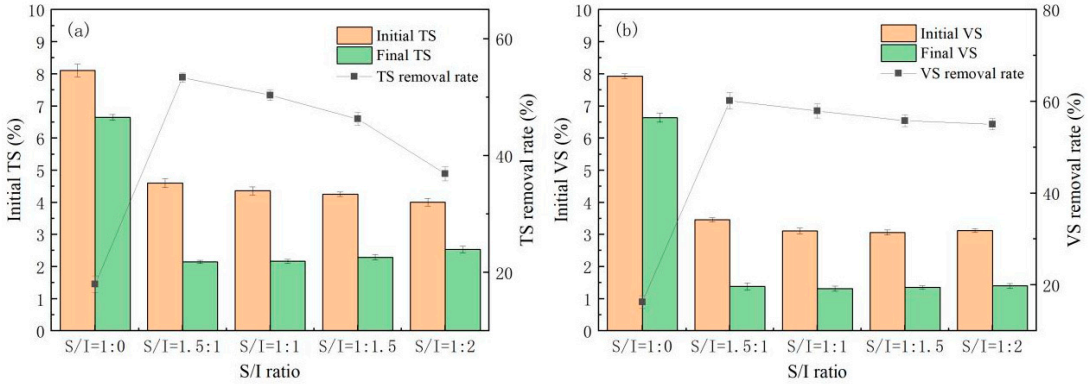

Figure 6. TS removal rate (**a**) and VS removal rate (**b**) at different S/I ratios.

Therefore, although the maximum solids removal was obtained at an S/I ratio of 1.5:1, the S/I ratio of 1:1 provided the best overall anaerobic digestion performance when methane production, substrate degradation efficiency, and process stability were considered together. Consequently, S/I = 1:1 was identified as the optimal operating condition in this study.

3.5. Variations in VFAs, TAN, and FAN at 1:1 S/I Ratio

As shown in Figure 7, in the 1:1 S/I group, the propionic acid concentration was low in the late fermentation stage, and the propionic acid ratio gradually stabilized, indicating strong propionic acid degradation capacity. The average concentrations of acetic acid and propionic acid in the system were 2398.06 mg/L and 502.26 mg/L, which is consistent with a previous study that showed that the system could tolerate acetic acid (≤2400 mg/L) and propionic acid (<900 mg/L) [34].

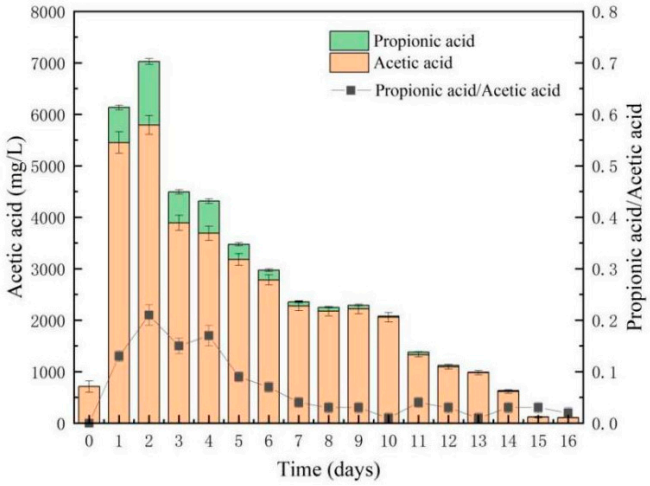

Figure 7. Changes in VFAs at 1:1 S/I ratio.

In the anaerobic digestion (AD) system, total ammonia nitrogen (TAN) undergoes a reversible reaction to form free ammonia nitrogen (FAN) when the system pH is above 7, as shown in the reaction equation:

$$NH_3 + H_2O \leftrightarrows NH_4^+ + OH^-$$

It should be noted that AD systems are rich in CO_2. Under low pH conditions, ammonium ions can combine with CO_2 to form diammonium carbonate [$(NH_4)_2CO_3$], thereby shifting the equilibrium between TAN and FAN. FAN is widely acknowledged as the main contributor to ammonia inhibition in AD processes.

However, there is a key clarification regarding the effect of high temperature on FAN and ammonia inhibition. On the one hand, elevated temperature conditions can enhance the growth rate and activity of microorganisms, as well as the metabolic performance of methanogens, which account for a relatively high proportion in the total microbial community [17]. On the other hand, high temperature promotes the dissociation of ammonium carbonate, leading to an excessive increase in FAN concentration, which may intensify ammonia inhibition.

Figure 8 shows the temporal variations in FAN and TAN concentrations under the S/I ratio of 1:1. Both FAN and TAN concentrations fluctuated dynamically throughout the digestion process. The average FAN concentration was 415.79 mg/L, while the average TAN concentration was 1240.16 mg/L. FAN reached a maximum value of 941.80 mg/L on Day 12, coinciding with the peak TAN concentration of 2296.85 mg/L.

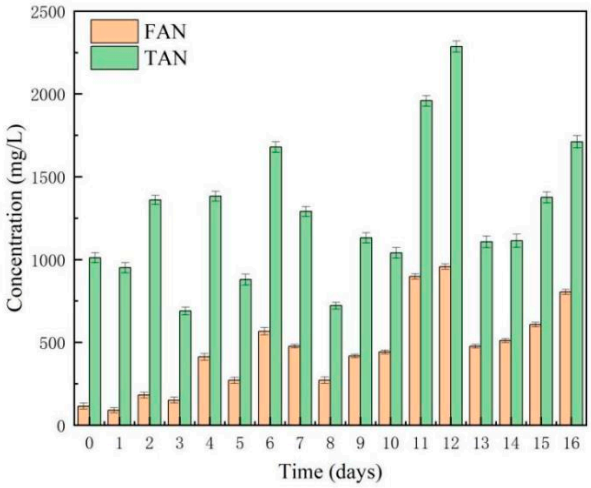

Figure 8. Changes in TAN and FAN concentration at 1:1 S/I ratio.

Notably, the FAN peak occurred during the later stage of digestion, after the major phase of biogas production had already taken place. Although transient increases in FAN and TAN concentrations were observed, cumulative methane production continued to increase thereafter. Meanwhile, VFAs were progressively consumed and pH remained within the range suitable for methanogenesis. These observations suggest that severe ammonia inhibition was not evident under the experimental conditions.

Previous studies have reported that ammonia inhibition thresholds may vary considerably depending on substrate characteristics, operating temperature, acclimation conditions, and microbial community composition. FAN concentrations in the range of 337–800 mg/L and TAN concentrations above approximately 1700 mg/L have been reported to potentially cause inhibitory effects in some anaerobic digestion systems [35,36]. However, since no direct ammonia tolerance assay was conducted in this study, a specific ammonia tolerance threshold for the microbial consortium cannot be established. Therefore, the interpretation presented here is based primarily on the observed process performance indicators rather than direct comparison with literature-reported inhibition thresholds.

4. Conclusions

This study implemented a two-stage evaluation framework to investigate the anaerobic digestion (AD) performance of kitchen waste (KW). First, archaeal community succession was characterized across a broad temperature gradient (25–60 °C) to identify temperature conditions favorable for methanogenic microorganisms. Subsequently, the digestion performance of KW using biogas slurry as an inoculum was evaluated under a selected

thermophilic condition (50 ± 1 °C) at five substrate-to-inoculum (S/I) ratios (1:0, 1.5:1, 1:1, 1:1.5, and 1:2).

Among the tested treatments, the S/I ratio of 1:1 exhibited the best overall digestion performance under thermophilic conditions. This treatment achieved the highest cumulative biogas production (1494.07 mL g^{-1} VS) and methane yield (746.28 mL g^{-1} VS), while maintaining relatively high TS and VS removal rates of 50.34% and 57.92%, respectively. In addition, the digestion process remained stable, as indicated by the maintenance of a neutral-to-alkaline pH (7.62–8.46). The average TAN (1240.16 mg L^{-1}) and FAN (415.79 mg L^{-1}) concentrations remained within ranges commonly reported for anaerobic digestion systems, suggesting a low risk of severe ammonia inhibition under the tested conditions.

Microbial community analysis indicated that *Methanoculleus* and *Candidatus Methanoplasma* were dominant methanogenic taxa under thermophilic conditions and were associated with enhanced methane production performance. The increased abundance of *Methanoculleus* may reflect the adaptation of hydrogenotrophic methanogens to thermophilic environments, whereas the occurrence of *Candidatus Methanoplasma* suggests a potential association with hydrogen-utilizing methanogenic pathways. However, these interpretations are based on 16S rRNA gene sequencing data and should be regarded as ecological associations rather than direct evidence of methanogenic activity.

Temperature screening revealed distinct shifts in archaeal community composition. As the temperature increased to thermophilic levels (≥50 °C), the relative abundance of *Halobacterota* increased, accompanied by an enrichment of hydrogenotrophic methanogens. Under the selected thermophilic condition (50 °C), the highest methane content (85.54%) was observed at the S/I ratio of 1.5:1. However, this treatment also exhibited a prolonged lag phase and lower methane yield than the 1:1 treatment, indicating that methane concentration alone was insufficient to represent overall digestion performance. In contrast, mesophilic conditions maintained relatively stable archaeal community structures.

The results also demonstrated the importance of inoculum addition during KW digestion. The reactor without inoculum (S/I = 1:0) exhibited extremely poor methane production performance, which was associated with insufficient methanogenic populations and volatile fatty acid accumulation. Conversely, increasing the inoculum proportion beyond the optimal level (S/I = 1:1.5 and 1:2) did not further improve methane production efficiency and may have reduced substrate availability per unit microbial biomass. Therefore, balancing substrate loading and inoculum addition was essential for achieving efficient and stable thermophilic digestion of KW.

Overall, the findings of this study suggest that an S/I ratio of 1:1 represents the most favorable operating condition among the tested treatments for thermophilic anaerobic digestion of kitchen waste. This study provides experimental evidence for optimizing inoculum loading and improving the thermophilic utilization of kitchen waste.

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