Investigation of Methane Production and Microbial Communities of Digested Sewage Sludge on Thermotolerant Anaerobic Digestion

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Abstract. The operating temperature of an anaerobic digestion (AD) process regulates CH_4 production and microbial communities. This study investigated the CH_4 production and microbial communities at the temperature range between mesophilic and thermophilic temperatures, which was defined as the thermotolerant region. Laboratory-scale anaerobic digestion was carried out using 160 ml vials with digested sludge as inoculum. The vials were then incubated under temperature conditions of 42 °C, 45 °C, and 48 °C. A higher temperature condition significantly enhanced the methane production from 0.21, 0.27, to 0.29 L/g COD at 42, 45, and 48 °C respectively. During incubation, the methanogen population was dominated by acetoclastic *Methanosaeta*, while only a few of hydrogenotrophic methanogens were found. One of the major competitors of *Methanosaeta* in craving available acetate to produce CH_4 is the syntrophic acetate-oxidizing bacteria (SAOB). The high population of SAOB suppressed the growth of *Methanosaeta* which consequently lower the CH_4 production by 12%.

Keywords: Anaerobic digestion, Methane production, Methanogen, *Methanosaeta*, Thermotolerant

1 Introduction

The current worldwide energy crisis triggered by inflated non-renewable energy sources price, due to their limited reserves, and the increasing generation of anthropogenic wastes are major concerns in several countries as these challenges may collapse their economies while also threatening environmental sustainability. Without proper handling and treatment, anthropogenic waste that was generated from human-caused activities, such as sewage sludge and municipal solid waste, may exhibit a detrimental effect on the environment. Currently, sewage sludge generated in the EU, China, and the USA reaches 10 Mt, 39 Mt, and 7.2 Mt, respectively, and increases in quantity have been expected due to population growth and the expansion of commerce and industry [1, 2]. The development of sludge management methods globally will entail novel approaches given the anticipated growth in sludge creation, the environmental cost of waste disposal, and strict environmental norms [2, 3]. As a result, the use of environmental wastes as feedstock for the production of fuel and chemicals has subsequently gained great attention [4, 5].

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Currently, significant amounts of sludge are being utilized in various chemical, thermochemical, and biological processes to produce bioenergy/biofuel and valuable products [6, 7]. Anaerobic digestion (AD) is a prominent biological process utilized as an end treatment for sewage, primary, and waste-activated sludge in many WWTPs as it is a promising eco-friendly method for recycling large volumes of sewage sludge [8–10]. AD has been extensively studied as a method of producing biogas from organic wastes such as primary sludge (PS), waste-activated sludge (WAS) [11], sewage sludge (SS) [12], and digested sewage sludge [13, 14]. AD has been regarded as one of the viable processes for waste bio-valorization predicated on the syntrophic mechanisms of microbial communities [6]. During the AD process, organic compounds are digested into soluble feedstocks through hydrolysis, which is then fermented by syntrophic bacteria to acetate and carbon dioxide (CO_2) via acidogenesis and acetogenesis. Syntrophic acetate-oxidizing bacteria (SAOB) are microbial communities that are responsible for the consumption of acetate and CO_2 to produce bio-hydrogen (H_2). Methanogens then consume these substances to produce methane (CH_4) via the hydrogenotrophic and acetoclastic pathways. To produce methane, hydrogenotrophic methanogens reduce CO_2 to CH_4 and water (H_2O) by using H_2 as an electron donor, while acetoclastic methanogens primarily consume acetate [15, 16].

In general, there are several temperature conditions where the AD system is frequently operated: psychrophilic (< 30 °C), mesophilic (30 – 40 °C), and thermophilic (50 – 60 °C) [17]. Mesophilic and thermophilic conditions are widely used in most commercial-scale AD systems, aiming to maintain a maximum methane production rate of 0.2 – 0.4 and 0.32 – 0.45 L/g COD, respectively [18]. There have been a number of previous study attempts to explain how mesophilic and thermophilic temperature leads to different biogas yield and regulates microbial communities in the AD process [19–21]. However, in between the mesophilic and thermophilic temperatures region, there is a range of temperatures that are neither classified as mesophilic nor thermophilic conditions. This temperature region ranges from 42 – 49 °C which in the present study we defined as a thermotolerant area since only several thermotolerant microorganisms, such as *Methanosaeta*, *Methanobacterium*, and *Methanosarcina* [13, 14, 21, 22] can possibly grow and survive in this temperature area. Tezel et al. [23] identified the thermotolerant temperature range may be above the maximum threshold for mesophile growth but not high enough to support the growth of thermophiles.
The potential for thermotolerant bacteria to generate biogas from the anaerobic digestion of digested sewage sludge has not been widely understood yet. It is necessary to conduct further studies on thermotolerant bacteria, particularly to examine their capacity to produce biogas. The goals of this study were to evaluate the potential for biogas generation (particularly CH_4) and to discover the microorganisms that produced the most biogas among the cultures after being exposed to various temperature in thermotolerant temperatures region.

2 Materials and methods

2.1 Inoculum and substrates

The inoculum source was a digested sludge from the mesophilic AD (37 °C) of the Eastern Ube Sewage Treatment Plant in Ube City, Yamaguchi Prefecture, Japan; Table 1 lists its properties. Before incubation, The inoculum samples and substrate solution were mixed in a 1:1 ratio. In

the present study, the carbon source for biogas production was glucose-based synthetic wastewater with 1.5 g/L of glucose. Additionally, glucose has been the primary carbon source for microbial growth in the AD process in various prior studies because of its biodegradability, which enables the destruction of difficult-to-degrade compounds in wastewater [24, 25]. Other nutrients were provided in the form of a mixture of 2 mg/L K_2HPO_4, 2 mg/L $NaHCO_3$, 1 g/L yeast extract, 0.7 g/L $(NH_4)_2HPO_4$, 0.75 g/L KCl, 0.85 g/L NH_4Cl, 0.42 g/L $FeCl_3{\cdot}6H_2O$, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 0.82 g/L $MgCl_2{\cdot}6H_2O$, 0.018 g/L $CoCl_2{\cdot}6H_2O$, and 0.15 g/L $CaCl_2{\cdot}2H_2O$ [14, 26].

Table 1 Characteristics of anaerobic sludge as inoculum.

Parameters	Anaerobic Sludge	Units
pH	8.17	$pH = -\log10[a(H^+)]$
Total Solid (TS)	8125	mg/l
Volatile Solid (VS)	3125	mg/l
Fixed Solid (FS)	5000	mg/l
VS/TS ratio	0.38	-

2.2 Experimental protocol

Laboratory-scale reactors with each volume of 160 ml were prepared. Then, these reactors were capped with butyl rubber stoppers and aluminum caps to prevent gas leakage from the reactors. As an initial treatment, batch experiments were performed by adding 40 ml of substrates to a reactor containing 40 ml of sludge as inoculum. To ensure obligate anaerobic conditions, reactors were purged with nitrogen gas to flush residual oxygen. The mixture was then incubated at three different temperature levels: 42 °C, 45 °C, and 48 °C with shaking speed at 50 rpm for one month. This approach was intended to assist microbial populations to acclimatize and eliminate any potential remaining intractable organic substances that might have been transported from the WWTP to the lab [27].

After the initial treatment, incubation of each reactor was continued for two months with similar temperature conditions. When the gas production showed a considerable decline, up to 2 ml of 1.5 g/l glucose was injected into each reactor. Consequently, the reactor system was changed from a batch system to a fed-batch system. During the incubation period, total gas volume and gas compositions of samples, including that of H_2, CH_4, and CO_2, were measured daily using gas chromatography (GC-8APT/TCD; Shimadzu, Kyoto, Japan) with a 60/80 activated charcoal mesh column (1.5 × 3.0 mm internal diameter) and argon gas as the carrier gas. Glass syringe method was utilized to quantify the volume of the biogas produced. During operation, the temperatures of the injector, column, and detector on the GC were adjusted to 50 °C, 60 °C, and 50 °C, respectively.

2.3 DNA extraction and sequencing

Following the instructions in the NucleoSpin® soil handbook, DNA was extracted using the NucleoSpin® kit. The DNA samples were extracted and then transferred to the Faculty of Medicine at Yamaguchi University in Japan for next-generation sequencing. NCBI BLAST was utilized to examine the sequence. Next-generation sequencing (NGS) was employed to collect a broad range of genes from phylum to genus by using 16S ribosomal ribonucleic acid (rRNA)

gene amplicons of the Illumina MiSeq System [14]. The Illumina 16S metagenomic sequencing library preparation instructions were applied in performing the 16S metagenomic analysis. In the present study, 16S rRNA amplicon sequencing was performed using 12.5 ng of DNA samples and two primer sets as follows:
16S amplicon forward primer:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S amplicon reverse primer:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

2.4 Microbial communities and statistical analysis

Every month, samples were taken at each temperature condition for NGS analysis to track how microbial communities responded to the different temperature levels and to understand how well these communities' characteristics changed over time in thermotolerant AD. The collected samples were divided into six groups based on the incubation conditions: 1 month at 42 °C (1M 42), 2 months at 42 °C (2M 42), 3 months at 42 °C (3M 42), 1 month at 45 °C (1M 45), 2 months at 45 °C (2M 45), 3 months at 45 °C (3M 45), 1 month at 48 °C (1M 48), 2 months at 48 °C (2M 48) and 3 months at 48 °C (3M 48). After acquiring monthly microbial communities' data, microbial diversity index analysis was performed to observe any differences in microbial abundance in every reactor under the thermotolerant temperature conditions. The diversity index is a metric that quantifies the number of distinct kinds (like species) that are present in a dataset (a community), as well as the richness, divergence, and evenness of evolutionary connections among individuals dispersed throughout those kinds [28]. Simpson's diversity index, Shannon's diversity index, and NGS-observed species were employed in the current work to evaluate microbial diversity. Analysis of variance (ANOVA), Tukey's multiple range test for mean comparisons, and Levene's test for homogeneity of variance ($p < 0.05$) were used to establish the statistical significance. In order to analyze the data, Origin Pro 2022 was used.

3 Result

3.1 Reactor performance in producing CH_4

The biogas production varied from the three different reactors with different incubation temperatures. As seen in **Figure 1**, in the reactor with an incubation temperature of 42 °C, the CH_4 production reach the maximum yield of 0.24 L/g COD, which was attained on day 68, with an average production of 0.20 L/g COD. Meanwhile, higher CH_4 production was recorded in the other reactors with higher temperature levels. At 45 °C, the reactor was observed to yield up to 0.27 L/g COD of CH_4 with an average production rate of 0.23 L CH_4/g COD, 12% higher than that of 42 °C. However, around day 60 – 70, CH_4 production in the 45 °C reactor, declined to around 0.20 – 0.23 L/g COD, which was the lowest level compared to the other reactor at the same time. The reactor showed quick recovery after day 70 and exhibited more stable performance upon that time. Furthermore, at 48 °C, the reactor produced 0.29 L CH_4/g COD with an average production yield of 0.24 L CH_4/g COD. This CH_4 yield is 21% higher compared to the reactor that was operated at 42 °C, and 7% higher than the 45 °C operated reactor.

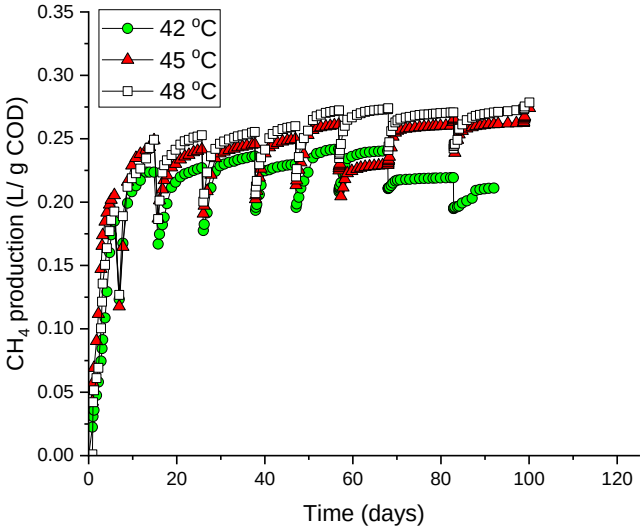

Fig. 1. CH_4 production from three different temperature conditions of 42 °C, 45 °C, and 48 °C.

Despite the 12 – 21% difference in CH_4 production following the higher operating temperature in the AD reactors, the biogas composition did not show any significant difference in every temperature condition. As shown in **Figure 2,** the highest CH_4 content was achieved by a reactor operated at 42 °C with 69% of CH_4 produced, while at 45 °C and 48 °C a maximum of 65% and 67% CH_4 was generated respectively. During the experiment day, there was a temporary drop observed every 10 days. This was caused by the feeding activities which was performed every time the biogas production declined sharply, with purpose to provide microorganism with sufficient carbon sources and maintain microbial communities that has been established during the incubation period. In the present study, after injecting the feed into the reactor, the biogas content would be reset by purging nitrogen gas to flush out the remaining biogas inside the reactor. Hence, the CH_4 content declined to nearly 0% and recover immediately after the methane-producing bacteria in the reactor started methanogenesis. Compared to 45 °C and 48 °C which show more stable CH_4 content over 100 days incubation period, a higher fluctuation of CH_4 production was exhibited at 42 °C, especially after day 70 when the CH_4 content fall to below 46%. The drop in CH_4 content following the decreasing CH_4 production signifies potential inhibition of methanogen during this period of incubation, as methanogen was the most prevalent micro that primarily produce methane through methanogenesis.

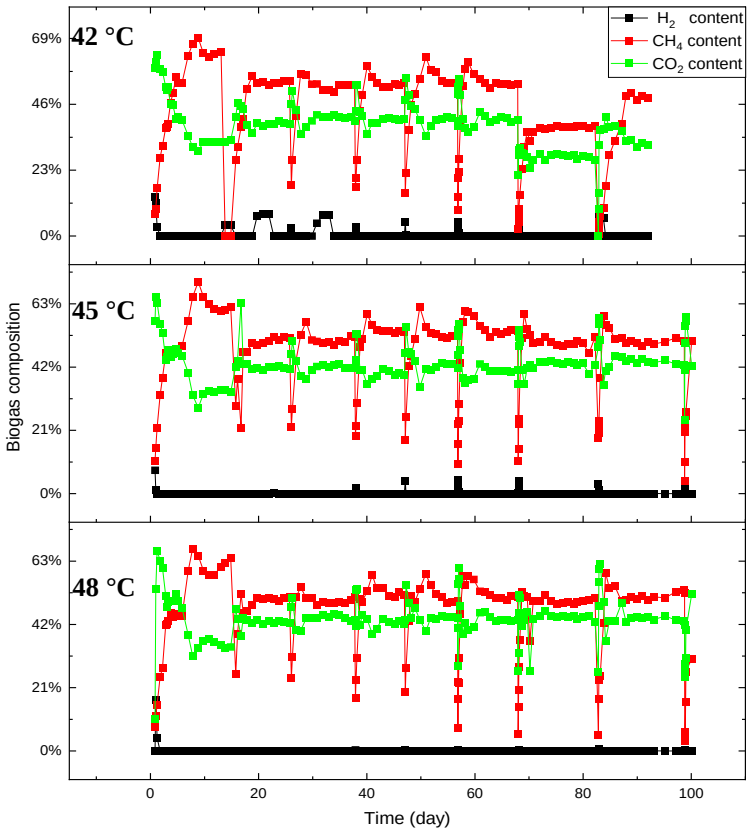

Fig. 2. Biogas composition in three different temperature conditions of 42 °C, 45 °C, and 48 °C.

3.2 The difference in microbial diversity in every reactor

In the present study, samples from the reactor were used for DNA isolation for bioinformatic analysis using NGS to analyze the difference in abundance of microbial communities in each temperature condition. The Shannon-Wiener's index and Simpson index were used to measure and compare the richness of the microbiota at a certain temperature. As illustrated by **Figure 3,** the diversity index value for Shannon-Wiener's and Simpson's index spread within the range of 3.15 – 3.71 and 0.86 – 0.95, respectively. Both diversity indexes show identical pattern of microbial diversity in each reactor and each incubation period, for instance, both Shannon-Wiener's and Simpson's index mentions the 2M48 reactor as the reactor with the lowest abundance (3.15 for Shannon-Wiener's index and 0.86 for Simpson's index) while 2M42, 2M45, and 3M45 shows nearly similar levels of abundance. However, from the Simpson's index perspective, the deviation of the diversity index between reactors was not significantly discerned, which signifies that each reactor has a close similarity of microbiota abundance and species to each other. Meanwhile, the discrepancies in microbial abundance between reactors can be seen clearly in Shannon-Wiener's index perspective.

According to Shannon-Wiener's index, the reactor with 48 °C presented the lowest diversity compared to the other reactors. This finding signified that at 48 °C the varieties of

microorganisms started decreasing and narrowing to the level where there are several specific microbial communities that dominates the ecosystem of the AD reactor. The decreasing varieties of microbial communities at 48 °C was associated to the capability of several types of microorganisms to acclimate in high temperature. Meanwhile, high diversity of microorganisms was observed during incubation in the reactor with lower operating temperature, at 42 °C and 45 °C. Using analysis of variance (ANOVA) method with significance of $p < 0.05$, the effects of different temperature conditions to diversity index was observed. The probability value (p-value) for Shannon-Wiener's and Simpson's index were $p < 0.001$, which signified that there were statistically significant differences in microbial diversity between several temperature conditions.

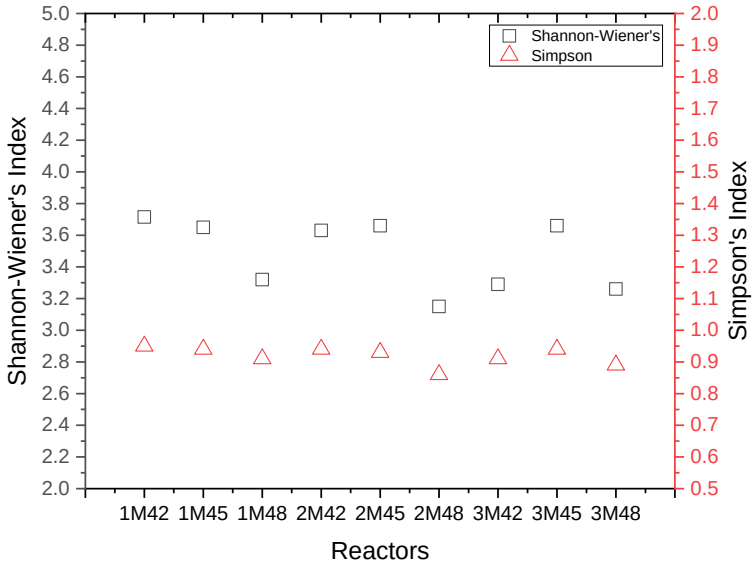

Fig. 3. Microbial diversity index in every temperature condition and incubation period

3.3 Microbial communities structure

As seen in **Figure 4(a)**, *Methanosaeta* was found as the most prominent methanogen in every reactor with various thermotolerant temperature and incubation period, signifying its capability to grow and dominate major methane producer communities in thermotolerant temperature conditions. *Methanosaeta* dominated as much as 75 – 89% of total methanogen population followed by several hydrogenotrophic methanogens such as *Methanoculleus* (represents 5 – 11% of total methanogens), *Methanobacterium* (3 – 10% of total methanogens), *Methanolinea* (1 – 3% of total methanogens) and *Methanobrevibacter* (< 1% of total methanogens). While the other acetoclastic methanogen, *Methanosarcina* only represented less than 1% of total methanogen population. Every reactor in the present study shows a significant decrease in the total methanogen population in 2 months of incubation, as presented in **Figure 4(b)**. This phenomenon indicates potential distress that inhibit the acclimatization of methanogen communities in thermotolerant temperature conditions.

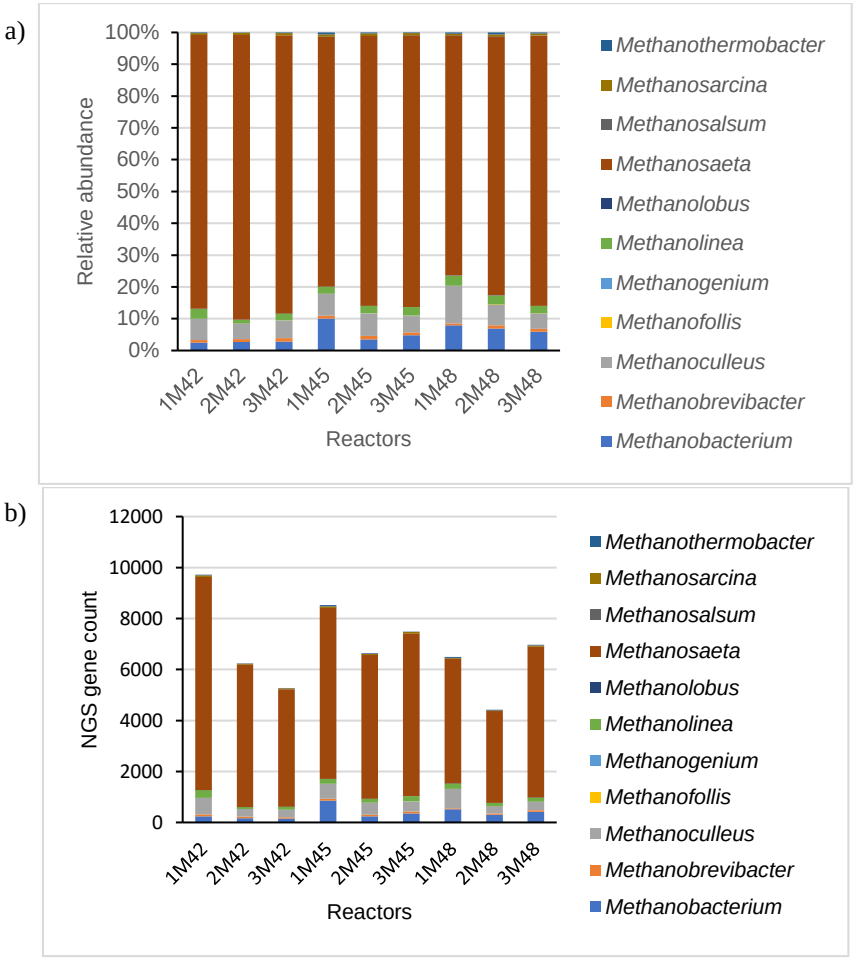

Fig. 4. Methanogen distributions in every incubation condition: (a) composition in %; (b) total abundance

At 45 °C and 48 °C, the total methanogen population recovered swiftly on the third month of incubation, denoted by a 13% increase in the *Methanosaeta* population from 2M 45 to 3M 45 and 36% from 2M 48 to 3M 48. However, the rapid recovery of the methanogen population seemingly did not occur at the 42 °C reactor as the methanogen population slumps 18% from 2M 42 to 3M 42 after a 33% decline from 1M 42 to 2 M 42. To understand how different types of microbial communities may affect the methanogenesis process in general, the distribution of non-methanogenic bacteria, such as the syntrophic acetate oxidizing bacteria (SAOB), needs to be determined. In the present study, the SAOB communities were identified to be the most abundant microbial communities in every reactor. As shown in **Figure 5(a),** *Clostridium* represents up to 89% of the total SAOB population at the 42 °C reactor (obtained in 3M 42). However, the domination of *Clostridium* gradually decreased and was replaced by *Tepidanaerobacter* along with the increase in operating temperature. On the third month of the 48 °C reactors, *Clostridium* only influences 11% of the total SAOB population, while *Tepidanaerobacter* dominates 85% of the SAOB population. The decrease in *Clostridium* along

with the increase in temperature conditions shrinks the total abundance of the SAOB population by more than 50%, as shown in **Figure 5(b).**

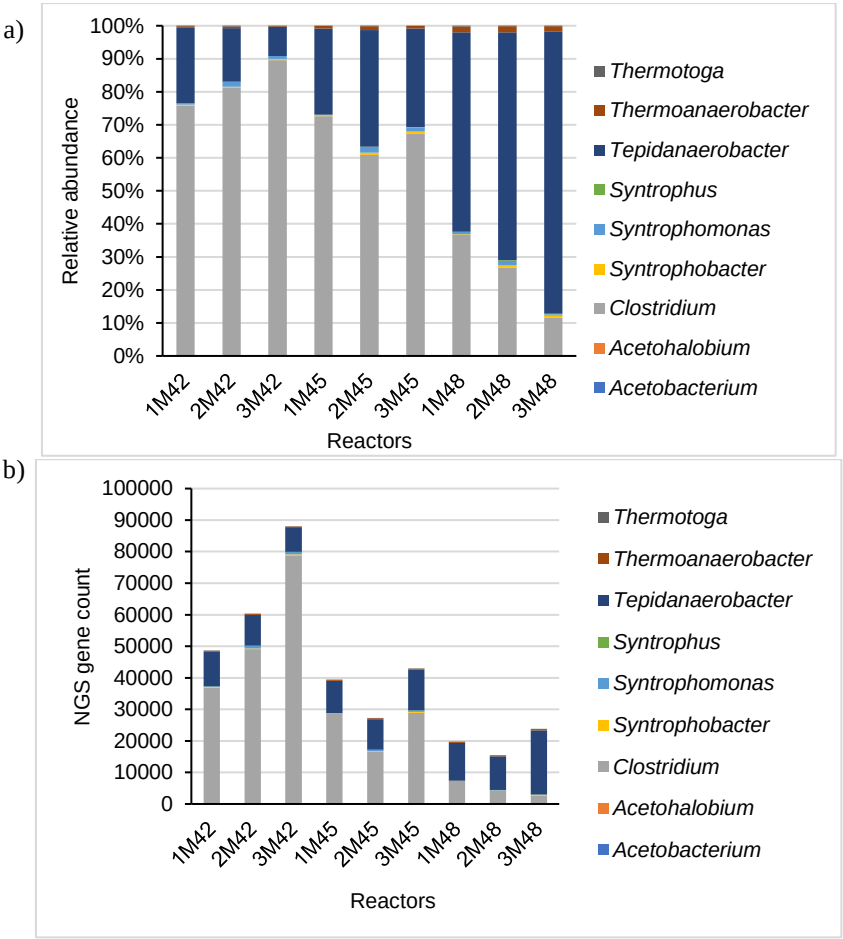

Fig. 5. SAOB distributions in every incubation condition: (a) composition in %; (b) total abundance

4 Discussion

In the AD process, temperature significantly affects the microbial community and production of biogas [29, 30]. The effects of temperature in mesophilic and thermophilic conditions have been the subject of numerous studies [31–33]. However, to our knowledge, the investigation of potential biogas production (especially CH_4) and microbial adaptation under several thermotolerant temperature regimes has not been extensively studied. Considering the results from the previous studies, we expected higher biogas yield would be generated when the temperature was adjusted close to the optimum temperature of mesophilic condition (37 – 39 °C) [34] and thermophilic condition (> 50 °C) [35]. Interestingly, we discovered that the CH_4

yield at 45 °C was 12% higher than that of 42 °C despite the 5 °C difference to the upper threshold of mesophilic optimum temperature. The CH_4 production further improved to a maximum of 0.30 L/g COD at 48 °C, close to the theoretical energy production (expressed as CH_4 production) recovered from digested wastewater sludge through the AD process, which was 0.38 L/g COD [36]. This result is consistent with previous studies that found higher temperature AD may enhance biogas production [31, 37–39].

Along with the higher temperature conditions for the thermotolerant AD, we discovered a decrease in microbial diversity as the temperature of the digester influenced the bacterial evolution from mesophilic communities to thermophilic communities. In the present study, the methanogen community was dominated by *Methanosaeta*, an acetoclastic methanogen, under all temperature conditions in this study. Some notable hydrogenotrophic methanogens that are usually found abundant in mesophilic AD such as *Methanoculleus, Methanobacterium,* and *Methanobrevibacter* [40, 41], cannot thrive and survive at thermotolerant temperatures of 42 °C, 45 °C, and 48 °C. This finding was supported by Lin et al. [42] that discovered the low potential activity of both *Methanobacterium* and *Methanobrevibacter* under the temperature of 45 – 55 °C. *Methanoculleus* however, was the most active hydrogenotrophic methanogen in all temperature conditions, though it cannot withstands the domination of acetoclastic *Methanosaeta*, which is also similar to the findings from Lin et al. [42].

The domination of acetoclastic methanogens over hydrogenotrophic methanogens indicates that the CH_4 production in the present study relied heavily on the concentration of acetate, instead of hydrogen. Acetoclastic methanogens consumed acetate, which was fermented from the acetogenesis process, to produce CH_4 while hydrogenotrophic methanogen tends to rely on the availability of hydrogen (H_2) as an electron donor to produce CH_4 [16]. In a previous research, acetoclastic methanogens largely dominated (87%) at a neutral pH, however when the pH dropped to 4.8, the hydrogenotrophic methanogen population grew to 65% and the acetoclastic methanogen population reduced [43]. According to the researchers, hydrogenotrophic methanogens prefer acidic pH, whereas acetoclastic methanogens prefer neutral pH. This was consistent with the current finding that acetoclastic methanogens predominated at all temperature conditions, where the pH had been buffered to almost neutral.

The high abundance of acetoclastic methanogens in the current study suggested that CH_4 production was predominantly directed through acetoclastic pathways. Consequently, it is important to understand the interaction between acetoclastic methanogens to their competitor, SAOB, in consuming available acetate [16]. According to Kurade et al. [44], optimum CH_4 generation through acetoclastic pathways can be achieved from a relatively low abundance of SAOBs and a high abundance of acetoclastic methanogens. In the present study, as seen in **Figure 6,** the high population of SAOB observed at the 42 °C reactor leads to a lower CH_4 production compared to the 45 °C and 48 °C reactor which shows gradual spikes in CH_4 production following the decline in SAOB population. This finding suggests that a high SAOB population may impair the ability of acetoclastic methanogens to produce CH_4 owing to the immense competition they encounter when attempting to consume available acetate. However, there is still unanswered question that, whether the high population of SAOB was the result of the declining population of acetoclastic methanogen or it was the SAOB that caused the deterioration of the acetoclastic methanogen communities. Due to several limitations in this study, further studies are required to determine the effect of several factors such as acetate level, pH, volatile fatty acid, and alkalinity to microbial communities (especially methanogens and

SAOB) and CH_4 production, so that the clearer image of the competition among microbial communities can be obtained.

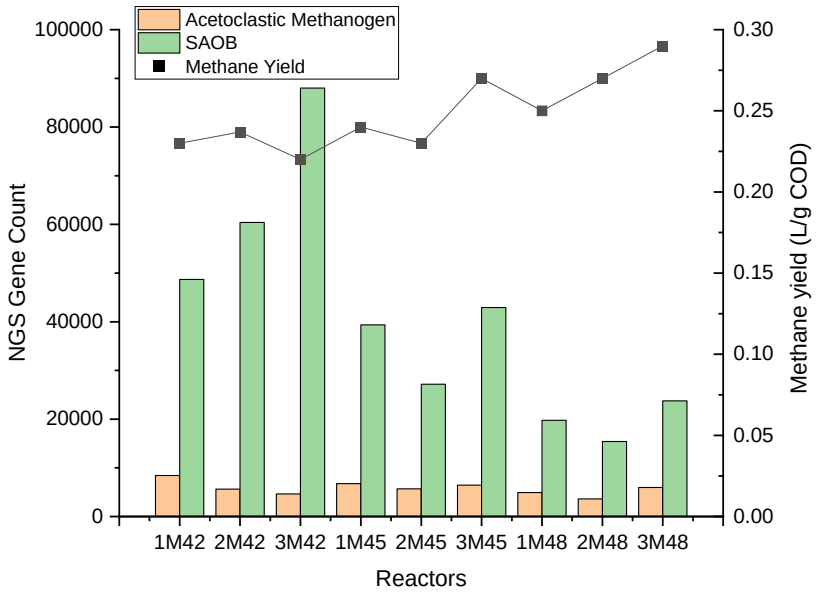

Fig. 6. Abundance comparison between acetoclastic methanogen community to SAOB and its influence on CH_4 yield

5 Conclusion

The effect of incubation of digested sewage sludge under several thermotolerant temperatures on AD performance, such as biogas production and microbial communities, were observed in the present study. Incubation under 42 °C deteriorated AD performance by showing a decline in CH_4 yield and methanogens community over months. In the higher temperature reactor, we discovered a 12% increase in CH_4 production at 45 °C and 21% at 48 °C. The higher temperature led to a significant decrease in SAOB population with simultaneous increase in CH_4 production signified SAOB population was negatively correlated to the CH_4 production due to competition with acetoclastic methanogens in consuming available acetate. Despite the competition with SAOB and different temperature conditions in each reactor, *Methanosaeta* was predominantly found in all temperature conditions, denoting its thermotolerance. A trivial presence of SAOB and hydrogenotrophic methanogens, and dominance of acetoclastic *Methanosaeta* suggested that CH_4 generated during thermotolerant AD was primarily conducted through acetoclastic pathways. These findings may provide a better understanding of microbial community functions in a complex AD process under thermotolerant conditions.

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