Doctoral Dissertation

Exploring Thermotolerant Anaerobic Digestion: Metagenomics Analysis and Biogas Production in Temperature Shift Scenario

（耐熱性嫌気性消化の探究：温度シフトシナリオにおけるメタゲノム解析とバイオガス生産）

March 2025

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ABSTRACT

The increasing concern over sludge generation in wastewater treatment plants (WWTPs), stemming from processes such as primary sedimentation (PS) and activated sludge (AS), has garnered global attention. Anaerobic digestion (AD) presents a viable solution, enabling the conversion of various residual biomass—including sewage sludge, anaerobic sludge, agricultural waste, and food waste—into bioenergy and bio-fertilizer. This process yields valuable by-products such as volatile fatty acids (VFAs) and biogas, which is primarily composed of methane (CH_4) (50–70%) and carbon dioxide (CO_2) (30–50%), along with trace gases like hydrogen sulfide and ammonia. AD is recognized as both cost-effective and environmentally sustainable, requiring less energy and producing a lower carbon footprint compared to traditional waste disposal methods like landfilling and incineration, thereby contributing to carbon neutrality goals.

CH_4 production through AD hinges on the activity of diverse microbial communities involved in hydrolysis, acidogenesis, acetogenesis, and methanogenesis, with temperature playing a critical role in maintaining metabolic stability and optimizing biogas output. AD is typically conducted within three temperature ranges: psychrophilic (20°C), mesophilic (20–43°C), and thermophilic (50–60°C), with commercial-scale operations favoring mesophilic and thermophilic conditions. Despite extensive research within these conventional ranges, challenges remain, particularly the limited exploration of the intermediate temperature range between mesophilic and thermophilic (40–45°C). There is a notable gap in the understanding of the microbial communities that thrive in this range and their adaptability to both mesophilic and thermophilic environments. Previous studies have suggested the potential for increased CH_4 production at 45°C; however, these studies were conducted under stable temperature conditions. Given that WWTP sludge is typically processed at mesophilic and thermophilic temperatures, transitioning to the 45°C range represents a viable approach to simulate potential temperature fluctuations in existing digesters.

Two temperature shift scenarios (upshift and downshift) were employed in the current study using fed-batch mode with anaerobically digested sewage sludge as inoculum and a glucose-based substrate. The temperature was shifted with intervals of at least 5°C. The first scenario involved acclimatization to an upshift from 42°C to 48°C, while the second involved a downshift from 55°C to 45°C. Both scenarios resulted in reduced biogas production, particularly at 45°C. The upshift scenario saw a maximum reduction of 83% in biogas production, while the downshift scenario experienced a 16-33% decrease in CH4 output. Next-generation 16S rRNA sequencing revealed that *Methanoculleus* dominated in the upshifted scenario, but the low correlation between its abundance and biogas production suggests inhibition of the hydrogenotrophic pathway. In contrast, the downshifted scenario demonstrated better biogas production, attributed to the dominance of acetoclastic *Methanosaeta* and the low presence of sulfate-reducing bacteria. These findings underscore the impact of temperature shifts on microbial communities and their consequent influence on biogas production performance.

The study was then focused on the upshift scenario, with an in-depth exploration of metagenomics aspects including functional analysis and microbial network dynamics. The study utilized digested sewage sludge as inoculum and a substrate consisting of 1.5 g/l glucose mixed with trace elements. The temperature was incrementally increased from 37°C to 45°C in 2°C intervals. Results indicated CH_4 production levels of 298 and 309 mL CH_4/g

COD_{fed} at 37°C and 39°C, respectively, with a decline observed at 41°C. However, an increase to 43°C restored CH_4 production to 260 mL CH_4/g COD_{fed}, accompanied by lower CO_2 output. At 45°C, the widening gap between CH_4 and CO_2 production suggested enhanced methanogenic activity due to prolonged high temperatures. *Methanothrix* emerged as the predominant methanogen, with *Methanobacterium* activity increasing at 43°C and 45°C, alongside the upregulation of key hydrogenotrophic pathway enzymes *(fwd, ftr, mch*). The presence of *Acetomicrobium* and *Defluviitoga* likely supported the activity of these enzymes. These results highlight the adaptability of microbial communities and potential syntrophic relationships in response to temperature changes, demonstrating the resilience of AD processes under varying environmental conditions.

In contrast, when a higher glucose level of 10 g/l was used, the results differed. During incubation at 37–41°C, similar disturbances in acetogenic bacteria and methanogen populations were observed, leading to a significant reduction in CH_4 production. However, as the temperature increased further, the activity of acetogens and methanogens rose, correlating with increased CH_4 and CO_2 production. *Methanothrix* remained the predominant methanogen, indicating the dominance of the acetoclastic pathway. Functional analysis revealed that acetate decarboxylation enzymes remained resilient at 43°C and 45°C, unlike the hydrogenotrophic pathway enzymes. Compared to the 1.5 g/l glucose scenario, the acetoclastic pathway played a more prominent role in CH_4 production under higher glucose levels, while the hydrogenotrophic pathway was more susceptible to inhibition. The characteristic high CH_4 followed by high CO_2 production is indicative of the acetoclastic pathway's dominance over the hydrogenotrophic pathway, highlighting that higher glucose levels favor the acetoclastic pathway and limit CH_4 production via the hydrogenotrophic pathway.

ACKNOWLEDGEMENT

I would like to extend my sincere and deepest gratitude to my supervisor, Professor Tsuyoshi Imai for the kind support and guidance during my PhD study. There are numerous lessons I have learnt during my study that further expedites my expertise and research experience.

I would also like to express my special thanks to Professor Alissara Reungsang from Khon Kaen University that relentlessly provided me remarkable advices and references whenever I experienced impasse during my “exploration” in anaerobic digestion field of expertise.

My deepest appreciation to my graduate committee, Professor Koichi Yamamoto, Prof. Takaya Higuchi, Professor Masakazu Niinae, and Professor Eiichi Toorisaka, who have helped me so much to improve the quality of my work with their constructive suggestions.

Sincere thanks to Professor Masahiko Sekine and Professor Ariyo Kanno for noteworthy suggestions and advice given to me during the English Seminar.

I gratefully acknowledge The Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) for providing me with notable scholarship.

Sincere thanks to my great colleagues from Khon Kaen University: Dr. Chonticha Mamimin, Dr. Srisuda Chaikitkaew, Dr. Nantharat Wongfaed, Dr. Kanathip Promnuan, Dr. Umarin Jommonkhaow, Dr. Worapong Wongarmat, Assist. Prof. Jutarop Phetcharaburanin, and from Mahidol University: Assist. Prof. Sureewan Sittijunda for providing me significant knowledge in methanogenesis process, data science and metagenomic analysis.

Sincere thanks to Prof. Stefano Campanaro et al. and Dr. Gavin Douglas et al. for providing me available references in understanding bioinformatics, especially functional annotations.

I acknowledge members of EISEI and visiting researchers: Yuko Yamamoto, Ganjar Samudro, Rafita Hasanah, Kai Sueda, Yuma Osaki, Hiroki Kagawa, Shingo Nakamura, Takuya Inoue, Ryota Jutori, Yuta Yakura, Riho Takahashi, Kanta Sugiyama, So Sakamoto, Hana Matsuo, Joaninha Belo Ornai, Dr. Jantima Teeka, Ajahn On, Dr. Edy Kurniawan. Also Dr. Amandangi Hastuti, Yuya Senba and the other colleagues from Nagai-Lab.

Many thanks to PPI Jepang-Yamaguchi for the support and entrusting me to serve the organization as vice chairman and general secretary for 2 consecutive periods.

To all my colleagues in Universitas Udayana, especially at the Environmental Engineering Department.

I heartily acknowledge my parents and my beloved long-distance girlfriend for the long-lasting understanding and support.

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CHAPTER I
INTRODUCTION

1.1 Rationale and background

The two pivotal challenges in the realm of global sustainable development revolve around the intensifying energy crisis and environmental deterioration resulting from inadequate management of wastewater, particularly arising from processes within wastewater treatment plants (WWTPs). The production of sludge associated with WWTPs, including primary sedimentation (PS) and activated sludge (AS), assumes considerable significance on a global scale. Notably, EU nations, the United States, China, Iran, Turkey, Canada, and Brazil collectively contribute to an annual cumulative generation of approximately 8910, 6510, 2960, 650, 580, 550, and 370 thousand metric tons of sewage sludge, respectively (Mateo-Sagasta et al., 2015). Considering the significant magnitude of sludge production, it is not unexpected that the control and elimination of sewage sludge have become critical global issues. Due to its high moisture content, constrained dehydration capability, and strict regulations governing the recycling and disposal of sludge, the handling and oversight of sludge present a formidable and complex challenge (Nazari et al., 2018). The paradigm of wastewater treatment has shifted towards an environmentally friendly approach, aiming to reduce the volume of discarded sludge and transform it into a bioenergy source.

Anaerobic digestion (AD) stands as an effective approach capable of generating bioenergy and bio-fertilizer from diverse residual biomass sources like sewage sludge, anaerobic sludge, agricultural waste, and food waste, aiming to address pertinent challenges. This process yields valuable products such as volatile fatty acids (VFAs) and biogas. Biogas typically comprises methane (CH_4) (50–70%), carbon dioxide (CO_2) (30–50%), alongside other compounds like hydrogen sulfide and ammonia (Porté et al., 2019). Methane synthesis in anaerobic digestion (AD) typically relies on a variety of microorganisms with different physiological characteristics, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The transformation of substrates into CH_4 is influenced by crucial elements like acetate (HAc), hydrogen (H_2), and CO_2, which take place through two main methanogenic pathways: acetoclastic and hydrogenotrophic pathways (Tian et al., 2018). The acetoclastic pathway is commonly influenced by *Methanosarcinaceae spp.* and

Methanosaetaceae spp., whereas the hydrogenotrophic pathway is mainly affected by *Methanobacteriales spp.* and *Methanomicrobiales spp.* (Kurade et al., 2019).

In both acetoclastic and hydrogenotrophic pathways, temperature plays a critical factor in preserving metabolic stability and biogas generation during the four stages of CH_4 synthesis since AD microbial communities are sensitive to perturbations brought on by temperature fluctuations (Nie et al., 2021). Several studies have revealed how temperature variations impact the overall performance of AD and lead to imbalances in microbial populations, particularly in the case of acetogenic and methanogenic microorganisms (Hupfauf et al., 2018; Kovalovszki et al., 2020; Madigou et al., 2019). Hydrolytic bacteria are generally more sensitive to temperature, according to studies by another group (Chen and Chang, 2020). However, even with the recognized influence of temperature and extensive research in this field, the specific consequences of consistent temperature fluctuations on the AD process are not thoroughly comprehended. Significantly, there is a gap in knowledge regarding how dynamic temperature changes affect the temperature adaptability (thermotolerance) of microorganisms involved in the AD process.

Anaerobic digestion (AD) is commonly carried out within three specific temperature ranges: psychrophilic (20 °C) (Fernández-Rodríguez et al., 2016), mesophilic (20–43 °C) with optimal temperatures ranging from 35–37°C (Wang et al., 2018), and thermophilic (50–60°C) (Madigou et al., 2019). Most commercial-scale AD systems operate predominantly at mesophilic and thermophilic temperatures, with CH_4 production rates averaging between 0.03–0.65 L/g volatile solid (VS)/day and 0.04–0.70 L/g-VS/day, respectively (Kasinski, 2020; Nie et al., 2021). Despite extensive research in these conventional temperature ranges, challenges persist in the operational practices of AD processes. This includes limited attention to the intermediate temperature range between mesophilic and thermophilic (40–45 °C) and a corresponding lack of understanding regarding the microorganisms inhabiting this range and their adaptability to both mesophilic and thermophilic conditions.

Current research on AD has mostly concentrated on maintaining constant temperatures inside individual reactors in an effort to minimize the possibility of instability in microbial communities (Hidaka et al., 2022; Roopnarain et al., 2021). As a result, there has been limited focus on processes in AD that account for temperature variations, and the specific effects of temperature fluctuations on both biogas production and the dynamics of methanogenic bacteria are not well understood. Additionally, the intricate microbial networks within AD have impeded a thorough exploration of microbial populations and

their interactions. Recognizing the crucial ecological niches in the AD process and understanding the metabolic traits of microbial communities within these niches could lead to innovative designs and operational techniques, ultimately improving methane recovery from organic waste. This study was prompted by the challenges mentioned above and aimed to examine the changes in biogas production and the relationships among microbial communities when subjected to shifts in temperature conditions. The findings from this investigation contribute to a more comprehensive comprehension of microbial behavior during temperature fluctuations in the context of methanogenesis.

1.2 Research objectives

This study primary aims to investigate the effect of temperature upshift in biogas production and microbial communities of digested sewage sludge AD process. Five sub-objectives have been formulated as follow to attain the goal aforementioned:

1. To evaluate the potential biogas production during upshifted temperature conditions.
2. To identify the predominant methane producing bacteria among microbial communities during upshifted temperature conditions.
3. To analyse the abundance of microbial communities in the AD process through Next-Generation Sequencing methods.
4. To investigate the behaviour and the interconnection of microbial communities' network that involved in the AD process under upshifted temperature conditions.
5. To analyse the effects of temperature shifts on the methane producing pathway through microbial metabolic analysis.

1.3 Structure of dissertation

This study is compiled and structured into six chapters according to the following list:

- **Chapter I Introduction** explains the background of the current study, elaborate the problem statement, aims of the research, rationale and potential contribution of the current study to science.
- **Chapter II Literature review** presents all of the relevant literatures that support the finding of the current study, including the fundamental analysis of methanogenesis process and metagenomic analysis.

- **Chapter III Metagenomics analysis of the effect of temperature shift in anaerobic digestion: upshifted and downshifted scenario.** This chapter describes how temperature shift affects microbial communities abundance, especially those involved in biogas production, and how the abundance of sulphate-reducing bacteria hindered biogas production in two different scenarios. The result obtained in this chapter, inspires the further investigation of potential stable biogas production by initially incubate the reactor at optimum mesophilic temperature.
- **Chapter IV Shift in temperature, shift in pathway? unravelling the effect of temperature upshift on methane production pathway.** According to the finding from Chapter III, this chapter unveils potential of achieving stable biogas production with less fluctuated microbial communities by initially incubate the reactor at 37 °C and gradually increased the temperature until 45 °C. With the addition of 1.5 g/l glucose, the current study observed possible shift in methanogenesis pathway, from acetoclastic to hydrogenotrophic at higher temperature levels and extended incubation periods.
- **Chapter V Higher glucose level preserve stable digestion process during stepwise temperature upshift through acetoclastic pathway.** In this chapter, in addition to temperature shift, higher glucose level of 10 g/l was added. The current study observed the long-preserved acetoclastic methanogenesis pathway despite temperature shift and extended incubation periods.
- **Chapter VI Conclusion** summarize the overall findings of the current study and provides potential engineering implications and limitations of the study.

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CHAPTER II
LITERATURE REVIEWS

2.1 Climate change and the importance of renewable energy

Global warming, propelled by the persistent rise in atmospheric carbon concentrations, is a worldwide occurrence leading to catastrophic events. Prolonged exposure to escalating carbon concentrations has significantly contributed to the heightened atmospheric temperature, adversely impacting the environment. Assuming the current trend persists, it is anticipated that the global average temperature will increase by 3 °C by the year 2100 compared to the preindustrial era (Statista, 2022). Nevertheless, the potential increase in temperature marked only the initial phase of the narrative. Subsequently, there were environmental concerns such as rising sea levels and sea surface temperature, and a heightened occurrence of natural disasters (such as floods, droughts, cyclones, forest fires, storm precipitations, heat waves, glacier melting, etc.). These phenomena substantiate that climate change is not a fabricated deception but rather an unfortunate reality.

With the rapid growth of the world's population and the urges for better living standards, these concerns inflicted a rising demand for global energy. Despite the massive growth and promotion of renewable energy usage, the vast majority of energy sources in the 21st century is still dominated by fossil fuels such as oil, coal, and natural gas (Figure 2.1). The usage of oil as energy has been decreasing in the past 21 years, while coal and natural gas were showing an increasing usage for energy on average. In Japan particularly, more than 50% of energy was still provided by non-renewable energy: 28.3% of provided by coal, 37.4% was provided by liquified natural gas (LNG), and 3.7% of energy was provided by oil (Figure 2.2). The basic energy plan issued by the Japanese Ministry of Economy, Trade, and Industry (METI) in 2021 demands that 22-24% of Japan's energy needs should be fulfilled by renewable sources while the reliance on nuclear energy is reduced from 25% to 20% and fossil fuel from 65% to 56%.

Fossil fuels provide energy to various sectors of society, including residential, commercial, industrial, and transportation. Burning fossil fuels for energy generation, emitted uncontrolled carbon emissions into the atmosphere. CH_4 and CO_2 represent the primary carbon-based greenhouse gases that have accumulated in the atmosphere as a result of human activities using energy derived from fossil fuels. CO_2 is predominantly generated through the combustion of fossil fuels, while CH_4 emissions are associated with fossil fuel extraction,

intensive agricultural practices, livestock, transportation, and the accumulation of untreated waste biomass. Presently, the atmospheric concentration of CO_2 reaches 412 ppm, and CH_4 is 1879 ppb, compared to the stable thresholds of approximately 285 ppm and 722 ppb, respectively, during the preindustrial era (USEPA, 2022). The ongoing rise in atmospheric CO_2, driven by the escalating demand for energy in the fast-paced economic development race, has disrupted the Earth's capacity to utilize CO_2 effectively.

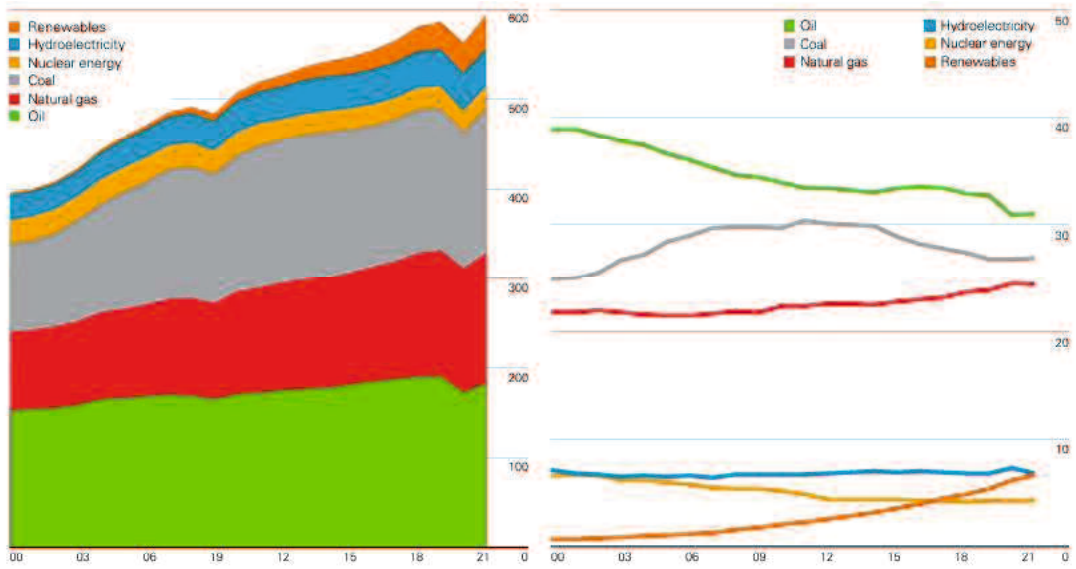

Figure 2. 1 World's energy consumption (left) and shares of global primary energy (right) (Dale, 2022)

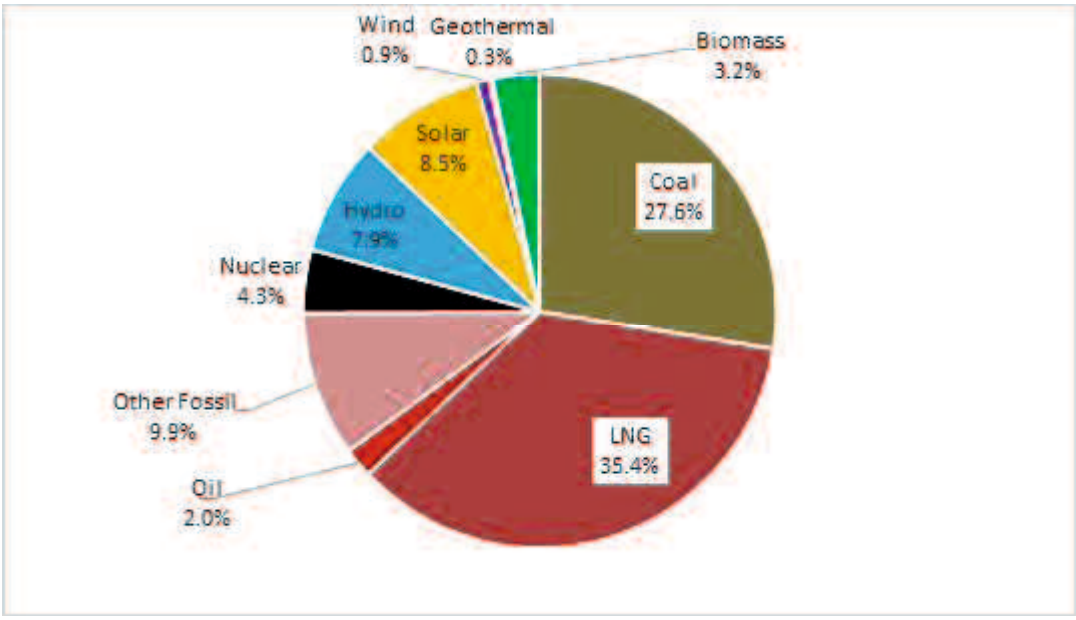

Figure 2. 2 Japan's total energy consumption 2020 (ISEP, 2021)

2.2 Anaerobic digestion and biogas recovery from waste

Transitioning to energy generation from renewables and concurrently reducing fossil fuel consumption by a minimum of 6% by 2030 could lead to a substantial decrease in carbon emissions (UNEP, 2020). Anaerobic digestion (AD) has been widely recognized as a versatile technology to decompose organic wastes with two significant by-products: biogas (composed mainly of CH_4, CO_2, hydrogen sulfide (H_2S), moisture, etc.) and bio-fertilizer (abundant in nitrogen, phosphorus, potassium, and all micro-nutrients essential for plant growth obtained from fundamental resources such as soil and water) (Isha et al., 2020). An additional benefit lies in the versatility of biogas applications, which can include thermal and electrical energy generation or serve as vehicular fuel, given that the biogas undergoes upgrading (CO_2 removal) or polishing (H_2S removal) (Kapoor et al., 2017).

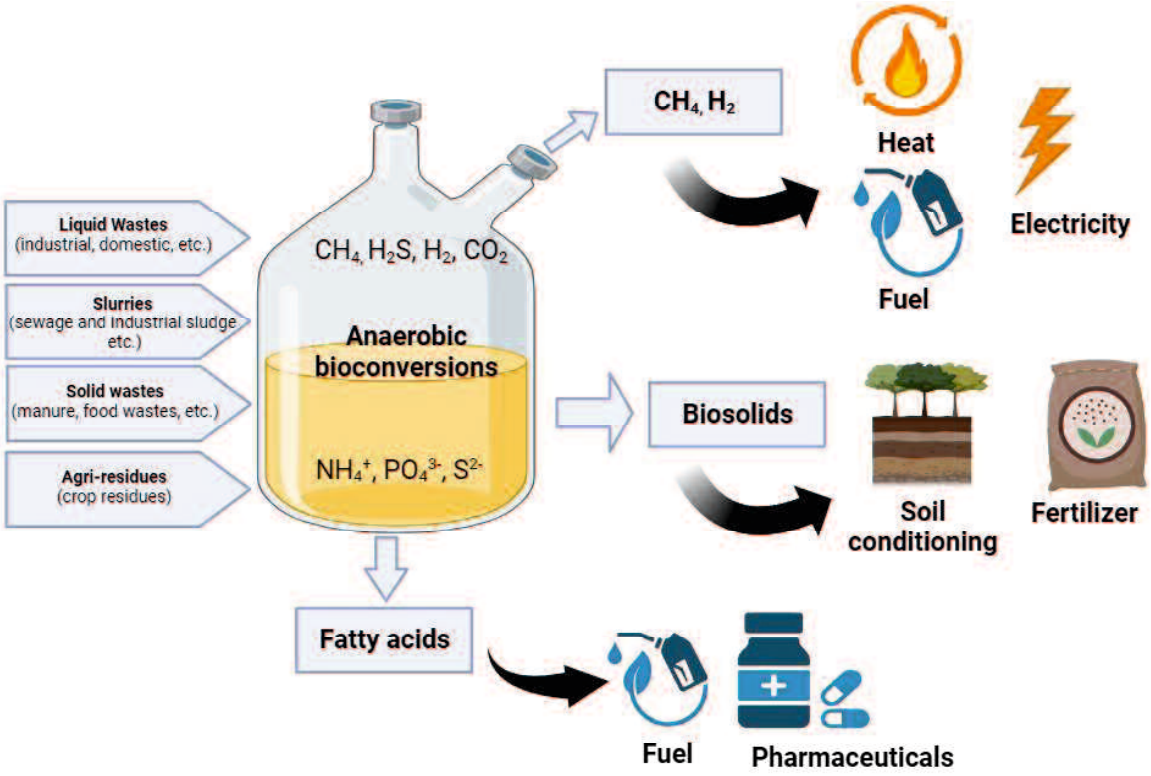

Figure 2. 3 Anaerobic digestion processes in recovery of resources from multiple waste streams. Reconstructed from explanation in (Khanal, 2008) and (Kurniawan et al., 2024).

Figure 2.3 demonstrates the potential of anaerobic digestion in extracting value-added products and biofuels from waste streams. These products encompass biofuels (hydrogen, butanol, and methane), electricity generated through microbial fuel cells (MFCs), fertilizers,

and various chemicals (including sulfur, organic acids, ethanol, etc.). The production of biogas (predominantly composed of CH_4 and CO_2) through anaerobic digestion of diverse feedstocks, primarily organic wastes like livestock manure, food wastes, sewage sludge, crop residues, agricultural byproducts, and the organic fraction of municipal solid wastes (OFMSW), relies on a complex microbial community, or microbiome, present in anaerobic digestion bioreactors.

There are several organic wastes that can be utilized as a source of microorganisms and feedstock for the AD process, one of which is the wastes discharged from wastewater treatment plants (WWTP). Concerns regarding the production of sludge from wastewater WWTPs, originating from processes like primary sedimentation (PS) and activated sludge (AS), have become significant on a global scale. This issue is particularly pronounced in various countries, including EU nations, the United States, China, Iran, Turkey, Canada, and Brazil, which collectively contribute to an annual cumulative output of sewage sludge. Specifically, these nations produce approximately 8910, 6510, 2960, 650, 580, and 370 thousand metric tons, respectively (Mateo-Sagasta et al., 2015). Given the substantial volume of sludge generated, the handling and disposal of sewage sludge have evolved into critical international issues (Nazari et al., 2018).

AD is a common approach used as an end treatment for sewage, primary, and waste-activated sludge in numerous WWPTs (Campo et al., 2018; Hanum et al., 2019; Young et al., 2013). Compared to other biological processes used in sludge treatment, AD offers various advantages, including ease of operation, the potential for producing an organic by-product that may be used in agriculture, low operational cost, and low energy consumption (Panepinto et al., 2019; Pramanik et al., 2019).

2.3 Potential of sewage sludge biomass in AD

Sewage sludge, a byproduct of wastewater treatment, poses a significant disposal burden, often associated with landfilling and incineration. These methods incur considerable costs and raise environmental anxieties related to air pollution and leachate contamination. In this context, AD emerges as a promising alternative, offering both waste reduction and resource recovery. Anaerobic digestion has been established as an effective technology for treating sewage sludge, allowing the processing of high-water-content sludge without the need for pretreatment (Ward et al., 2008).

The characteristics of sewage sludge undergo modifications during anaerobic digestion, resulting not only in the production of biogas but also in several favourable outcomes for subsequent sludge management. The process enhances sludge stabilization, reduces pathogens and odour emissions, and decreases the dry matter of the sludge, leading to a significant reduction in its final volume. As the source of biomass for AD process, sewage sludge potentially generates biogas with CH_4 content up to 40-60% depending on the pre-treatment method and substrate mixture (D' Silva et al., 2021; Hallaji et al., 2019, 2018). The CH_4 production rate in conventional digesters treating sewage sludge usually falls within the range of 0.19 to 0.24 Nm^3/kg VS (Bachmann, 2015), and this is contingent on the sludge retention time (SRT) employed in the wastewater treatment process (Bolzonella et al., 2005).

Table 2.1 presents the total biogas production and specific data from wastewater treatment plants (WWTPs) in various countries, based on statistics from the International Energy Agency (Bioenergy, 2001) and the World Bioenergy Association (IEA, 2017). Germany stands out as the leader in global biogas production, boasting 10,431 plants generating 55,108 GWh/y of electricity, including 1,258 sewerage plants contributing 3,517 GWh/y. In contrast, India, despite having 83,540 biogas plants, primarily focuses on household-level bioenergy production, particularly in rural areas, with limited utilization of small-sized biogas plants.

In Japan, strategic initiatives like SPIRIT21 (Sewage Project, Integrated and Revolutionary Technology for Twenty First Century) and Sewerage Vision 2100 have been in effect for the past decade, focusing on enhancing wastewater treatment plants (WWTPs) and optimizing sewage sludge utilization. These policies have spurred active efforts in sewage sludge utilization, including energy recovery through anaerobic digestion. As reported by the Japan Sewage Works Agency, 280 out of 2,150 WWTPs are equipped with operational anaerobic digestion systems, producing an average of 0.4–0.6 m3 CH_4/kg VS (Hanum et al., 2019). Notably, a standout example is a successful WWTP in Yamagata Prefecture, where anaerobic digestion is applied to treat sewage sludge. This facility generates a daily biogas output of 4,082 m3, resulting in the production of 7,226 kWh/d of electricity. Remarkably, this output accounts for 48.7% of the plant's total energy consumption (Li and Kobayashi, 2010).

Table 2. 1 Biogas production in several countries

Country	Year	Total biogas production (from sewage sludge, landfills, industrial wastewater, agricultural, biowastes)		Biogas production in WWTPs (sewage sludge only)	
		Number of plants	[GWh/y]	Number of plants	[GWh/y]
Australia	2017	242	1587	52	381
Austria	2017	291	3489	39	18
Argentina	2016	62	n.a.	n.a.	n.a.
Belgium	2015	184	955	n.a.	n.a.
Brazil	2016	165	5219	10	210
China	2014	11500	90	2630	n.a.
Czech Republic	2015	554	2611	n.a.	n.a.
Denmark	2015	156	1763	52	281
Finland	2015	88	623	16	152
France	2017	687	3527	88	442
Germany	2016	10431	55108	1258	3517
India	2015	83540	22140	n.a.	n.a.
Ireland	2015	28	202	n.a.	n.a.
Italy	2015	1491	8212	n.a.	n.a.
Japan	2015	n.a.	30200	2200	n.a.
United States	2017	2100	1030	1240	n.a.
United Kingdom	2016	987	26457	162	950

n.a. : Data not available

2.4 Organics conversion in AD system

The success of microbial-mediated anaerobic digestion (AD) processes hinges significantly on a thorough comprehension of process microbiology, particularly the examination of microscopic organisms involved in waste breakdown and byproduct generation. Unlike

aerobic processes, anaerobic processes are notably intricate due to the participation of a diverse array of microorganisms and a sequence of interdependent metabolic stages. Ensuring stable operation requires careful process control, given the low growth rate, specific nutrient and trace mineral needs of methane-producing bacteria (methanogens), and their vulnerability to changes in environmental conditions. The biochemical aspects entail enzyme-mediated chemical transformations, the types of substrates (wastes/residues) microorganisms can break down or convert into new compounds, and the stepwise pathway of degradation.

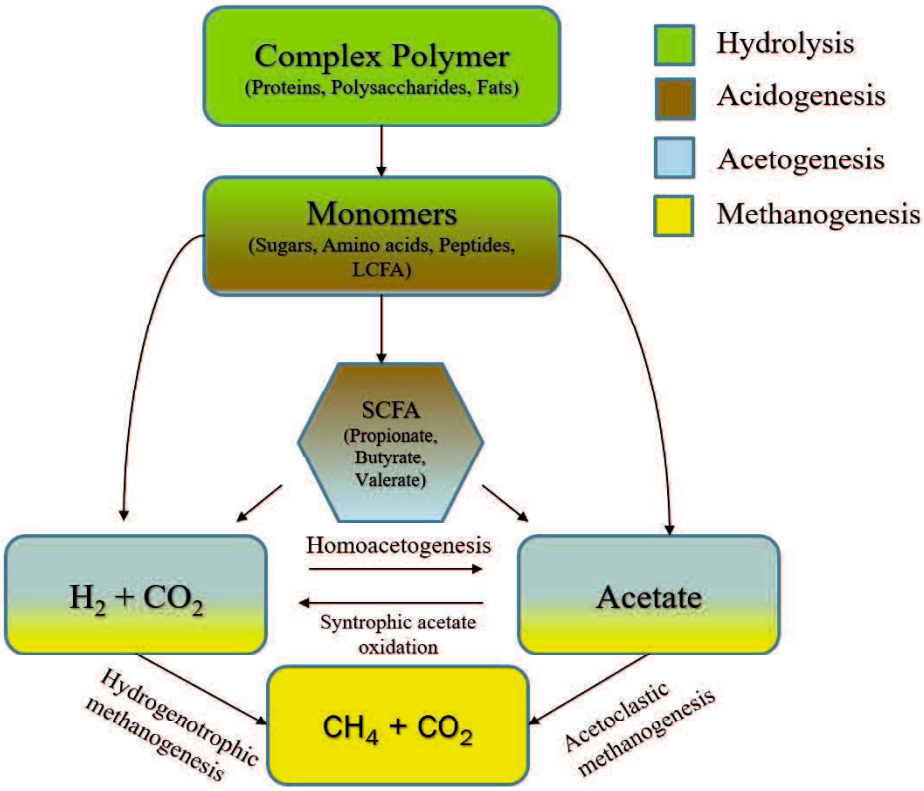

Figure 2. 4 Schematics of conversions stages in anaerobic digestion of complex organic compounds into biogas and other byproducts. Reconstructed from explanation in (Gujer and Zehnder, 1983; Khanal, 2008).

The process of converting compound macromolecules, such as proteins, carbohydrates (polysaccharides), and lipids (fats), found in wastewater or solids, into biogas like CH_4 and CO_2 involves a series of metabolic phases facilitated by various sets of microbial communities. Figure 2.4 depicts the schematics outlining the diverse stages and the bacterial categories participating in the anaerobic breakdown of intricate wastes. The action of extracellular enzymes secreted by the fermentative bacteria converts complex organic substances like proteins, carbohydrates, and lipids into simple soluble products such as

sugars, amino acids, peptides, and long-chain fatty acids. This process is sometimes referred to as liquefaction or hydrolysis. In the overall anaerobic treatment procedures, hydrolysis may be the rate-limiting step (step that controls biochemical reactions in AD) for wastes such sewage sludge, animal manure, and food waste that include lipids and/or a large amount of particle matter (Henze and Harremoës, 1983; Khanal, 2008; van Haandel and Lettinga, 1994).

The soluble products resulting from the initial phase are subjected to fermentation by fermentative bacteria, generating organic acids, hydrogen, and carbon dioxide (Lim et al., 2020). Acidogenesis specifically involves the formation of volatile fatty acids (VFAs) (C > 2), including propionic and butyric acid. Subsequently, VFAs and ethanol undergo conversion to acetic acid, hydrogen, and carbon dioxide, facilitated by a distinct category of bacteria referred to as hydrogen-producing acetogenic bacteria. This stage, recognized as acetogenesis, is considered thermodynamically unfavorable unless the hydrogen partial pressure is maintained below 10^{-3} atm through the effective elimination of hydrogen by organisms with hydrogen-consuming capabilities, such as hydrogenotrophic methanogens and/or homoacetogens (Khanal, 2008; Lim et al., 2020; O-Thong et al., 2020). Elevated hydrogen partial pressure has been documented to impede the degradation of propionate (O-Thong et al., 2020; Speece, 1996).

The major substrates for methanogenesis are CO_2, H_2, and acetate. Acetate decarboxylation accounts for roughly 72% of CH_4 synthesis based on chemical oxygen demands (COD), with CO_2 reduction contributing for the remaining portion (Khanal, 2008). The microorganisms referred to as acetotrophic or acetoclastic methanogens are those that convert acetate into CH_4. The hydrogenotrophic methanogens produce the remaining CH_4 from H_2 and CO_2. The majority of the biochemical reaction in the anaerobic wastewater treatment process is regulated by a process known as acetoclastic methanogenesis as CH_4 is mostly produced from acetate. Few studies have been conducted on homoacetogens' production of acetate from H_2 and CO_2. In a digester for cattle manure, acetate synthesis via homoacetogenesis pathway only makes up 1-2% of the overall acetate generation at 40 °C and 3-4% at 60 °C (Mackie and Bryant Marvin, 1981).

In anaerobic digestion, there exists a syntrophic relationship between acetogens and methanogens. The syntrophic relationship keep the anaerobic system well balanced. One of the most important tests to judge this balance is the determination of individual VFAs in the effluent. VFAs in this context are the short-chain organic acids, which are the intermediates

formed during anaerobic fermentation of complex organic materials. For a normal operating anaerobic system, the effluent VFA concentration ranges from 50 – 250 mg/L acetic acid. When the syntrophic relationship is disturbed, due to either overloading, toxicity, nutrient deficiency, or biomass washout, there is an accumulation of VFAs, and their levels continue to increase. This may cause an abrupt drop in pH and subsequent souring of the anaerobic reactor.

2.5 Microbial communities in AD process

The anaerobic digestion of complex organic matter is carried out by different groups of bacteria as indicated in Fig. 2.4. There exists a coordinated interaction among these bacteria. The process may fail if one group is inhibited.

2.5.1 Hydrolytic/Fermentative Bacteria

Fermentative bacteria play a crucial role in the initial stages of anaerobic digestion (AD), encompassing hydrolysis and acidogenesis. The predominant anaerobic species associated with these processes belong to the phyla *Firmicutes* and *Bacteroides* as well as genera *Bacteroides, Clostridium, Butyrivibrio, Eubacterium, Bifidobacterium*, and *Lactobacillus* (Lim et al., 2020; Novaes, 1986). Notably, Bacillaceae, Lactobacillaceae, and Enterobacteriaceae are all found in the digested sludge, with Bacillaceae being the prevailing family (Novaes, 1986). During the hydrolysis phase, polymeric substrates, predominantly polysaccharides (including hemicellulose, cellulose, and starch), lipids, and proteins undergo hydrolysis. This process is facilitated by extracellular hydrolase enzymes secreted by hydrolytic bacteria. The enzymatic action leads to the breakdown of these polymeric substrates into monomers or oligomers. For instance, cellulose is transformed into glucose and cellobiose, starch yields glucose and maltose, hemicellulose releases xylose, proteins are broken down into amino acids, and lipids result in long-chain fatty acids (LCFA) and glycerol (Lim et al., 2020; Roopnarain et al., 2021). In general, hydrolytic bacteria have a rapid rate of growth and are less susceptible to temperature and pH variations in the environment (Sudiartha et al., 2023; Zhang et al., 2022).

In the acidogenesis phase, fermentative bacteria, including Clostridia, undergo a transformative process where hydrolysed products of proteins, such as peptides and amino acids, are converted into VFAs, CO_2, H_2, NH^{4+}, and S^{2-}. During this stage, the hydrolytic products undergo additional fermentation, leading to the production of short-chain fatty acids

(SCFA). The primary SCFA generated in this process include acetate, propionate, butyrate, valerate, and isobutyrate, synthesized by the acidogens. Acidogenesis takes place swiftly, and when readily digested feedstocks, like food wastes, overwhelm AD bioreactors, it can lead to the buildup of SCFA, and an abrupt pH drop then followed. The AD process may get disrupted or perhaps fail as a result of SCFA build-up (Lim et al., 2020).

The ultimate outcomes of metabolism are contingent upon the diverse array of substrates and prevailing environmental conditions. One critical factor in this regulation is the management of H_2 levels. Specifically, the thermodynamic favourability of producing key methanogenesis substrates like acetate, CO_2, and H_2 occurs only under conditions of low hydrogen partial pressure. Conversely, when the partial pressure of H_2 is elevated, the synthesis of propionate and various other organic acids takes place (Novaes, 1986).

2.5.2 Acetogenic Bacteria

Acetogenic bacteria carry out the metabolism of three-chained carbon (C3) or higher organic acids, ethanol, and specific aromatic compounds, converting them into acetate, H_2 and CO_2. When considering the anaerobic oxidation of these compounds in a pure culture of acetogenic bacteria, the thermodynamics are unfavourable, as indicated by positive changes in Gibbs free energy, as outlined in Table 2.2.

Table 2. 2 The anaerobic oxidation of propionate, butyrate, benzoate, and ethanol by hydrogen-producing acetogenic bacteria in pure cultures under standard circumstances results in free energy changes (Dolfing and Tiedje, 1988).

Reactions	**$\Delta G^{\circ'}$ (kJ/reaction)**
Propionate → acetate $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$	+ 76.1
Butyrate → acetate $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+ 48.1
Benzoate → acetate $C_7H_5CO_2^- + 7H_2O \rightarrow 3CH_3COO^- + 3H^+ + HCO_3^- + 3H_2$	+ 53
Ethanol → acetate $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+ 9.6

However, in a syntrophic relationship within a co-culture involving hydrogen-producing acetogenic bacteria and hydrogenotrophic methanogens, a notable synergy emerges. The process of hydrogen production and consumption are important event occurs in AD process (Hungate, 1967). Hydrogenotrophic methanogenic bacteria efficiently consume hydrogen, maintaining an exceptionally low level of hydrogen partial pressure (Harirchi et al., 2022; Khanal, 2008; O-Thong et al., 2020). This creates a thermodynamically favorable environment for hydrogen-producing acetogenic bacteria, enabling them to break down the mentioned organic compounds into acetate, H_2, and CO_2, as evidenced by negative changes in Gibbs free energy (Table 2.3).

Table 2. 3 The anaerobic oxidation of propionate, butyrate, benzoate, and ethanol by hydrogen-producing acetogenic bacteria in coculture of hydrogen-producing methanogens (Dolfing and Tiedje, 1988).

	Δ G°'			
	Hydrogenotrophic Methanogen		**Hydrogenotrophic Sulfate-Reducing Bacteria**	
Substrate	Substrate (kJ/mol)	CH_4 (kJ/mol)	Substrate (kJ/mol)	CH_4 (kJ/mol)
Propionate	- 25.6	-34.1	-37.8	-50.4
Butyrate	-19.7	-39.4	-27.9	-55.7
Benzoate	-10.7	-14.3	-22.9	-30.6
Ethanol	-58.2	-116.4	-66.4	-132.7

The conversion of propionic acid to acetate is only thermodynamically preferable when hydrogen partial pressures are less than 10^{-4} atm, and less than 10^{-3} and 1 atm for the oxidation of butyrate and ethanol, respectively. (Blonskaja et al., 2003; Malina and Pohland, 1992). It is noteworthy to mention that up to 30% of the electrons involved in the anaerobic treatment of complex wastes, particularly sewage sludge, are involved in the propionate oxidation process (McCarty and Smith, 1986). Propionate oxidation thus seems to be more important than oxidation of other organic acids and solvents (Khanal, 2008). Propionic acid buildup is significantly more severe in thermophilic environments than it is in mesophilic environments (Gannoun et al., 2009).

2.5.3 Homoacetogenic Bacteria

Homoacetogenic bacteria, or widely known as homoacetogens, are bacteria responsible for production of acetate as the primary precursor to produce CH_4 from AD process. Homoacetogens can be divided into autotrophs and heterotrophs. Autotrophic homoacetogens employ both H_2 and CO_2 for their metabolic processes, in which utilizing CO_2 as the primary carbon source for cellular synthesis (Khanal, 2008; Song et al., 2021). Some homoacetogens can alternatively utilize carbon monoxide as a carbon source. In contrast, heterotrophic homoacetogens utilize organic substrates such as formate and methanol, generating acetate as the final product (Kai and Volker, 2016; Song et al., 2021).

Among mesophilic homoacetogenic bacteria identified in sewage sludge, *Clostridium aceticum* and *Acetobacterium woodie* are notable examples (Novaes, 1986). Homoacetogens demonstrate a high thermodynamic efficiency, preventing the accumulation of H_2 and CO_2 during their growth on multicarbon compounds (Zeikus et al., 1982). There is a potential competition in craving available H_2 between homoacetogens and hydrogenotrophic methanogens as both bacteria had identical kinetic parameters of H_2 consumptions: specific growth rate of 0.4 – 1.9/day; growth yield of 0.35 – 0.85 g dry weight/g hydrogen; and substrate utilization rate of 1.4 mg H_2/g. min (Dolfing and Tiedje, 1988). This close competition was also confirmed through expression of Gibb's free energy changes in Eq. 2.1 and 2.2 (Khanal, 2008).

Hydrogenotrophic methanogens

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O,\ \Delta G^{\circ'} = -135.6\ \text{kJ/reaction} \quad (2.1)$$

Homoacetogenic bacteria

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O,\ \Delta G^{\circ'} = -104.6\ \text{kJ/reaction} \quad (2.2)$$

2.5.4 Methanogenic Bacteria

Methanogenic bacteria are no longer classified as bacteria but an archaea due to the unique properties such as the presence of membrane lipids, absence of peptidoglycan, and different ribosomal RNA compared to bacteria (Boone and De Brabander, 1993). These archaea, commonly referred to as methanogens, play a crucial role in the methanogenesis process. Methanogens can be classified into three groups based on their methanogenesis substrates and pathways: (a) acetotrophic (or acetoclastic) methanogens, which utilize acetate to

produce CH_4 through the acetoclastic pathway; (b) hydrogenotrophic methanogens, which use formate and H_2 to reduce CO_2 to CH_4 via the hydrogenotrophic pathway; and (c) methylotrophic methanogens, which generate CH_4 from methyl compounds such as methanol, methylamines, and methyl sulfides through the methylotrophic methanogenesis pathway (Anderson et al., 2009; Khanal, 2008).

Under certain circumstances, an alternate mechanism may generate methane. This pathway combines the conversion of H_2 and CO_2 to CH_4 by hydrogenotrophic methanogens with the syntrophic oxidation of acetate to H_2 and CO_2 by syntrophic acetate-oxidizing bacteria. In the majority of anaerobic digestion (AD) bioreactors, the pathway described is not the primary route for biogas production. This is because syntrophic acetate-oxidizing bacteria face stiff competition and are not as competitive as acetoclastic methanogens (Lim et al., 2020). Nevertheless, under specific conditions such as high ammonia concentration and fluctuations in operational temperature, inhibiting acetoclastic methanogens, primarily from the genera *Methanothrix, Methanosarcina*, and *Methanosaeta*, can make this pathway crucial for biogas production (Lim et al., 2020; Sudiartha et al., 2023). The CH_4 production process involves multiple groups of microorganisms, and it is essential to maintain a balanced microbial community to ensure a stable and robust AD process.

Cicerone and Oremland (1988) classified CH_4 production pathways in depth as shown in Table 2.4. Most methanogens can grow by using H_2 as a source of electrons via hydrogenase process as shown in Reaction (A) of Table 2.4 (Khanal, 2008). The hydrogenotrophic pathway plays a role in contributing up to 28% of CH_4 generation. It is noteworthy that numerous H_2-consuming methanogens have the capability to utilize formate as an electron source for reducing CO_2 to CH_4, as illustrated in Reaction (B). Some particular group of methanogens can even oxidize primary or secondary alcohols as part of the process of reducing CO_2 to CH_4. In contrast, the acetoclastic pathway is a dominant catabolic process, accounting for up to 72% of the total methane generation (Gujer and Zehnder, 1983). As illustrated in Reaction (C), in acetoclastic pathway, acetate is converted to CH_4. The two primary genera of acetoclastic methanogens are *Methanosarcina* and *Methanosaeta* (formerly identified as *Methanothrix).* Compared to *Methanosaeta* that only grow only on acetate, *Methanosarcina* can employ multiple substrates from different pathway to produce CH_4 such as methanol (Reaction (D)), methylamines (Reaction (E)), and in some cases shift to hydrogenotrophic pathway (Zabranska and Pokorna, 2018). *Methanosarcina* has a typical

doubling time of 1-2 days on acetate while *Methanosaeta*, on the other hand, has doubling time of 4-9 days (Monica and Zinder, 1988; Nie et al., 2021).

Table 2. 4 Substrates preferences for methanogenesis and energy yield by methanogens. (Cicerone and Oremland, 1988)

Reactions	**Δ G (kJ/mol)**
(A) $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-139
(B) $4HCOO^- + 2H^+ \rightarrow CH_4 + CO_2 + 2HCO_3^-$	-127
(C) $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-28
(D) $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-103
(E) $4CH_3NH_2 + 2H_2O + 4H^+ \rightarrow 3CH_4 + CO_2 + NH_4^+$	-102
(F) $(CH_3)_2S + H_2O \rightarrow 1.5CH_4 + 0.5CO_2 + H_2S$	-74

Given the longer decoupling time, when an anaerobic reactor operates with a short solids retention time (SRT), it is highly probable that *Methanosaeta* would be washed out from the system (Khanal, 2008). Consequently, *Methanosarcina* is likely to become the predominant genus in such conditions. Biokinetic parameters, including the half-velocity constant, yield coefficient, specific growth rate, and specific substrate utilization rate, serve as valuable indicators for assessing the prevalence of *Methanosaeta* and/or *Methanosarcina* in a system (Conklin et al., 2006). According to Conkin et al. (2006), the reported values for *Methanosaeta* tend to be lower than those observed for *Methanosarcina* across these parameters. This clearly demonstrates that, at lower substrate concentrations, *Methanosaeta* outcompetes *Methanosarcina*. *Methanosaeta* frequently predominates at low acetate concentrations of 100 to 150 mg COD/L (Kurade et al., 2019).

2.6 Environmental parameters governing AD

Anaerobic microorganisms, particularly methanogens, exhibit a high sensitivity to perturbations in environmental conditions. Given the considerable susceptibility and low growth rate of methanogens in anaerobic treatment systems, meticulous attention is required for the maintenance and monitoring of environmental conditions. Key factors influencing methanogenic activity include temperature, nutrient and trace mineral concentrations, pH levels, toxicity levels, and maintaining optimal redox conditions.

2.6.1 Temperature

Anaerobic processes exhibit a strong dependence on temperature. In an anaerobic system, three optimal temperature ranges for methanogenesis are recognized: psychrophilic, mesophilic, and thermophilic. Correspondingly, methanogens are categorized as psychrophiles, mesophiles, and thermophiles based on their preferred temperature range. Anaerobic conversion rates generally show an increase with temperature up to 60 °C (Malina and Pohland, 1992). The highest efficiency for anaerobic conversion is observed at 5 – 15 °C for psychrophiles, 35 – 40 °C for mesophiles, and around 55 °C for thermophiles, with decreased rates between these optimum temperature ranges (Lettinga et al., 2001; van Haandel and Lettinga, 1994).

AD systems are typically operated within three optimal temperature ranges: psychrophilic (less than 20 °C)(Fernández-Rodríguez et al., 2016), mesophilic (20–43 °C) with optimum temperatures approximately at 35–37 °C, and thermophilic (50–60 °C) (Madigou et al., 2019; Wang et al., 2018). In many commercial-scale AD systems, mesophilic and thermophilic temperatures are commonly employed, with maximum CH_4 production ranging from approximately 0.03–0.65 L/g volatile solid (VS)/day for mesophilic and 0.04–0.70 L/g-VS/day for thermophilic conditions (Kasinski, 2020; Nie et al., 2021). Despite extensive studies in these conventional temperature scenarios, operational challenges persist in AD processes. Notably, the temperature range between mesophilic and thermophilic (40–55 °C) is often overlooked in practice, and there is limited understanding of the microorganisms present in this range and their adaptability to both mesophilic and thermophilic conditions. Previous research in AD has primarily focused on biogas production under stable temperature conditions for each reactor to mitigate potential instability in microbial communities, treating the microbial aspect as a "black box" in the AD process (Hidaka et al., 2022; Roopnarain et al., 2021). Maintaining stable temperatures in the reactor during AD, particularly in large-scale wastewater treatment plants, may involve substantial energy consumption, leading to increased operating costs, especially in regions with distinct seasons (Sudiartha et al., 2022).

2.6.2 Operating pH and Alkalinity

The performance of anaerobic treatment is negatively impacted by even slight deviations in pH from the optimum range. Among the microorganisms within the microbial community,

methanogens exhibit higher susceptibility to pH variations (Kim and Whitman, 2014; O-Thong et al., 2020). Anaerobes can be categorized into two distinct pH groups: acidogens and methanogens. The optimum pH is 5.5–6.5 for acidogens and 7.8–8.2 for methanogens. The ideal pH for combined cultures falls within the range of 6.8 to 7.4, with a neutral pH being considered optimal. Given that methanogenesis is deemed the rate-limiting step, it is crucial to maintain the reactor pH close to neutral. Acidogens display significantly lower sensitivity to low or high pH values, and under such conditions, acid fermentation tends to dominate over methanogenesis. This dominance may lead to increased acid production in the content of the AD reactor (O-Thong et al., 2020; van Haandel and Lettinga, 1994).

According to Speece (1996), in acetoclastic pathway, methanogenic activity reached the highest activity level around pH of 6 – 8 and decreased significantly on pH 9. The substantial decrease in methanogenic activity observed at pH 8.0 and above could be attributed to a shift of NH_4^+ to its more toxic unionized form, NH_3 (Seagren et al., 1991). In the anaerobic treatment process, a decline in pH is frequently triggered by the buildup of VFAs and/or an excessive production of carbon dioxide (Khanal, 2008). As methanogens are sensitive to sudden deviations in pH away from the optimal value, it is essential for the anaerobic treatment system to have adequate buffering capacity (alkalinity) to counteract and alleviate pH changes. The pH within an anaerobic system, when operating within an acceptable range, is mainly regulated by self-produced alkalinity or natural alkalinity. Wastes that contain high levels of organic nitrogen are effective contributors to alkalinity. However, many carbohydrate-rich wastes may not contribute to alkalinity as they lack organic nitrogen (Khanal, 2008; O-Thong et al., 2020).

2.6.3 Nutrients

Similar to all biochemical operations, both macronutrients and micronutrients are essential for anaerobic processes to facilitate the synthesis of new biomass. The quantities of nitrogen and phosphorus needed for biomass synthesis can be determined by assuming the empirical formula for an anaerobic bacteria cell is $C_5H_7O_2N$ (Speece and McCarty, 1964). The cellular mass typically comprises about 12% nitrogen, indicating a requirement of approximately 12 g of nitrogen for every 100 g of anaerobic biomass produced, while the demand for phosphorus is approximately 1/7 to 1/5 of the nitrogen demand (Khanal, 2008). As a general rule, it is assumed that about 10% of the Chemical Oxygen Demand (COD) removed during an

anaerobic process is utilized for biomass synthesis, providing a basis for calculating the nitrogen and phosphorus needs (Li and Khanal, 2016). Another approach for determining macronutrient requirements is based on wastewater strength. The theoretical minimum COD/N/P ratios of 350:7:1 for highly loaded (0.8 – 1.2 kg COD/kg VSS.day) and 1000:7:1 for lightly loaded (<0.5 kg COD/kg VSS.day) anaerobic systems can be utilized to calculate the nitrogen and phosphorus needs (Henze et al., 2019; Henze and Harremoës, 1983).

2.6.4 Ammonia

Ammonia may be present in the influent or produced during the anaerobic degradation of organic nitrogenous compounds such as proteins or amino acids. Protein usually contains 16% nitrogen. Substrates with elevated nitrogen concentrations, such as agricultural livestock manure, have the potential to release ammonia during the AD process and leading to the severe inhibition of functional microorganisms, consequently, reduction in biogas production (Moestedt et al., 2017). The primary mechanisms of ammonia inhibition include the diffusion of free ammonia molecules into the cells of anaerobic microorganisms. This can result in potassium insufficiency, alterations in intracellular pH, proton imbalances, and the suppression of specific enzymatic reactions (Wang et al., 2016). These effects collectively contribute to the adverse impact of ammonia on the microbial activity essential for efficient anaerobic digestion.

It is widely acknowledged that methanogenic archaea are the functional category of microorganisms that is most affected by high ammonia levels among the other groups of microbes engaged in the AD process (Capson-Tojo et al., 2020). In particular, the presence of ammonia has a pronounced inhibitory effect on acetoclastic methanogens, which are more sensitive to ammonia compared to hydrogenotrophic methanogens (Westerholm et al., 2018). This inhibition disrupts the process of acetate conversion to methane, specifically through syntrophic acetate oxidation (SAO). Generally, total ammonia concentrations within a broad range of 1000 to 3000 mg/L can impede the activity of anaerobic digestion (AD) systems, with the inhibitory effect depending on pH levels with concentrations surpassing 3000 mg/L have been demonstrated to be toxic to the operation of AD systems, irrespective of the pH range (Gaspari et al., 2024; Rajagopal et al., 2013). An ammonia level of 3.3 g NH_4^+- N/L was found in a prior study to be associated with an impeded AD process, particularly for the phase of generating CH_4 (Rajagopal et al., 2013). Acetoclastic methanogens are able to

withstand quantities of free ammonia as high as 700 mg N/L by adaptation (Angelidaki and Ahring, 1994).

2.6.5 Short-Chain Fatty Acid (SCFA)

Anaerobic treatment system functionality is determined by the amount VFAs. The expression "volatile" implies that they are recoverable through atmospheric pressure distillation. Complex organic matter is hydrolyzed and fermented into low-molecular-weight molecules, such as SCFA, during anaerobic digestion (Khanal, 2008; Li and Khanal, 2016). SCFA, which include acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic acid, are short-chain (C2–C6) organic acids. These acids function as a carboxylate platform, providing building blocks for use in the chemical industry (Llamas et al., 2020).

The SCFA content in the effluent of a healthy anaerobic system is typically in the range of 50–250 mg HAc/L (Sawyer, 2003). When the syntrophic relationship between acidogens and methanogens deteriorates, SCFA accumulate. The inhibition of methanogens, whether due to toxicity, changes in environmental factors, or nutrient deficiencies, leads to the accumulation of acetate and hydrogen. Elevated hydrogen partial pressure can severely inhibit syntrophic propionate-consuming bacteria, ultimately causing the buildup of propionate (Khanal, 2008; Li and Khanal, 2016; Lim et al., 2020). This breakdown in the syntrophic balance can disrupt the normal flow of anaerobic digestion processes and result in the accumulation of propionate.

A prior study indicates that concentrations of acetate and butyrate at 2400 and 1800 mg/L, respectively, did not cause significant inhibition of methanogenic activity (Wang et al., 2009). However, a propionate concentration of 900 mg/L led to a notable inhibition of methanogens. These findings highlight the varying sensitivities of methanogens to SCFAs, with propionate being more inhibitory compared to acetate and butyrate at the specified concentrations.

2.7 Next-generation sequencing (NGS)

The landscape of DNA sequencing has evolved considerably from the days of two-dimensional chromatography in the 1970s. The breakthrough of the Sanger chain termination method in 1977 brought about a reliable and reproducible approach to DNA sequencing

(Sanger et al., 1977b) (Figure 2.5). Subsequently, Applied Biosystems introduced the initial automated, capillary electrophoresis (CE)-based sequencing instruments—the AB370 in 1987 and the AB3730xl in 1998—which played pivotal roles in the NIH-led and Celera-led Human Genome Projects (Collins et al., 2003). Although considered high throughput in their time, these "first-generation" instruments were surpassed by the Genome Analyzer in 2005, elevating sequencing runs from 84 kilobase (kb) per run to 1 gigabase (Gb) per run.

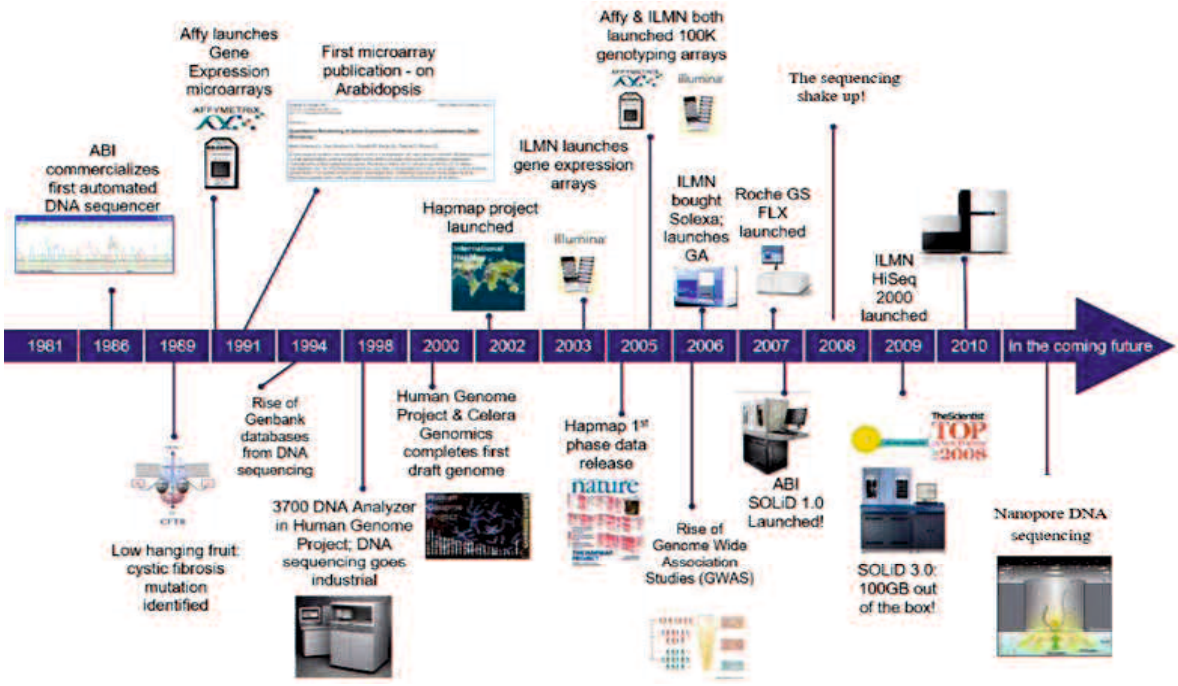

Figure 2. 5 History of NGS (CRG, 2020)

NGS platforms generate large quantities of DNA reads—ranging from millions to billions—with read lengths typically between 25 and 400 base pairs (bp). These reads are shorter than those produced by the traditional Sanger sequencing method, which ranges from 300 to 750 bp. However, recent advancements in NGS technology have enabled the production of DNA reads exceeding 750 bp. The development of NGS traces back to the discovery of the DNA double helix structure in 1953 by James Watson, an American geneticist, and Francis Crick, a British physicist (WATSON and CRICK, 1953). The first nucleic acid sequencing was achieved by American biochemist Robert Holley and his colleagues in 1964 and 1965, who developed sequencing methods for tRNA (Holley et al., 1964). In 1977, independent methods for sequencing longer DNA were successfully developed by British biochemist Frederick Sanger and American biochemist/physicist Walter Gilbert (Maxam and Gilbert, 1977; Sanger et al., 1977a).

In 2000, Lynx Therapeutics (USA) introduced the first next-generation sequencing (NGS) technology with the launch of Massively Parallel Signature Sequencing (MPSS). This company was later acquired by Illumina. In 2004, 454 Life Sciences (Branford, CT, USA) introduced a parallel version of pyrosequencing, marking the second generation of sequencing technologies following MPSS. The initial version of their machine reduced sequencing costs by sixfold compared to automated Sanger sequencing. 454 Life Sciences was subsequently acquired by Roche, headquartered in Basel, Switzerland. Pyrosequencing offers intermediate read lengths and costs per base, positioned between Sanger sequencing and the technologies developed by Illumina and SOLiD. In 2005–2006, Roche introduced the 454 GS 20 sequencing platform, which revolutionized DNA sequencing by producing 20 million bases (20 Mbp). This platform was succeeded by the GS FLX model in 2007, capable of producing over 100 Mbp of sequence data in just four hours, which increased to 400 Mbp in 2008. The GS FLX model was later upgraded to the 454 GS−FLX+ Titanium sequencing platform, capable of generating over 600 Mbp of sequence data in a single run, with Sanger-like read lengths of up to 1,000 bp. Roche also produced the GS Junior, a compact, benchtop platform that delivers long sequencing reads of 400 bp with rapid sequencing runs (Barba et al., 2014).

In 2005, Solexa launched the Genome Analyzer (GA), utilizing sequencing by synthesis (SBS) technology based on reversible dye-terminator chemistry. Solexa was acquired by Illumina in 2007. The GAIIx platform from Illumina generates up to 50 billion bases (50 Bbp) of usable data per run, with the latest models achieving up to 85 Bbp per run. Over the last 3–4 years, Illumina has developed the HiSeq platform series, including HiSeq® 2500, HiSeq 2000, HiSeq 1500, and HiSeq 1000 sequencing platforms. These platforms vary in output, run time, cluster generation, paired-end reads, and maximum read lengths, with the HiSeq 2500 offering the longest read length of 200 bp and the HiSeq 1000 the shortest. The HiSeq 2500 platform can sequence a human genome in approximately 24 hours, often referred to as "Genome in a day." It can also sequence 20 exomes in a day or 30 RNA samples in as little as five hours, generating 120 billion bases (120 Bbp) of data in 27 hours. The standard HiSeq 2000 produces 600 billion bases (600 Bbp) per run, and the HiSeq 2500 can achieve outputs of up to 600 Gbp. In 2011, Illumina also released the MiSeq, a benchtop platform that shares many technologies with the HiSeq series and generates 1.5 Gbp per run in approximately 10 hours (Illumina, 2017).

According to Illumina (2017) the underlying principle of Next-Generation Sequencing (NGS) technology shares similarities with CE sequencing. DNA polymerase facilitates the integration of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand through successive cycles of DNA synthesis. In each cycle, the fluorophore excitation identifies the nucleotides at the incorporation point. The crucial distinction lies in NGS's ability to extend this process concurrently across millions of fragments in a massively parallel manner, diverging from the single DNA fragment sequencing of CE. Illumina sequencing by synthesis (SBS) chemistry, contributing to over 90% of the world's sequencing data, is prominent in NGS (Illumina, 2017). This method ensures high accuracy, a substantial yield of error-free reads, and a significant proportion of base calls surpassing Q30 (Bentley et al., 2008; Nakazato et al., 2013; Ross et al., 2013). Illumina NGS workflows consisted of four fundamental stages:

1. **Library Preparation** → The sequencing library is prepared by random fragmentation of the DNA or cDNA sample, followed by 5' and 3' adapter ligation (Figure 2.5 (A)). Adapter-ligated fragments are then PCR amplified and gel purified.
2. **Cluster Generation** → For cluster generation, the library is loaded into a flow cell where fragments are captured on a lawn of surface-bound oligos complimentary to the library adapters. Each fragment is then amplified into distinct, clonal clusters through bridge amplification (Figure 2.5 (B)). When cluster generation is complete, the templates are ready for sequencing.
3. **Sequencing** → Illumina SBS technology uses a proprietary reversible terminator-based method that detects single bases as they are incorporated into DNA template strands (Figure 2.5 (C)).
4. **Data analysis** → During data analysis and alignment the newly identified sequence reads are aligned to a reference genome (Figure 2.5 (D)). Following alignment, many variations of analysis are possible, such as single nucleotide polymorphisms (SNP) or insertion-deletion identification, read counting for RNA methods, phylogenetic or metagenomic analysis.

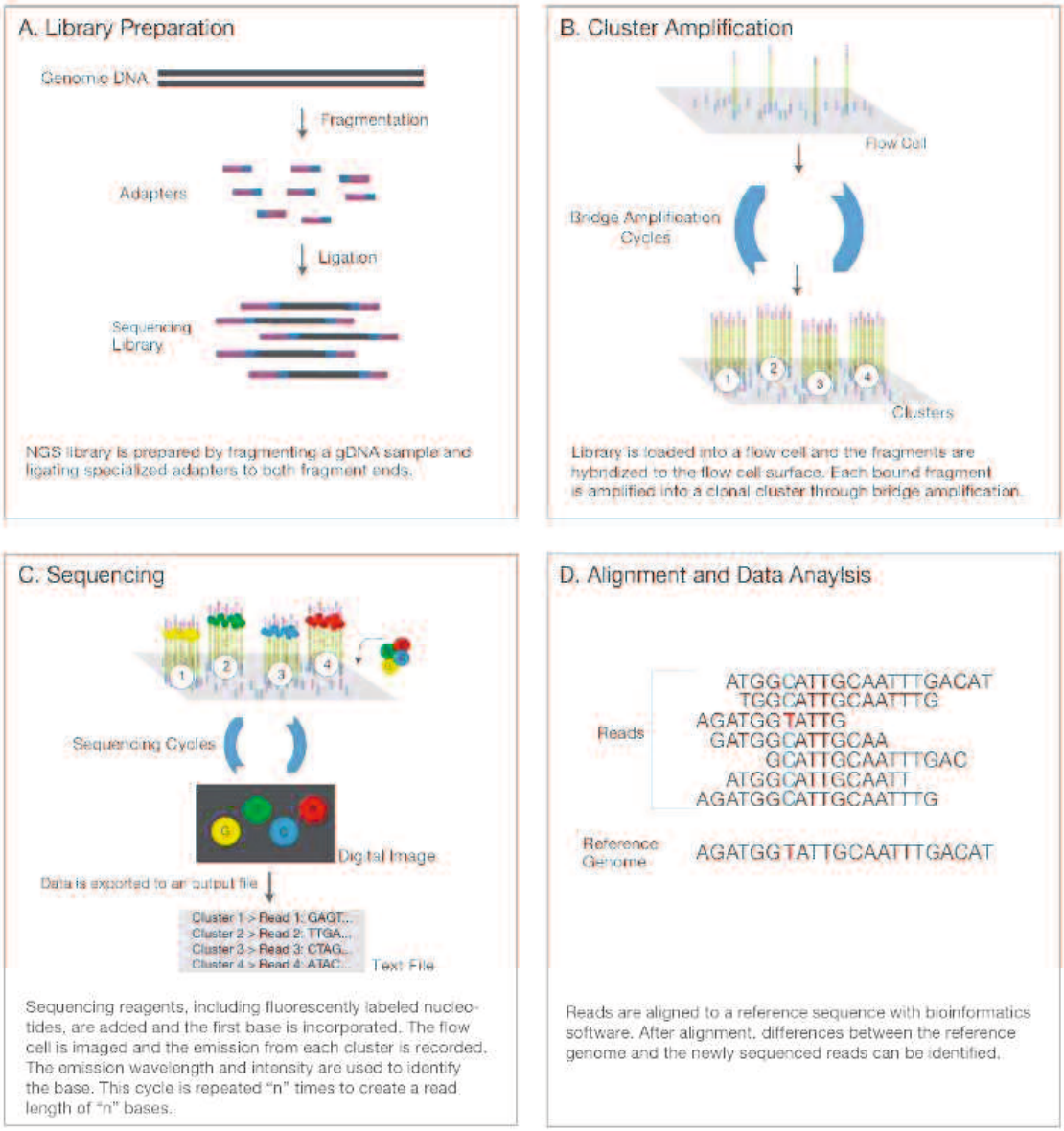

Figure 2. 6 Illumina Next-Generation Sequencing workflows overview including (A) library preparation, (B) cluster generation, (C) sequencing, and (D) alignment and data analysis.

2.8 Metagenomic analysis on AD

Metagenomic analysis can be a useful tool for examining the activity of the entire microbiome during AD since the process of AD resembles to the gut digestion process, which involves a diverse range of microbial communities. Historically, microbe identification in anaerobic digestion (AD) relied on inoculation and cultivation techniques. Subsequently, more advanced molecular biology methods, such as DGGE, PCR, cloning on marker genes, molecular fingerprinting, and qPCR, were employed to delve deeper into understanding microbial communities in AD (Lim et al., 2020). While these molecular biology techniques

paved the way for insights, they yielded relatively rudimentary findings compared to recent advancements. The substantial progress in comprehending the AD process in recent years is attributed to the emergence of multi-omic techniques and analyses, notably metagenomic analysis (Zhang et al., 2017, 2019).

The standard metagenomics workflow is depicted in Figure 2.6. Metagenomic study generally begins with collecting samples (Step 1) and extracting environmental DNA (Step 2). This DNA is then either directly sequenced using next-generation sequencing (NGS) (Step 3) or cloned into a host to create a metagenomic library (Step 3′). The metagenomic library undergoes further analysis through genetic/functional screening such as PCR amplification, hybridization, or metabolite degradation (Step 4′). Upon PCR amplification, selected DNA is then sequenced by NGS (Step 5′), and the results are analysed in the same manner as bioinformatic analysis of direct sequencing data. In direct sequencing (Step 3), many reads are generated, which are then assembled into contigs in the assembly stage. Taxonomic binning can be applied either before or after assembly to sort reads into taxonomic bins, thereby reducing the complexity of metagenome assemblies.

Subsequently, genes within contigs are predicted and annotated using annotation service (software or web servers). Functions are then assigned to annotated genes, and metabolic pathways and microbial interactions may be inferred or modelled from the obtained data. Besides functional analysis, the taxonomic composition of a microbial community can be determined through similarity-based classification of 16S rRNA gene fragments obtained from the metagenome, or through composition-based classification of the metagenomic sequences. Tools used for taxonomic similarity-based classification are also applicable for analyzing 16S rRNA PCR amplicons sequenced by NGS (another “metagenomic” method not illustrated in this flow chart). Orange rectangles highlight the main steps of a metagenomic study, dashed frames indicate optional steps and green solid rectangles represent bioinformatics analysis steps and genetic/functional screening methods.

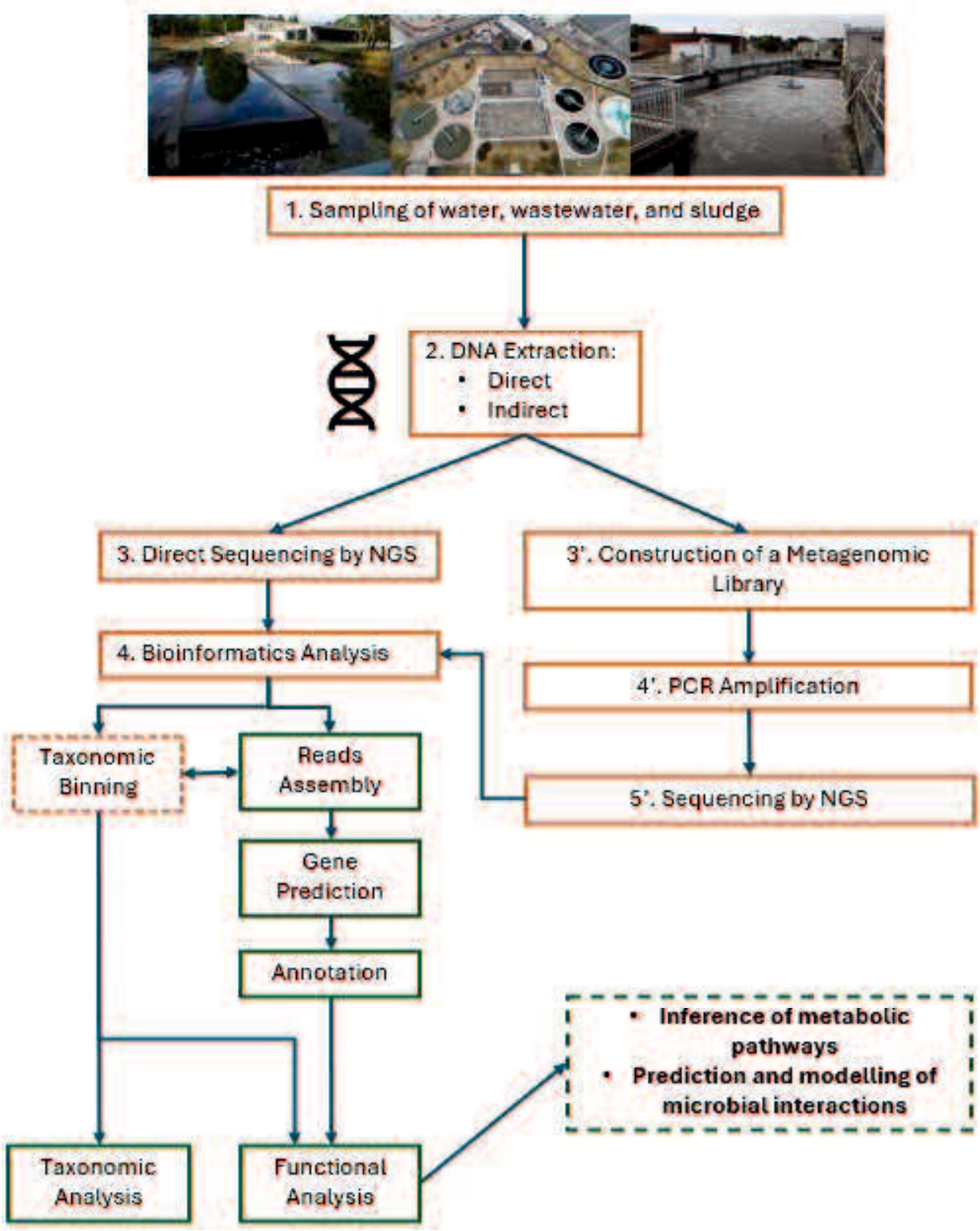

Figure 2. 7 Flow chart of a standard sequencing and metagenomics methodology. Reconstructed from previous study (Bouhajja et al., 2016).

Metagenomics involves the examination of genomic data retrieved from environmental samples, offering predictions about all the genes present within one or more genomes. There

are several programs (commonly referred to as pipelines) that can be used to perform metagenomic functional annotations, as follows:

1. *PROKKA*

   PROKKA is a rapid annotation tool designed for prokaryotic genomes (Seemann, 2014). It annotates FASTA sequences, providing concise annotations that include the name and, in some cases, a brief description of the protein. Unidentified proteins are labeled as 'hypothetical protein'. The genomic annotation involves several bioinformatic tools independent of PROKKA, including Prodigal for coding sequences (Hyatt et al., 2010), RNAmmer for ribosomal RNA genes (Lagesen et al., 2007), Aragorn for transfer RNA genes (Laslett and Canback, 2004), SignalP for signal leader peptides (Teufel et al., 2022), and Infernal for non-coding RNA (Nawrocki and Eddy, 2013). Prodigal identifies feature coordinates, and subsequent tools predict the function based on these coordinates. Typically, protein function prediction involves comparing genomic sequences with extensive databases. In PROKKA, this comparative approach utilizes a hierarchical system where sequences are first compared with a small, reliable database, followed by larger ones. PROKKA generates annotations in 10 different formats, facilitating further analysis.

2. *EggNOG-mapper*

   EggNOG-mapper, as introduced by Cantalapiedra et al. (2021), functions as a tool for functional annotation, relying on the KEGG Orthology (Kanehisa et al., 2016) and the KEGG database (Cantalapiedra et al., 2021). In contrast to NCBI BLAST+ (Camacho et al., 2009) and InterProScan (Camacho et al., 2009), which use homologs for comparison, EggNOG-mapper predicts protein functions by comparing against orthologs. Orthologs are more likely to maintain their functions post-speciation events, enhancing the accuracy of functional annotations provided by EggNOG. Predictions of orthology are made at various taxonomic levels, offering a broader scope for annotating genes within genomes. When predictions are at lower taxonomic levels (closer to species-level), the function can be more specifically targeted to the genome. EggNOG-mapper provides more extensive annotations for Open Reading Frames (ORFs) compared to PROKKA. Each ORF receives a functional Clusters of Orthologous Groups (COG), a KEGG number, taxonomic origin, and often a name along with a free

description. The free text description is generated using a text mining machine learning pipeline (Huerta-Cepas et al., 2019). Functional COGs, known as orthologous groups, consist of approximately three homologous sequences that have evolved divergently from a single speciation event. This aligns with the theory that orthologs are more likely to retain their function over extended evolutionary distances. EggNOG-mapper underwent benchmarking during development to ensure its performance compared favorably with similar existing tools. The tool's Gene Ontology (GO) predictions have been compared against NCBI BLAST+ and InterProScan, which provide homology-based predictions of function, making them suitable for benchmarking (Huerta-Cepas et al., 2017). As with many bioinformatic tools, EggNOG is continuously evolving and improving, with a current focus on enhancing the analysis of metagenomic sequences.

3. *KofamScan*

   KofamScan is a command line tool that, similar to EggNOG, serves as a functional annotation tool (Aramaki et al., 2020). It predicts functions by comparing nucleotide sequences against the KEGG Orthology database. The tool offers information such as an E-value, a KEGG number, and a protein description in its annotation, although it may lack some details compared to EggNOG. However, KofamScan provides an option, using a command line flag, to generate an output file directly compatible with KEGG-Decoder. This facilitates the generation of a heatmap for visualizing KofamScan outputs. KofamScan operates as a homology-based search tool, akin to BLAST+ and GHOSTX, differing from other bioinformatic tools developed by Kanehisa et al., such as GhostKOALA and blastKOALA (Kanehisa et al., 2016). In contrast to GhostKOALA and BlastKOALA, KofamScan compares the predicted KEGG numbers against a database of profile hidden Markov models (pHMM). This distinctive feature adds a layer of specificity and differentiation to its functional predictions.

4. *NCBI Blast*

   NCBI Protein Blast+ is often the research standard for protein and microorganism identification, as it is routinely used as a benchmark for other homologybased tools such as KofamScan (Camacho et al., 2009). Blast+ is a homology based search tool that compares query amino acid sequences to the BLAST database, by aligning query sequences to reference sequences from the database. An E-value

(expected value) is then given to each matching sequence by calculating the number of matches that are expected to be found within the database (Camacho et al., 2009). An NCBI accession number is given to each matching sequence to provide a summarised report about each protein from its individual function, to the exact position in the plasmid/chromosome of the query sequences.

5. *PICRUSt2*

   PICRUSt was established in 2013 to forecast the microbial community's functional potential based on marker gene sequencing patterns (Langille et al., 2013). Since 2020, PICRUSt2 has been developed to enhance the primary functional annotation features (Douglas et al., 2020). In particular, PICRUSt2 enables interoperability with any operational taxonomic unit (OTU)-picking or denoising technique, has a more recent and expanded library of gene families and reference genomes, and permits phenotype predictions. Profiling bacterial populations is most commonly achieved by sequencing the conserved 16S rRNA gene. Because strain heterogeneity makes it impossible to directly identify functional profiles from 16S rRNA gene sequence data, a number of techniques have been developed to predict the functions of microbial communities based only on taxonomic profiles (amplicon sequences) (Aßhauer et al., 2015; Douglas et al., 2020; Iwai et al., 2016). The 41,926 bacterial and archaeal genomes from the IMG database (8 November 2017) serve as the foundation for the PICRUSt2 default genome database (Markowitz et al., 2012). This represents a >20-fold increase over the 2,011 IMG genomes used by PICRUSt1. Numerous more genomes come from strains within the same species and share the same 16S rRNA genes.

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CHAPTER III
METAGENOMICS ANALYSIS OF THE EFFECT OF TEMPERATURE SHIFT IN ANAEROBIC DIGESTION: UPSHIFTED AND DOWNSHIFTED SCENARIO

3.1 Introduction

The two critical issues with global sustainable development are the escalating energy crisis and environmental degradation. Anaerobic digestion (AD) is a successful strategy that can produce bioenergy and bio-fertilizer from a wide array of residual biomass (such as sewage sludge, anaerobic sludge, agriculture waste, and food waste) to tackle those issues. A few examples of products that can be recovered through this process are volatile fatty acids (VFAs) and biogas, which typically consist of methane (CH_4) (50–70%), carbon dioxide (CO_2) (30–50%), and other compounds, such as hydrogen sulfide, and ammonia (Porté et al., 2019). It is also cost-effective and environmentally friendly for waste disposal, requiring less energy input or carbon footprint than landfilling and incineration, which both significantly aided in achieving carbon neutrality (Ma and Liu, 2019; Xu et al., 2021). In general, CH_4 synthesis from AD often relies on the involvement of physiologicaly various microorganisms involved in hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Wu et al., 2022). In order to generate methane, acetate, H_2, and CO_2 are the primary components, with two main methanogenic pathways: the acetoclastic and the hydrogenotrophic pathways (Tian et al., 2018b). Acetoclastic methanogens utilize acetate, while hydrogenotrophic methanogens use H_2 and CO_2 to produce CH_4. The acetoclastic pathway is strictly regulated by *Methanosarcinaceae spp.* and *Methanosaetaceae spp.* In contrast, the hydrogenotrophic pathway is mainly driven by *Methanomicrobiales spp.*, *Methanobacteriales spp*., *Methanococcales spp.*, *Methanopyrales spp.*, and *Methanocellales spp.* (Kurade et al., 2019; Sudiartha et al., 2022).

The AD process is vulnerable to perturbation from variations in operational or environmental parameters, such as pH, temperature, organic loading rate (OLR), and hydraulic retention time (HRT), which could disturb the balance of interaction among AD microorganisms and lead to poor reactor performance (Sudiartha and Imai, 2022; Wu et al., 2022). Among those parameters, the temperature is an essential parameter that heavily

influences the metabolic activity of the microbial community involved in methanogenesis and subsequently determines the stability of the community (Nie et al., 2021). According to Madigou et al.(Madigou et al., 2019), temperature changes can have a substantial impact on microbial populations and the performance of AD. Previous studies have reported that the different temperature levels inflicted the population imbalance between acidogenic and methanogenic communities, which led to the instability of the output of the AD process (Hupfauf et al., 2018; Kovalovszki et al., 2020). Beale et al. (Beale et al., 2016) experienced a drop in biogas production due to significant microbial competition after increasing the temperature from 32 °C to 45 °C. Chen et al. (Chen and Chang, 2020) demonstrated the different behavior of microbial communities in different methanogenesis phases. The researchers found that the microbial communities involved in hydrolysis were more susceptible to temperature conditions. In contrast, the microbial communities showed more stability in the rest of the AD process. However, despite the known influence of temperature and the breadth of study on the topic, it is still unclear how dynamic temperature changes affect the AD process, especially regarding the temperature adaptability (thermotolerance) of microorganisms involved in the AD process.

The AD system is typically operated at three optimal temperature ranges: psychrophilic (20 °C) (Fernández-Rodríguez et al., 2016), mesophilic (20–43 °C), with optimum temperatures around 35–37 °C (Wang et al., 2018), and thermophilic (50–60 °C) (Madigou et al., 2019; Wang et al., 2018). Most commercial-scale AD systems frequently employ mesophilic and thermophilic temperatures with maximum CH_4 production varying around 0.03-0.65 L/g volatile solid (VS)/day and 0.04-0.70 L/g-VS/day, respectively (Kasinski, 2020; Nie et al., 2021). However, despite the extensive studies in these conventional temperature scenarios, there are still many challenges regarding operational practices in these temperature AD processes. For example, there is little consideration given to the range of temperatures between mesophilic and thermophilic (40-55 °C) in practice, and limited understanding of the microorganisms present in this range and their ability to adapt to both mesophilic and thermophilic conditions. Furthermore, previous research in AD primarily focused on biogas production under stable temperature conditions for each reactor (with pre-treatment conditions (Hidaka et al., 2022)) to reduce the potential of instability in microbial communities which was the “black box” for the AD process (Roopnarain et al., 2021). In some cases, preserving stable temperatures in the reactor during AD would involve substantial energy in a large-scale wastewater treatment plant (WWTP), increasing operating costs, particularly in four-seasoned nations (Sudiartha et al., 2022).

To date, few researchers worked on temperature-adjusted AD (Westerholm et al., 2018). However, the effect of each temperature change on biogas production and the dynamics of methanogenesis-related bacteria have not been presented. Furthermore, to our knowledge, the full extent of microbial populations and their interactions in AD has not been fully explored due to the complexity of microbial networking. Improving our understanding of the essential ecological niches in this process and the metabolic characteristics of the microbial populations that inhabit them could aid in developing novel process designs and operational techniques for enhancing the recovery of CH_4 from organic waste. This issue served as the impetus for the current work, which concentrated on investigating the fluctuation of biogas production and understanding the interconnection between microbial communities under several temperature shifts while at the same time designing active microbial communities by adjusting the operating temperature.

Therefore, the main objectives of this study were to determine the impact of two temperature shift scenarios (upshifted and downshifted) on biogas production and to demonstrate their effects on microbial compositions. Results led to a broader understanding of the behavior of microorganisms during temperature changes in methanogenesis.

3.2 Materials and methods

3.2.1 Inoculum and Substrates

Anaerobically digested sludge from the Eastern Ube Wastewater Treatment Plant, Ube City, Yamaguchi Prefecture, Japan, was used as the inoculum source. The properties of the inoculum are presented in Table 3.1. This study used a glucose-based substrate containing 100 g/L glucose as the carbon source for biogas generation. The substrate solution was added to the inoculum samples before they were incubated and cultured at upshifted and downshifted temperatures. The glucose was mixed with the following essential nutrients required for bacterial growth (all in g/L): 4 $NaHCO_3$, 4 K_2HPO_4, 0.1 yeast extract, 85 NH_4Cl, 81 $MgCl_2{\cdot}6H_2O$, 75 KCl, 350 $(NH_4)_2HPO_4$, 42 $FeCl_3{\cdot}6H_2O$, 25 $MgSO_4{\cdot}7H_2O$, 1.8 $CoCl_2{\cdot}6H_2O$, and 150 $CaCl_2{\cdot}2H_2O$ (Zhou et al., 2006).

Table 3. 1 Characteristics of anaerobically digested sludge as inoculum

Parameters	Anaerobic Sludge	Units
pH	8.17	-
Total Solid (TS)	8	% w/v
Volatile Solid (VS)	3	% w/v
Fixed Solid (FS)	5	% w/v
VS/TS ratio	0.6	-

3.2.2 Experimental Procedures

Serum vials with a capacity of 160 ml with a 72 ml of working volume, were used in a fed-batch mode to produce methane (Sudiartha and Imai, 2022). First, the serum bottles were added with 2 ml substrate and 70 ml sludge. Then, they were flushed with nitrogen gas to remove any remaining oxygen to create anaerobic conditions and covered with aluminum covers and butyl rubber stoppers. This study was subsequently divided into two scenarios, i.e., a) upshifted and b) downshifted temperature scenarios with various conditions. For the upshifted temperature scenario, the serum vial was initially incubated at 42 °C with shaking at 50 rpm in a shaking incubator (BT 101 and BT 300; Yamato Scientific Co., Ltd. Japan) for self-fermentation to allow microbial communities to acclimatize appropriately and eliminate recalcitrant organic contaminants that had remained during the transition from the wastewater treatment plant (WWTP) to the laboratory (Sakaveli et al., 2021) for 1 month.For the downshifted temperature scenario, the self-fermentation was conducted at 55 °C.

Upon self-fermentation process, 2 ml of glucose-based substrate solution with a glucose concentration of 100 g/L was regularly injected into the vial everytime biogas production showed a considerable decline for both temperature shifts scenario. Finally, the serum vials were treated in three sets of temperature conditions. For the upshifted scenario, the temperature was increased from 42 °C to 45 °C and finally to 48 °C. With the similar pattern, the downshifted scenario initially incubated at 55 °C and then the temperature decreased to 48 °C and finally to 45 °C. The datasets, such as biogas production and microbial communities, from each temperature condition was labelled as described in Table 3.2. For each temperature shift, the incubation period was 100 days.

Table 3. 2 Labels for each datasets obtained from temperature shifts

Conditions	Labels	Scenario
42 °C	U42	Upshifted
42 °C ⟶ 45 °C	U45	Upshifted
45 °C ⟶ 48 °C	U48	Upshifted
55 °C	D55	Downshifted
55 °C ⟶ 48 °C	D48	Downshifted
48 °C ⟶ 45 °C	D45	Downshifted

3.2.3 Data collection and analysis

The total gas volume and composition were monitored regularly during the incubation period using gas chromatography. The volume of biogas generated was measured using a glass syringe. Gas chromatography (GC-8APT/TCD; Shimadzu Corp. Japan) with a 60/80 activated charcoal mesh column (1.5 m x 3.0 mm internal diameter) and argon as the carrier gas was used to analyse the gas composition of the samples, such as H_2, N_2, CH_4, and CO_2. During operation, the injector, column, and detector temperatures were set to 50 °C, 60 °C, and 50 °C, respectively. The biogas production was standardized to STP conditions (273.15 K, 101.325 Kpa).

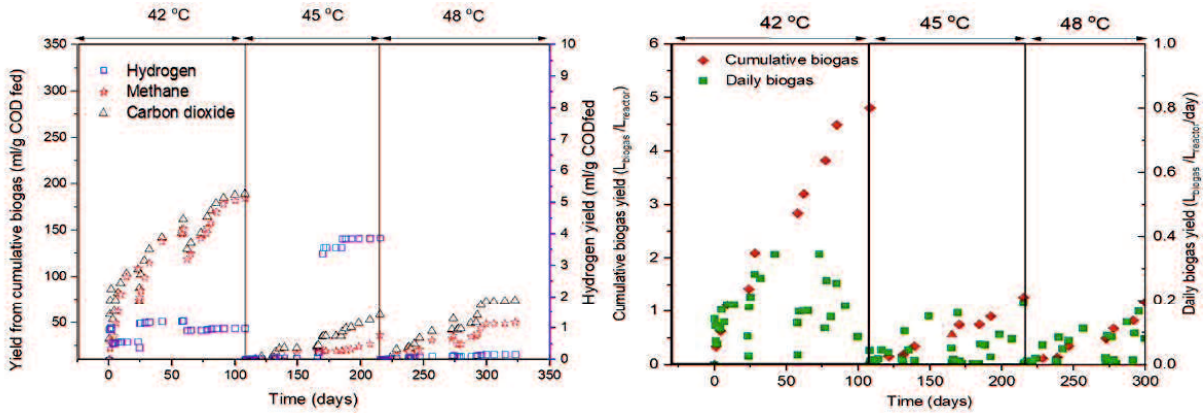
3.2.4 NGS and Microbial Diversity Analysis

As much as 1.5 ml of DNA samples were taken following the shifts in temperature. DNA was isolated using the NucleoSpin® kit according to the NucleoSpin® Soil Manual. After extraction, the DNA samples were sent to the Faculty of Medicine, Yamaguchi University, Japan, for 16S rRNA using NGS analysis. Notably, using these approaches, the genome (all 3 billion base pairs), all coding genes (exome; 1% of the genome or 30 million base pairs—that is, 20,000 genes made of 180,000 exons), all RNA produced from genes (transcriptome), and any subset of these can be sequenced. Methanogen diversity analysis was performed to determine any changes in methanogen populations during AD under shifted temperature conditions using the Shannon diversity index, Simpson's index and Inverse Simpson's Index with R studio. Statistical significance was determined using Principal Component Analysis (PCA) with correlation matrix analysis. Heatmap were analysed using MeV 4.9.0. The other data analysis was accomplished using Origin Pro 2022.

3.3 Results

3.3.1 Biogas Production under Temperature Shifts

The cumulative biogas production during the temperature shift in AD is shown in Figure 3.1. Each vial was incubated for 100 days, as the AD process to convert sewage sludge into biogas involves several metabolic pathways, including hydrolysis, acetogenesis, acidogenesis, and methanogenesis. Previous research has proposed incubation periods of 21 days (Ángeles et al., 2021; Tetteh and Rathilal, 2022; Zhao et al., 2021). However, in the case of temperature shift conditions, a more extended operating period is preferable, allowing microorganisms to acclimatize to harsh conditions and achieve more stable operation (Moestedt et al., 2014; Sun et al., 2015; Westerholm et al., 2015). After start-up, in the upshifted temperature scenario, the performance of the vial at 42 °C showed better biogas yield compared to those after upshifted to 45 °C and 48 °C. The vial yielded 184 mL CH_4/g COD and 189 mL CO_2/g COD. There was a roughly 77% decline in CO_2 and 83% in CH_4 production after the temperature shifted from 42 °C to 45 °C. As seen in Figure 3.1 (a), the cumulative biogas yield at 45 °C decreased to 36 mL CH_4/g COD and 58 mL CO_2/g COD at the end of the incubation period. The decline in biogas production is also confirmed by the decline in cumulative biogas yield from producing 4.8 L_{biogas}/L_{vial} at 42 °C to 1.26 L_{biogas}/L_{vial} at 45 °C presented in Figure 3.1(b). However, there was no significant difference in CH_4 and CO_2 production after the increased temperature to 48 °C. CH_4 production showed a slight increase of 33% to 50 mL CH_4/g COD, while CO_2 emissions increased to 74 mL CH_4/g COD in U48 during the 100 days incubation period.



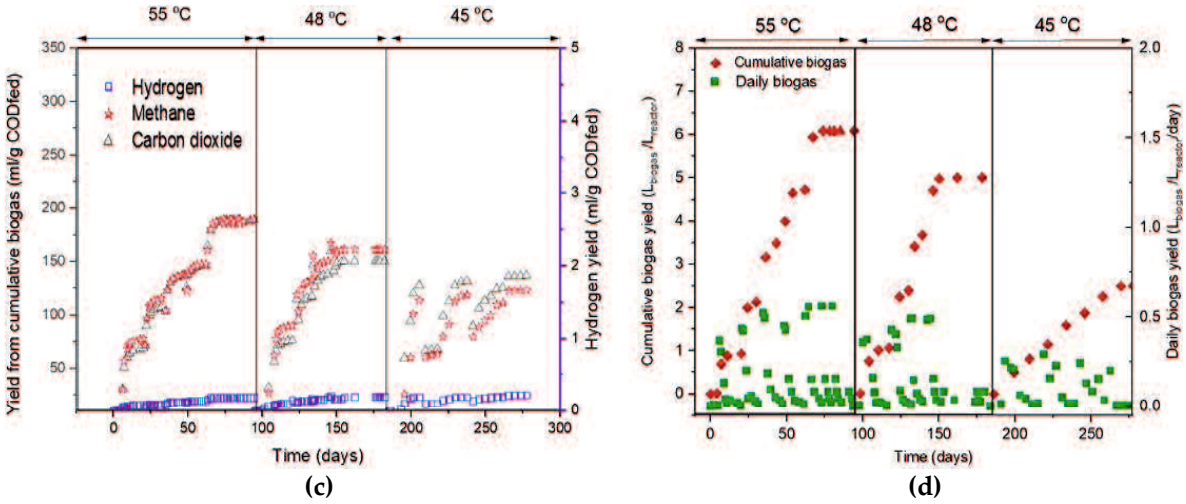


Figure 3. 1 Biogas production in response to temperature shift conditions: (a) CH_4, CO_2, H_2 yield with (b) volumetric biogas yield in the upshifted temperature, and (c) CH_4, CO_2, H_2 yield with (d) volumetric biogas yield in the downshifted temperature.

In contrast, the downshifted temperature scenario showed a more stable biogas production in the first temperature shift. As shown in Figure 3.1 (c), the vial produces 190 mL CH_4/g COD and 189 mL CO_2/g COD at 55 °C, which after temperature shift to 48 °C, the biogas production decreases 16% to the level of 160 mL CH_4/g COD and 150 mL CO_2/g COD Despite the lower biogas production, incubation at 48 °C yields more CH_4 than CO_2, indicating potential CH_4 production from hydrogenotrophic pathways where the methanogens convert CO_2 to CH_4 by using H_2 as an electron donor. However, after further temperature downshift to 45 °C, biogas production outpaced the performances at 48 °C and 55 °C. When incubated at 45 °C, the vial generated 123 mL CH_4/g COD and 136 mL CO_2/g COD, 30% lower than that produced on 55 °C and 48 °C vials. The downturn of the biogas production at 45 °C was also confirmed by the diminishing volume of biogas yield per vial volume, as shown in Figure 3.1(d).

Figure 3.2 confirmed the inhibition in methanogenesis process occurred in upshifted temperature scenarios after the temperature leaving 42 °C. Meanwhile the downshifted temperature scenario showed a rather stable biogas production rate at 55 °C and 48 °C, yet started to deteriorate at 45 °C. The trend profile in CH_4 and CO_2 production were also confirmed by the gradual changes in the biogas composition with temperature shifts. As can be seen in Figure 3.3 (a), the CH_4 and CO_2 contents produced at 42 °C fluctuated within the ranges 4-61% and 23-46%, respectively, then decreased significantly along with the

temperature shift to 45 °C in which the CH_4 and CO_2 contents varied within the ranges 0-30% and 6-24%, respectively. Interestingly, at 45 °C, there is a modest spike in H_2 composition to 8-9%, followed by increasing H_2 production (Figure3.3(a)) signifying the potential absence of hydrogenotrophic methanogen activity, which consumed CO_2 and H_2 to produce CH_4. High CO_2 and H_2 in the AD process indicate that the CH_4 was primarily produced from acetate via acetoclastic pathways. After the temperature was further shifted to 48 °C, other than a sharp decline in H_2 production, there was no noticeable difference in biogas composition.

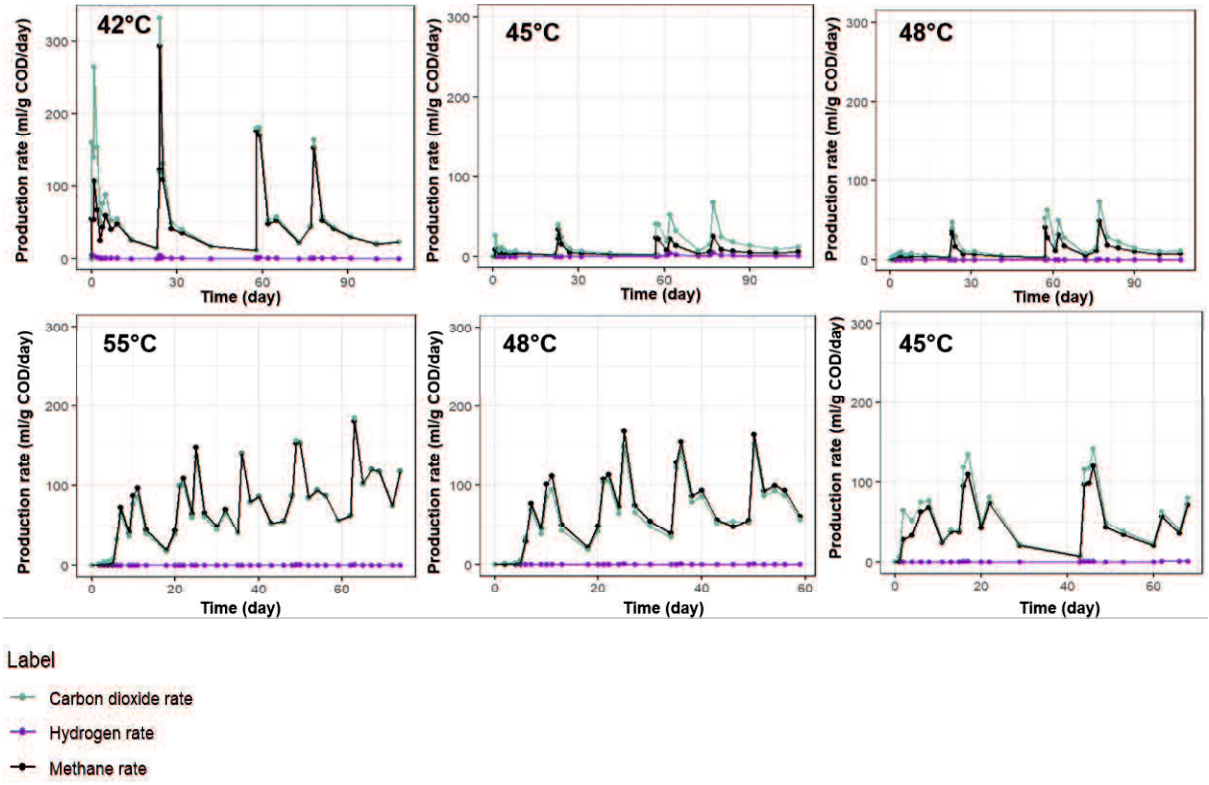
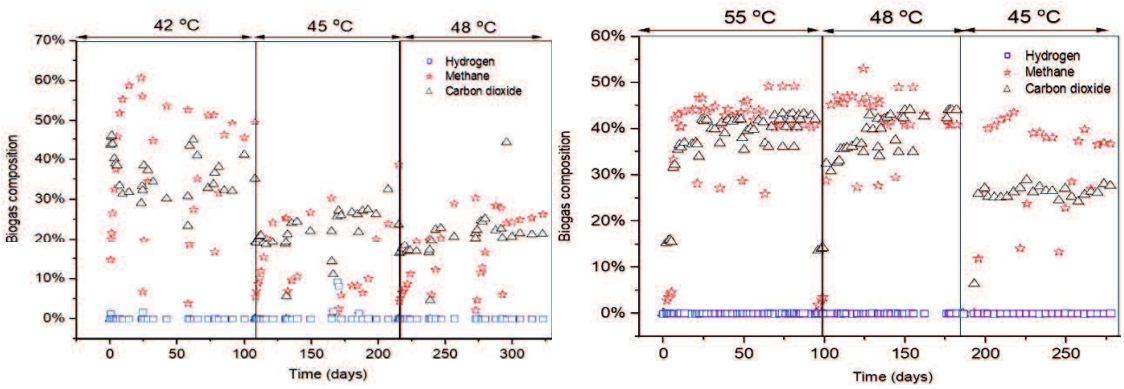


Figure 3. 2 Production rate in different temperature scenarios

Meanwhile, in the downshifted temperature scenario, as Figure 3.3(b) illustrated, a stable biogas composition was generated from the vial, especially during incubation at 55 °C and 48 °C. The vial consistently yielded around 30-53% CH_4 and 30-44% CO_2 over time. However, at 45 °C, the CH_4 and CO_2 composition fluctuated in a broader range below 50% and could not recover during the 100 days incubation periods. In the downshift scenario, no detectable level of H_2 was produced, which denotes that the CH_4 production was mainly conducted in an acetoclastic pathway.

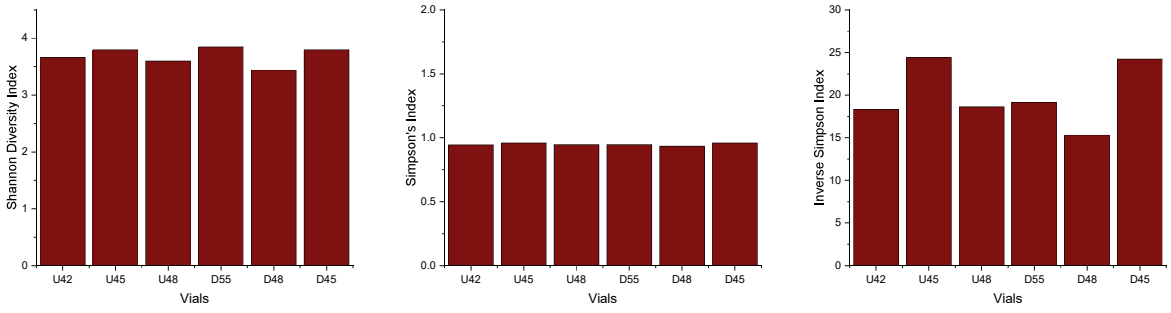


(a) **(b)**

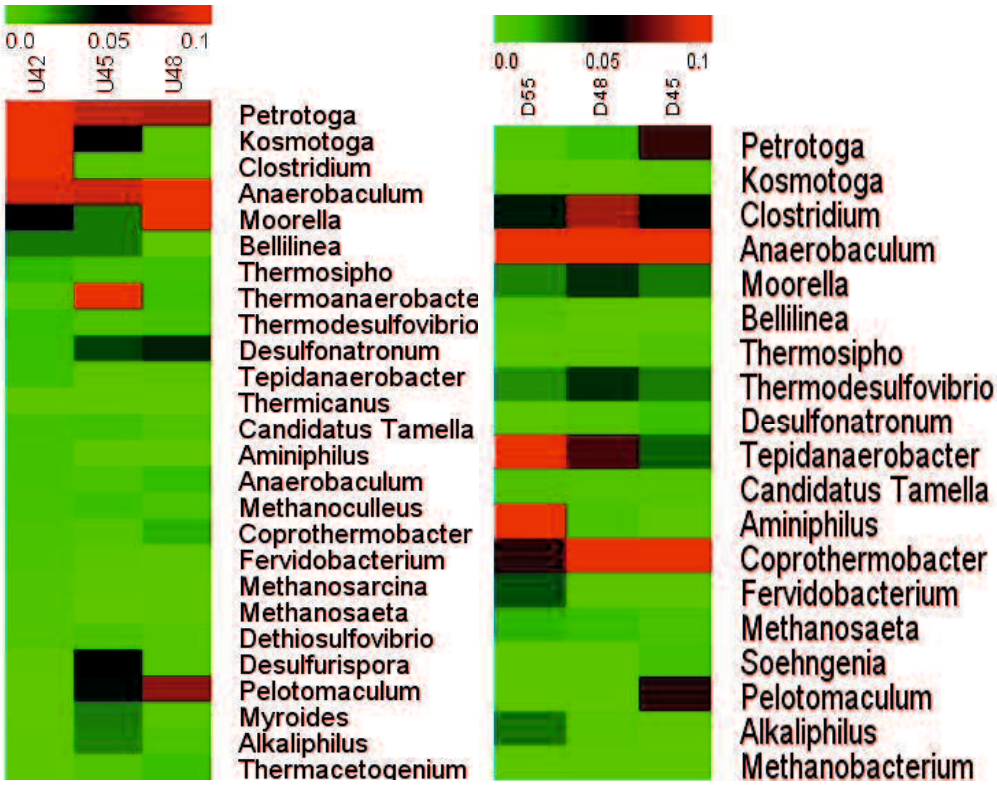
Figure 3. 3 Biogas composition in two different temperature shift scenarios: (a) upshifted and (b) downshifted.

3.3.2 Microbial response on the temperature shift conditions

To understand the influence of the temperature shifts on microbial community, the alpha diversity analysis was performed on each vial using Shannon Diversity Index, Simpson's Index and Inverse Simpson's Index. The diversity index has been used in several earlier studies to analyze microbial communities' abundance at various temperatures to understand better how the communities responded to temperature changes (Arelli et al., 2021; Shaw et al., 2019; Sudiartha et al., 2022). As shown by Figure 3.4 (a), all three-diversity indexes in the upshifted temperature scenario show the highest diversity score at U45 despite the significant downfall of biogas production. Meanwhile, both Shannon's and Simpson's indices display equal diversity scores at D55 and D45 in the downshifted temperature scenario. However, Inverse Simpson's index provides a clearer picture, demonstrating that D45 has greater microbial diversity than D55. Interestingly, despite the decrease in biogas generation at 45 °C, the trends in the alpha diversity indices indicated that the microbial communities grew significantly at the same temperature for both scenarios, suggesting that the diversity of microbial communities had no significant correlation to the biogas production.



(a)



(b) **(c)**

Figure 3. 4 Microbial diversity analysis (a); and microbial communities with > 0.5% relative abundance heatmap (scale) in upshifted temperatures scenario (b) and downshifted temperatures scenario (c).

Since biogas production, particularly CH_4 was mainly generated by methanogens via multiple methanogenesis pathways, the abundance of methanogens in the communities may be a reliable indicator to explain the fluctuation of biogas production levels. As seen in Figure 3.4 (b), in the upshifted temperature conditions, the methanogens community was dominated by *Methanoculleus*, *Methanosaeta, Methanosarcina,* and *Methanobacterium*. Nevertheless, at 42 °C, the relative abundance of methanogens only comprises 3.3% of the total microbial communities and decreases with the temperature shifts to 45 °C (2.9%) and 48 °C (2.1%), followed by the depletion of biogas production. In this community, *Anaerobaculum, Petrotoga,* and *Moorella* belong to phylum *Synergistetes*, *Thermotogae*, and *Firmicutes*, respectively, were dominant microorganisms in all three temperature conditions. There was a significant difference in the composition of microbial communities among the three temperature conditions. The abundance of *Clostridium* (*Firmicutes*) as syntrophic acetate-oxidizing bacteria (SAOB), which supports the CH_4 production through the hydrogenotrophic pathway, fell sharply when the temperature was shifted to 45 °C and 48 °C. Meanwhile, the

abundance of sulfate-reducing bacteria (SRB) such as *Desulfonatronum, Desulfurispora,* and *Desulfotomaculum* increased significantly and became dominant bacteria when the temperature was upshifted to 45 °C. The rising abundance of SRB, followed by a sharp decline in CH_4, may indicate a potential negative correlation between SRB's growth and biogas production.

Desulfonatronum was the only species predominated in the SRB community in the downshifted temperature scenario. As shown by Figure 3.4 (c), this microorganism' abundance escalated once the temperature was downshifted to 45 °C, while the other SRB was detected below 0.05% in all temperature conditions. The microbial community was dominated by *Anaerobaculum, Coprothermobacter, Clostridium, Tepidanaerobacter, Thermodesulfovibrio*, and *Methanosaeta*. The dominance of *Methanosaeta* over the SRB community at 55 °C and 48 °C helps stabilize the vial to produce less fluctuated biogas yield compared to the upshifted temperature vial. However, when the temperature was lowered to 45 °C, *Methanosaeta* abundance decreased, whereas *Desulfonatrum* grew substantially at the same temperature, leading to a considerable decline in biogas production. In summary, both temperature-shift conditions showed different microbial diversity and abundance. However, it is unclear whether the temperature shift increases the microbial diversity in the vials as the diversity index fluctuates in every temperature shift. Nevertheless, our findings discovered that the temperature shifts might disrupt methanogen populations that primarily regulate the CH_4 production while mildly affecting the population of SRB due to its thermotolerance.

3.3.3 Correlation analysis between methanogen and SRB population on biogas production

The correlation between the methanogen and SRB populations on biogas production and temperature influences was analyzed using PCA (Figure 3.5). Biogas production in the upshifted temperature scenario had a strong negative correlation to temperature changes (Figure 3.5 (a)), indicating that the temperature shift severely inhibits biogas production, such as CH_4, H_2, and CO_2. The population of SRBs, such as *Desulfonatronum* and *Desulfurispora,* also exhibits an inverse influence on biogas production and the growth of several prominent methanogens, such as *Methanosaeta* and *Methanobrevibacter*. Figure 3.5 (a) shows that *Methanoculleus w*as the most prominent methanogen among microbial communities; however, there was almost no correlation between the growth of *Methanoculleus* and biogas production. Other methanogens, such as *Methanothermobacter* and *Methanobacterium*, positively correlated with temperature shifts as they favor higher incubation temperatures.

However, their opposite position to biogas production suggests they are not prominent producers.

In the downshifted temperature scenario, both CH_4 and CO_2 production positively correlated to the temperature shift in which the biogas production declined along with the decrease of the incubation temperature (Figure 3.5 (b)). In this condition, the growth of acetoclastic methanogens, *Methanosarcina* and *Methanosaeta,* had a strong positive influence on the CH_4 production, which signifies that the CH_4 was primarily produced through the acetoclastic pathway. This finding was confirmed by the inverse position of major hydrogenotrophic methanogens such as *Methanoculleus, Methanobacterium,* and *Methanothermobacter* to the CH_4 production, denoting a negative or no correlation. The abundance of SRB almost had no (or weak) correlation to the biogas production due to their low abundance in downshifted temperatures conditions.

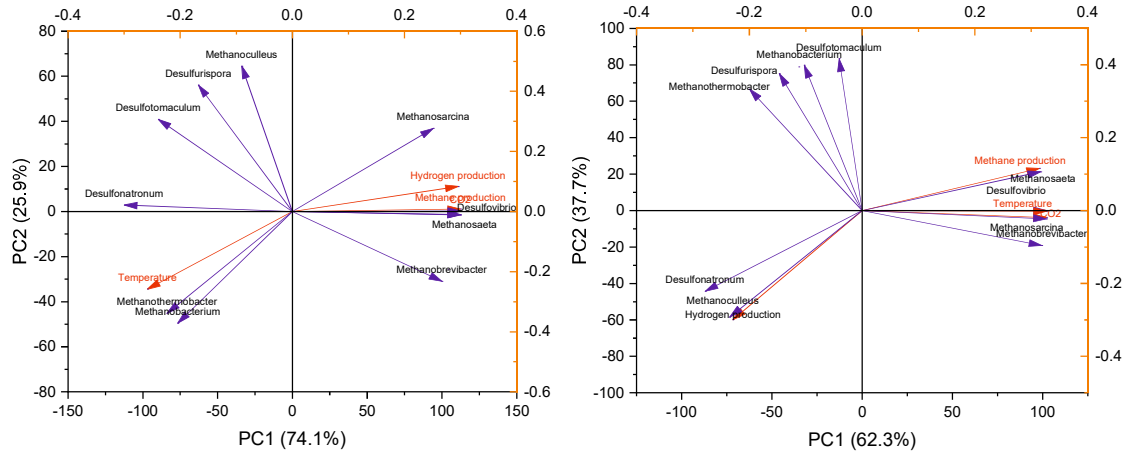


Figure 3. 5 PCA of methanogens, SRB, and biogas production for (a) upshifted temperatures scenario, (b) downshifted temperatures scenario. Microorganisms were selected at the genus level of each group. The blue lines and red lines represent bacteria and biogas production factors, respectively.

3.3.4 Effect of upshifted and downshifted temperatures on CH_4 metabolism

The present study utilized the Kyoto Encyclopedia of Gene and Genomes (KEGG) database to annotate various genes and examine the genes encoding important enzymes associated with methanogenesis to determine how temperature changes may affect the process. CH_4 was produced through four different modules, such as CH_4 production from CO_2 (M00567), CH_4 from acetic acid (M00357), CH_4 from methanol (M00356), and CH_4 from methylamine, dimethylamine, and trimethylamine (M00563). Figure 3.6 (a), (b), (c), (d) shows that the microbial communities involved in all four methanogenesis modules exhibited less

fluctuation in the downshifted temperature scenario compared to the upshifted temperature scenario. This indicates that the transition from thermophilic to mesophilic conditions reduced the likelihood of unstable methanogenesis processes caused by sudden thermal changes. The increasing temperature has disturbed the methanogenesis process. Reduced methanogen levels at 45 °C incubation heightened the instability risk in CH_4 production. The abundance of *Methanomicrobiales* in module M00567 exhibited a significant decline from 1.57% at 42 °C to 0.25% at 45 °C, followed by a gradual recovery to 1.35% at 48 °C. The *Methanobacteriales*, a significant hydrogenotrophic methanogen responsible for regulating the M00567 modules, exhibited a decline in abundance from 0.17% to 0.02% at 45 °C, but increased to 0.67% at 48 °C. The dominant methanogen in module M00357, *Methanosarcinales*, encountered a significant decline in relative abundance as temperature increased from 42 °C to 45 °C and 48 °C, dropping from 1.62% to 0.018% and 0.145%, respectively. Module M00357 exhibited the highest microbial abundance among the four modules, indicating that acetoclastic methanogenesis was the primary methanogenic pathway in both upshifted and downshifted temperature scenarios. According to the enzymes involved in the process, the microbial community abundance in the four methanogenesis modules is outlined in Figure 3.7. Both upshifted and downshifted temperature scenarios showed low abundances in hydrogenotrophic, methanol, and methylamine pathways. In the hydrogenotrophic pathway (M00567), the highest abundance was achieved during the formyl-MFR dehydrogenase (EC:1.2.7.12) enzyme reaction, which reduces CO_2 and methanofuran through N-carboxymethanofuran (carbamate) to N-formylmethanofuran (Hochheimer et al., 1995; Vorholt and Thauer, 1997). The abundance decreased in the reaction to produce N-formylmethanofuran via 5,6,7,8-tetrahydromethanopterin 5-formyltransferase enzyme (E.C.: 2.3.1.101) significantly due to fewer types of microorganisms being involved in this process. Both upshifted and downshifted temperature scenarios demonstrated a decreasing number of abundances in each temperature during the 5,6,7,8-tetrahydromethanopterin 5-formyltransferase process, suggesting that the temperature changes inflicted an adverse impact on the enzyme reaction. Since the synthesis of formyl-MFR is essential for the energy metabolism of hydrogenotrophic methanogens, as they derive energy from CO_2, inhibition in this process may explain the low correlation between hydrogenotrophic methanogens abundance to the biogas production in both temperature shift conditions.

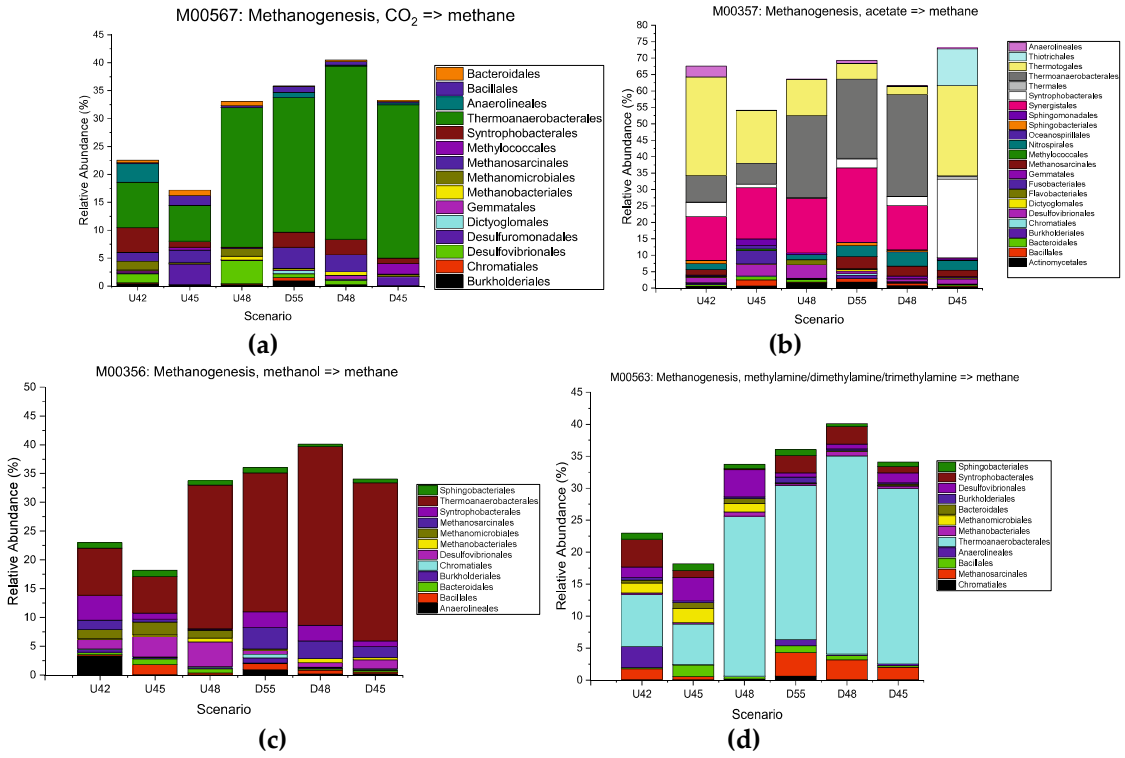


Figure 3. 6 The relative abundance of genes associated with methanogenesis in U42, U45, U48, D55, D48, D45 in four methanogenesis pathway modules: (a) M00567, (b) M00357, (c) M00356, (d) M00563.

In the acetoclastic methanogenesis pathway (M00357), two enzymes play an essential role in methane metabolism: acetate phosphotransferase (EC: 2.7.2.1) and acetyl-CoA synthetase (EC:6.2.1.1). Acetate phosphotransferase/acetate kinase (Ack) is responsible for switching and catalyzing acetate to acetyl phosphate and adenosine diphosphate (ADP) (Barnhart et al., 2015). At the same time, acetyl-CoA synthetase is also a crucial component of the large multienzyme complex known as acetyl-CoA decarbonize/synthase (ACDS) and is responsible for breaking down acetate and producing methyl and reducing equivalents (Grahame, 2011; Zhang et al., 2022). Both upshifted and downshifted temperature scenarios demonstrated a relatively high abundance of Ack genes. However, downshifted temperatures scenario had a higher and less oscillating abundance of ACS genes than the upshifted temperature scenario, contributing to a more stable biogas production. The resiliency of the downshift temperature scenario was also exhibited by the high abundance of genes involved in CH_4 production through methylamine, dimethylamine, trimethylamine (M00563), and methanol (M00356) pathways. *Methanosaeta* and *Methanosarcina* were the only

methanogens known to produce CH_4 via coenzyme-M methyltransferase for M00563 and M00356 synthesis, and they dominated the methanogens community in downshifted temperature scenario.

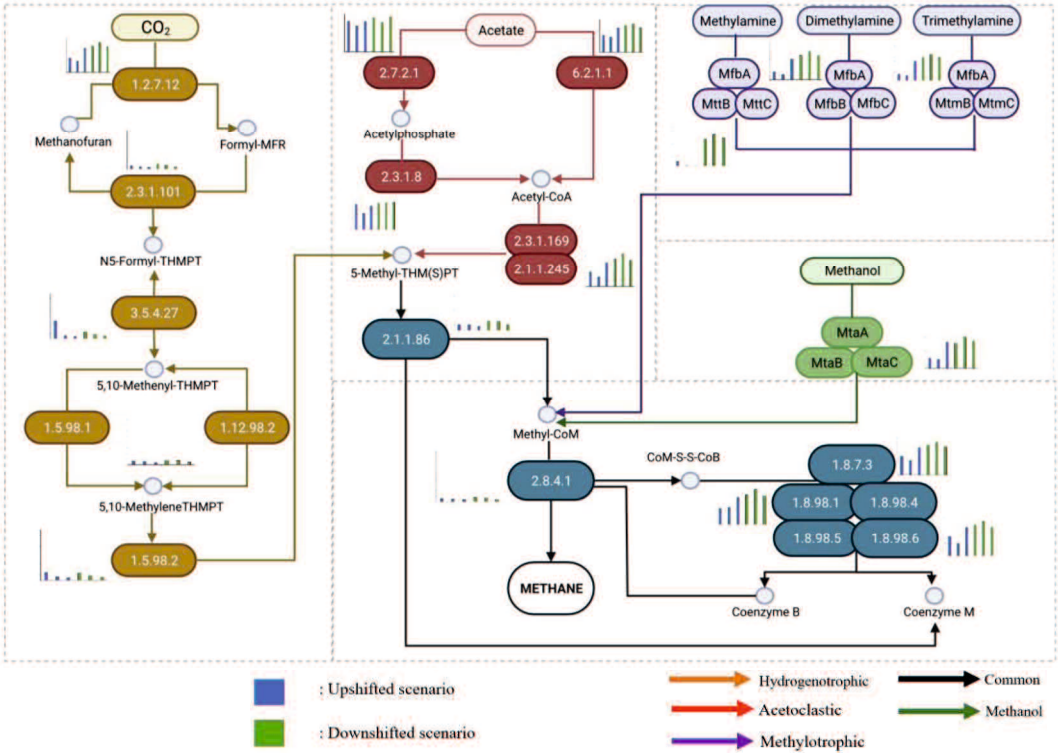


Figure 3. 7 The relative abundance of genes in each enzyme involved in four KEGG methanogenesis modules.

3.4 Discussion

The microbial community is a complicated system in which numerous species constantly adapt their ecological features or interactions in response to perturbations or changes in the environment (Shaw et al., 2019). In the AD process, the temperature changes significantly influence the abundance and the species of microbial communities, which may also impact biogas production (Nie et al., 2021; Sudiartha et al., 2022; Sudiartha and Imai, 2022). Previously, we attempted to investigate the effect of temperature upshifts (Sudiartha et al., 2022) and downshifts (Sudiartha and Imai, 2022) using a similar method as demonstrated in the present study. However, the microbiome analysis was briefly performed, and the intercorrelation between microbial communities during temperature shift conditions was not widely elucidated. We realized that microbial communities were the 'black box' of the AD process, hence in the present study, we performed extensive metagenomic analysis by

determining how the microbial communities involved in the methanogenesis process reacted to the temperature shifts and how the changes in microbial communities affect the methane production pathway (causing inhibition on one specific pathway). A higher glucose concentration of 100 g/l was used in this study. Nevertheless, the biogas production decreased after the temperature was gradually downshifted and upshifted within 100 days of incubation for each temperature shift. These findings were supported by previous studies that showed a considerable decrease in biogas production (mainly CH_4) when the reactor was given a shock temperature raise (Beale et al., 2016; Westerholm et al., 2018; Ziembińska-Buczyńska et al., 2014) and a temperature decrease (Babaei and Shayegan, 2019; Sudiartha and Imai, 2022; Wang et al., 2019), despite the different thermal changing methods and source of inoculum and substrates used in these studies.

NGS was employed as the DNA sequencing method in this study, with all reads passing the quality filter. The study utilized the relative abundance approach to compare microbial abundance in each temperature shift scenario, as the total number of reads in each temperature condition was not similar. NGS-based microbial network analysis demonstrated distinct microbial communities at upshifted and downshifted temperatures. In upshifted temperatures, Methanoculleus from the family Methanomicrobiaceae was the community's most predominant methanogen. *Methanoculleus* is a hydrogenotrophic methanogen that requires CO_2 and H_2, provided by syntrophic acetate-oxidizing bacteria, to produce CH_4 (Babaei and Shayegan, 2019). However, as previously mentioned in Figure 4 (a), the abundance of *Methanoculleus* had a weak correlation to biogas production, suggesting that the biogas was less likely to be produced predominantly via a hydrogenotrophic pathway. The relative rates of CH_4 production between the two pathways can vary depending on the specific conditions and the availability of acetate and hydrogen. CH_4 from H_2/CO_2 synthesis accumulates more slowly than acetate (Conrad, 1999; Metje and Frenzel, 2007). Previous studies supported this finding by demonstrating that lower CH_4 production was obtained when the methanogenesis pathway shifted, indicated by the low abundance of acetoclastic methanogen and increasing abundance of hydrogenotrophic methanogen (Conrad, 1999; Liebner et al., 2015). Furthermore, from the CH_4 metabolism pathway shown by Figure 5 (b), it was further demonstrated that acetoclastic methanogens were the primary contributors to the methane metabolism pathway during upshifted temperature conditions since the abundances of critical enzymes in the process of acetoclastic methanogenesis were much higher than the abundances of key enzymes in the hydrogenotrophic pathway. This finding was also supported by a previous study (Ni et al., 2022).

The domination of acetoclastic genes can be clearly shown in the downshifted temperature conditions. From the KEGG database, order *Methanosarcinales* is involved in all vital enzyme reactions such as Ack, ACS, phosphate acetyltransferase, acetyl-CoA decarbonylase and tetrahydromethanopterin S-methyltransferase. *Methanosaeta* was the most prevalent methanogen in the downshifted temperature microbial community, representing the dominant *Methanosarcinales*. *Methanosaeta* often predominates at low acetate concentrations of 100 to 150 mg COD/l, despite having a stronger affinity for acetate than the other acetoclastic methanogen, *Methanosarcina*, which also belongs to the *Methanosarcinales* order (Kurade et al., 2019). With the higher acetate affinity, *Methanosaeta* can efficiently adsorb acetic acid on its surface and utilize it as its energy source to grow and produce CH_4 faster than *Methanosarcina* (Ni et al., 2022). The domination of *Methanosaeta* from the beginning of the incubation at 55 °C and after downshifting to 48 and 45 °C can indicate strong thermotolerance. This finding negates previous studies that reported that acetoclastic methanogens (*Methanosaeta* and *Methanosarcina*) are mostly predominant at mesophilic temperatures.

In contrast, at thermophilic temperature, the methanogen composition will be shifted to hydrogenotrophic (*Methanoculleus, Methanobacterium, Methanothermobacter*) (Ho et al., 2013; Nie et al., 2021). In the present study, we demonstrated the capability of *Methanosaeta* to exist in both mesophilic and thermophilic conditions and its resiliency in surviving temperature changes while becoming the most predominant biogas producer via an acetoclastic pathway. This finding strengthens the earlier discovery that the abundance of acetoclastic methanogen (such as *Methanosaeta*) can provide reliable CH_4 production and stability indicators (Krohn et al., 2022). Instead of temperature, the metabolic shift of abundance from *Methanosaeta* to *Methanosarcina* may occur if the *Methanosaeta* cannot produce methane due to specific environmental changes like acetate availability, decreasing pH, increasing organic loading rates, high levels of salts and ammonia nitrogen (Kurade et al., 2019; Tian et al., 2018a; Vrieze et al., 2012).

To explain the downfall in biogas production in both temperature shifts conditions, the SRB community plays a crucial role that may interrupt the methanogenesis process and decrease biogas production. These bacteria compete with methanogens for the same hydrogen and acetate substrates required to decrease sulfate into sulfide (Demirel and Yenigu, 2002; Harirchi et al., 2022). The present study detected a high abundance of SRB, led by *Desulfonatronum*, *Desulfurispora*, and *Desulfotomaculum*, in an upshifted temperature scenario. Along with the increase in temperature, the abundance of SRB increased

significantly, followed by a sharp reduction in biogas production. The existence of SRB in anaerobic digestion is disadvantageous primarily to the growth of methanogens as they consume similar substrates as methanogens, and they can obtain the substrates faster than the methanogens.

Furthermore, *Desulforispora, Desulfotomaculum, Desulfonatronum,* and the other *Desulfovibrionales* order were one of the SRBs that can reduce sulfate to hydrogen sulfide (H_2S) (Harirchi et al., 2022; St-pierre, 2017; Thuy et al., 2021). This poisonous and corrosive gas may slow down the rate of methane production and cause odors in the AD reactor. It also has an inhibiting influence on the growth of acetogens and methanogens (Harirchi et al., 2022). Unlike methanogens that heavily rely on the availability of acetate or H_2, SRB can live in an extended range of temperatures and pH without sulfate by competing with methanogens in the consumption of available acetate and H_2 (St-pierre, 2017; Thuy et al., 2021; Wang et al., 2022).

However, despite being able to survive in a broad range of temperatures, the SRB communities exhibited slower growth in the thermophilic scenario. In the downshifted temperature conditions that started from 55 °C, the SRB detected in lower than 0.05% even after downshifting to 48 °C. *Desulfonatronum* abundance began to rise noticeably at 45 °C, followed by a decrease in methanogen abundance and a steep fall in biogas production. This finding indicates that most SRBs flourished only in mesophilic temperature conditions and struggled to survive in thermophilic conditions for 100 days. Hence, the present study suggests that high-temperature early incubation or pre-treatment treatment will potentially help eliminate several types of SRBs that inhibit the growth of methanogens. This measure may create a stable methanogenesis process and enhance biogas production. This study has taken a step toward understanding the relationship between the temperature shift representing ecosystem change and a long-term disturbance. The comprehensive strategies for understanding the impact of the other environmental factors such as VFA, higher loading rate, pH changes, and addition of free ammonia in multiple temperature shift conditions can also be applied to future research in this field or the other research field that also works to explore the dynamic transitions in a microbial population under several circumstances.

3.5 Conclusions

Our findings highlight the effect of temperature upshift and downshift on biogas production and how microbial communities respond to temperature changes every 100 days. The

upshifting and downshifting of the temperature during AD results in the deterioration of biogas production and changes in microbial communities. The increase in temperature from 42 °C to 45 °C depletes up to 83% of the CH_4 production, followed by the decrease in *Methanosaeta* and *Methanosarcina*. The CH_4 production increased by 33% after the further shift to 48 °C. The hydrogenotrophic *Methanoculleus* dominates the methanogen community in the upshifted temperature scenario. However, it weakly influenced biogas production, signifying inhibition via a hydrogenotrophic pathway. The increased SRB population may cause perturbations of biogas production due to the intense nutrient competition as they also crave available acetate and H_2 to survive. The downshift temperature scenario facilitated a better AD performance by experiencing a maximum of 33% drawdown of CH_4 production only at 45 °C. Despite the multiple temperature shifts, the treatment exhibited a resiliency of the *Methanosaeta* that predominantly produced biogas through the acetoclastic pathway. In addition, the SRB abundance was found to be minimum in the downshift temperature treatment. Hence, we propose that early incubation or pre-treatment at high temperatures may assist in removing various SRBs that impede the growth of the methanogens. This action could stabilize the methanogenesis process and increase the generation of biogas.

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CHAPTER IV
SHIFT IN TEMPERATURE, SHIFT IN PATHWAY? UNRAVELLING THE EFFECT OF TEMPERATURE UPSHIFT ON METHANE PRODUCTION PATHWAY

4.1 Introduction

Global water resource management and environmental protection policies have universally stipulated the meticulous treatment of wastewater before its release into water bodies (Nika et al., 2020). The generation of sludge from wastewater treatment plants (WWTP), such as primary sedimentation (PS) and activated sludge (AS), is of considerable magnitude on a global scale, with quantities reaching noteworthy levels: EU nations, the United States, China, Iran, Turkey, Canada, and Brazil contribute to an annual cumulative production of approximately 8910, 6510, 2960, 650, 580, 550, and 370 thousand metric tons of sewage sludge, respectively (Mateo-Sagasta et al., 2015). Given the substantial scale of sludge generation, it is unsurprising that the management and disposal of sewage sludge have emerged as subjects of essential international concern. Owing to its elevated moisture content, limited capacity for dehydration, and stringent stipulations pertaining to the recycling and disposition of sludge, the treatment and management of sludge pose a formidable and intricate challenge (Nazari et al., 2018). The paradigm of wastewater treatment has evolved to an eco-friendly approach that minimizes the quantity of sludge disposed of and converts it into a bioenergy source.

To tackle the issues linked to WWTP sludge, a viable solution involves the utilization of anaerobic digestion (AD). This process has the potential to produce both bioenergy and bio-fertilizer by treating diverse forms of residual biomass, such as sewage sludge, primary sludge, digested sludge, and waste-activated sludge (Campo et al., 2018; Hanum et al., 2019; Young et al., 2013). The AD process yields various recoverable products, including volatile fatty acids (VFAs) and biogas. The biogas comprises methane (CH_4) in the 50–70% range, carbon dioxide (CO2) ranging from 30 to 50%, and additional components like hydrogen sulfide and ammonia (Porté et al., 2019). The synthesis of methane in AD commonly hinges upon the involvement of diverse microorganisms possessing distinct physiological attributes encompassing hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

The conversion of substrates into methane relies on principal constituents such as acetate, hydrogen, and carbon dioxide. The facilitation of this transformation occurs through two main methanogenic routes: the acetoclastic pathway and the hydrogenotrophic pathway (Tian et al., 2018). In the acetoclastic pathway, acetate functions as the substrate for acetoclastic methanogens, while hydrogenotrophic methanogens employ hydrogen and carbon dioxide to produce methane. Importantly, the primary control over the acetoclastic pathway is largely attributed to *Methanosarcinaceae spp.* and *Methanosaetaceae spp.*, whereas the hydrogenotrophic pathway is primarily governed by *Methanomicrobiales spp*., *Methanobacteriales spp.*, *Methanopyrales spp.*, and *Methanocellales spp.* (Kurade et al., 2019).

The three ideal temperature ranges for the AD system are generally used for operation: psychrophilic (< 20 °C) (Fernández-Rodríguez et al., 2016), mesophilic (20 °C–40 °C), with optimum temperatures about 35 °C–37 °C (Wang et al., 2018), and thermophilic (50 °C–60 °C) (Madigou et al., 2019; Wang et al., 2018). The maximum methane production for the vast majority of commercial-scale AD systems varies between 0.03-0.65 L/g volatile solid (VS)/day and 0.04-0.70 L/g-VS/day, on the mesophilic and thermophilic temperatures, respectively (Kasinski, 2020; Nie et al., 2021). However, despite numerous studies investigating the production of biogas in various temperature levels, the behaviour of microbial communities during the methanogenesis process is still hardly understood especially when the temperature increased in the middle of the incubation period. In certain circumstances, the operating temperature of AD reactor may change significantly due to seasonal changes, potentially causing deterioration in methanogenesis and raising operational costs because sustaining constant reactor temperatures during AD may require a lot of energy in a large-scale WWTP.

Few scientists including the authors of the present study, have studied temperature-adjusted AD up to this point (Sudiartha et al., 2023, 2022; Sudiartha and Imai, 2022; Westerholm et al., 2018). There is a need to enhance our understanding of the effects of temperature changes on both biogas production and the dynamics of bacteria involved in methanogenesis. Furthermore, the intricate nature of microbial networks has impeded a thorough exploration of the extensive microbial communities and their interactions in anaerobic digestion (AD). This challenge has motivated the present study, which focuses on examining the fluctuations in biogas production, understanding the connections among microbial communities under varying temperature conditions, and simultaneously identifying active microbial communities by adjusting the operational temperature.

In Chapter III, the study successfully examined the complex microbial interactions and variations in biogas production under both temperature upshift and downshift scenarios. Findings from the Downshift Scenario (Chapter III) indicate that the abundance of methanogen competitors remains low at the optimal thermophilic temperature (55 °C). This suggests that incubation at this temperature may favor methanogenesis and enhance the adaptability of microorganisms involved in methane production. Given that improved adaptability could lead to increased biogas yields, the Chapter IV seeks to investigate the effects of maintaining an optimal mesophilic temperature on biogas production. Specifically, it aims to elucidate how microbial communities respond and adapt during the methanogenesis process when subjected to a gradual temperature transition.

4.2 Materials and methods

4.2.1 Inoculum and Substrates

The inoculum for this study was sourced from anaerobically digested sludge obtained from the Eastern Ube Wastewater Treatment Plant in Ube City, Yamaguchi Prefecture, Japan. The characteristics of the inoculum source are detailed in Table 4.1. A glucose-based substrate, containing 1.5 g/l of glucose, served as the carbon source for biogas production in this investigation. Before initiating the anaerobic digestion (AD) process, the inoculum sample was blended with the substrate at a ratio of 2:1 (inoculum to substrate). The substrate solution was prepared by mixing glucose with essential nutrients necessary for bacterial growth: 4 g/l $NaHCO_3$, 4 g/l K_2HPO_4, 0.1 g/l yeast extract, 85 g/l NH_4Cl, 81 g/l $MgCl_2{\cdot}6H_2O$, 75 g/l KCl, 350 g/l $(NH_4)_2HPO_4$, 42 g/l $FeCl_3{\cdot}6H_2O$, 25 g/l $MgSO_4{\cdot}7H_2O$, 1.8 g/l $CoCl_2{\cdot}6H_2O$, and 150 g/l $CaCl_2{\cdot}2H_2O$ (Zhou et al., 2006).

Table 4. 1 Characteristics of anaerobically digested sludge as inoculum

Parameters	Anaerobic Sludge	Units
pH	6.09	-
Total Solid (TS)	5714	mg/l
Volatile Solid (VS)	3809	mg/l
Fixed Solid (FS)	1905	mg/l
Chemical Oxygen Demand (COD)	1520	mg/l
Ammonia	408.74	mg NH_4-N/l

4.2.2 Experimental Procedures

The experiment utilized 500 mL serum vials with a working volume of 300 mL. A fed-batch fermentation method was employed implementing a 20-day hydraulic retention time (HRT) estimated using the first-order kinetic equation, as detailed in previous studies (Caillet and Adelard, 2021; Wongarmat et al., 2022). During the initial incubation phase, 100 mL of substrate was introduced into the serum bottles. The vials were then tightly sealed with aluminium covers and butyl rubber stoppers, undergoing thorough nitrogen gas flushing to eliminate any residual oxygen, ensuring the establishment of anaerobic conditions. Incubation commenced at a temperature of 37 °C (referred to as U37) and continued for a duration of 60 days. The incubation setup was agitated at 100 rpm utilizing the EYELA SLI-700 incubator with a shaker. The selection of 37 °C as the initial acclimatization temperature was based on the prevalence of a diverse range of microorganisms with optimal growth temperatures in this vicinity, particularly favoring methanogens (Arikan et al., 2015; Song et al., 2023). While previous studies suggested incubation times of 21 days (Ángeles et al., 2021; Tetteh and Rathilal, 2022), a longer operating period was chosen in the context of temperature variations. This extended period allows microorganisms to adapt to challenging conditions, promoting more stable operation (Sudiartha et al., 2023; Sun et al., 2015; Westerholm et al., 2015). The temperature was incrementally increased from 37 °C to 39 °C (U39), 39 °C to 41 °C (U41), 41 °C to 43 °C (U43), and finally to 45 °C (U45). The current study was mainly operated at 80 days interval, however there were 40 days extension at 37 °C due to self-fermentation process for acclimatization purposes, and additional 20 days extension at 41 °C due to significant inhibition.

4.2.3 Data collection and analysis

Regular monitoring of the total gas volume and composition was performed throughout the incubation period using gas chromatography. The biogas volume produced was quantified using a glass syringe method. Gas chromatography analysis of the samples, encompassing H_2, N_2, CH_4, and CO_2, was conducted using a GC-8APT/TCD instrument (Shimadzu Corp., Kyoto, Japan) equipped with a 60/80 activated charcoal mesh column (1.5 m × 3.0 mm internal diameter) and argon as the carrier gas. The injector, column, and detector temperatures were set at 50 °C, 60 °C, and 50 °C, respectively, during the analytical procedure. Volatile fatty acid (VFA) concentration was analysed using GC-8APF with FID detector (Shimadzu Corp., Kyoto, Japan).

4.2.4 NGS and Microbial Diversity Analysis

As much as 1.5 mL of DNA samples were collected from each temperature condition and stored at -22 °C. The DNA was extracted using the NucleoSpin® kit following the instructions provided in the NucleoSpin® Soil Manual. DNA concentration was assessed using Qubit® dsDNA Assay Kit in Qubit® 4.0 Fluorometer (Life Technologies, CA, USA). PCR amplification was performed in two stages. The 1st PCR was conducted on the V4 region of the 16S rRNA gene using 515F/806RC primers and amplified using KAPA HiFi Hot Start ready mix (TaKaRa Bio Inc., Shiga, Japan). Upon denaturing process, the clean-up process was performed using NucleoSpin® Gel and PCR Clean-up to remove free primers and primer dimer species. The 2nd PCR was then conducted to attach the dual indices and Illumina Sequencing Adapters using Nextera XT Index Kit. Denaturing process for the 2nd PCR has similar stages as 1st PCR with less cycles was carried out (30x cycles for 1st PCR and 12x cycles for 2nd PCR). To remove the index that cannot be included to the PCR product and remove any clusters that may form during the sequencing process, 2nd PCR clean-up was performed using AMPure XP Beads, 10 mM Tris pH 8.5 and EtOH 80%. Following the final PCR clean-up, high throughput sequencing was conducted using the Illumina MiSeq platform with iSeq 100 cartridge at the Department of Environmental Engineering, Yamaguchi University, Japan. The raw FASTQ data were then subjected to processing utilizing the Dragen Metagenomics Pipeline. Alpha diversity analysis, employing the Shannon Diversity Index, Simpson's Index, and Inverse Simpson's Index in R studio, was carried out to identify any alterations in microbial communities during anaerobic digestion under shifted temperature conditions. Principal component analysis (PCA) was performed to analyze how distinct the microbial communities amongst temperature conditions using STAMP. The microbial heatmap was made and subsequently analysed using MeV 4.9.0, while MiniTab 21.4.1.0 was utilized for other data visualization purposes.

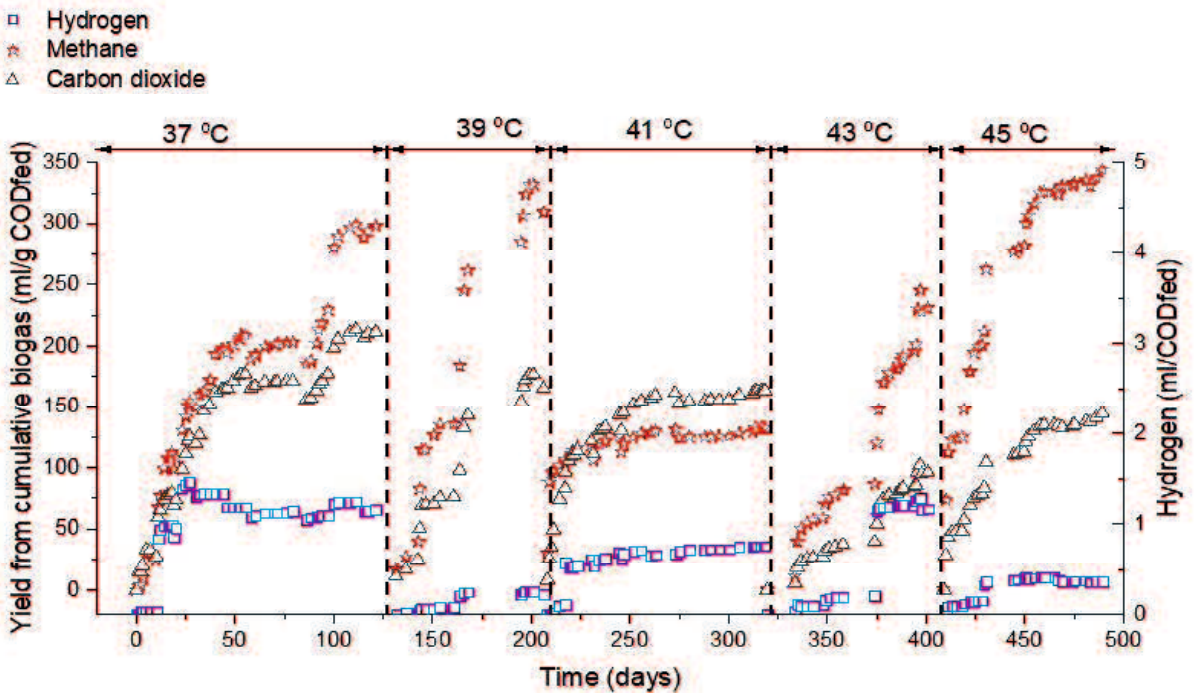
4.3 Results and discussion

4.3.1 AD performance under temperature shifts scenarios

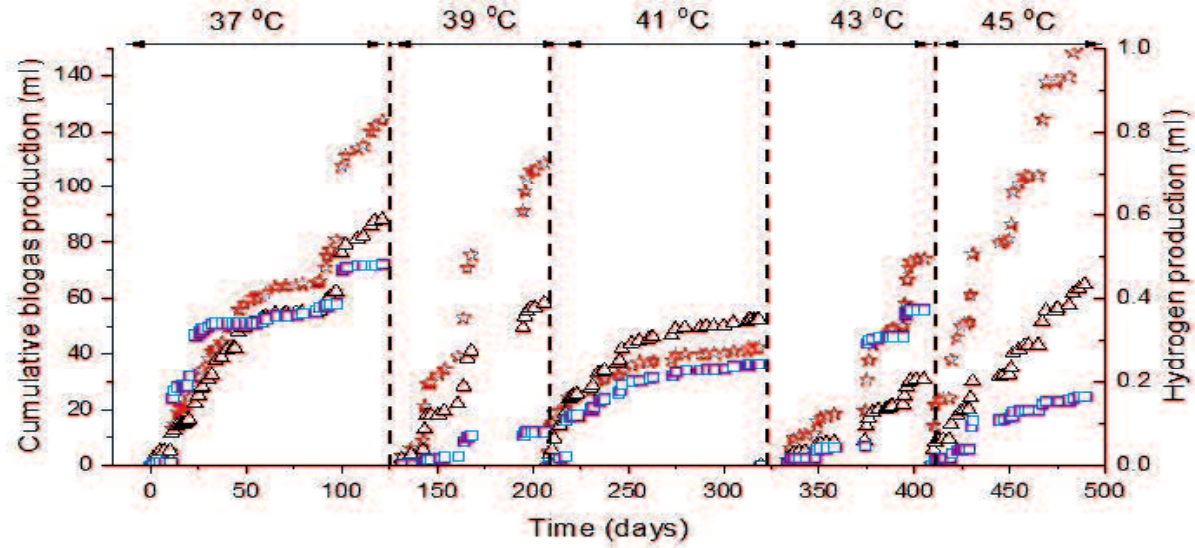
Biogas yield and production during the incubation under temperature shift scenarios are presented in Figure 4.1(a) and (b). In order to allow the microbial communities to adapt to temperature changes and establish stable metabolic pathways, such as hydrolysis, acetogenesis, acidogenesis, and methanogenesis, to convert sewage sludge into biogas, the

incubation period was set in the range of 80-120 days. Several previous research also suggested an extended incubation period of AD during harsh conditions to provide sufficient time for microorganisms to adapt (Moestedt et al., 2014; Sun et al., 2015; Westerholm et al., 2015). Incubation at 37 ºC was conducted in two phases: the self-fermentation phase (40 days) and the running phase (80 days). Self-fermentation refers to the initial process of fermentation where the vials are incubated for several days to allow the microbial communities to perform "self-cleaning" of recalcitrant compounds that get carried along during sludge sampling in WWTP. This process ensures the AD process (especially acetogenesis and methanogenesis) to occur naturally. Previous study performed this process as pre-treatment with purpose of acclimatizing microorganisms to the new environment (vials) and establishing a sustainable anaerobic condition inside the vials (Signorini et al., 2016).

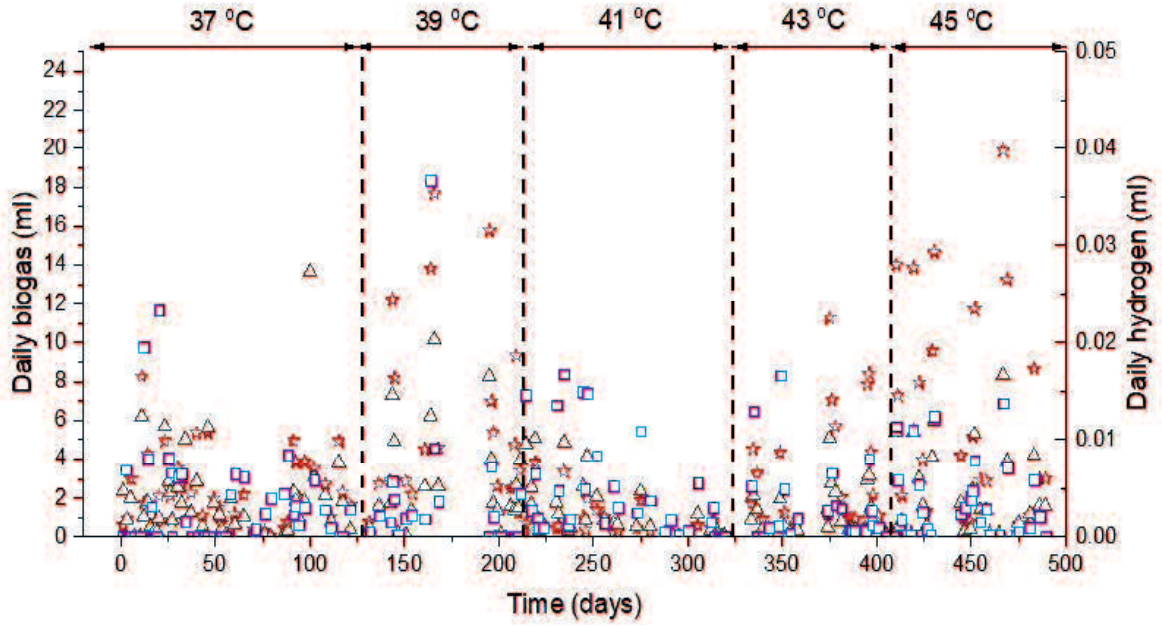
In the first 10 days upon start-up, under incubation at 37 ºC, the vial demonstrated a higher production of carbon dioxide (32.95 ml CO_2/g COD_{fed}) compared to CH_4 (29.62 ml CH_4/g COD_{fed}) which indicated the establishment of acetogenesis process and early phase of methanogenesis process. On the 11^{th} day, the CH_4 production started to outpace the carbon dioxide by the ratio of 1.2:1. a total of 123 ml CH_4 (298 ml CH_4/g COD_{fed}) was produced, followed by 87.95 ml CO_2 (211 ml CO_2/g COD_{fed}). Despite being incubated at optimum mesophilic temperature, the CH_4 was produced less than 10 ml daily (Figure 4.1(c)) with CH_4 content fluctuating around 0 – 42% and CO_2 content in the range of 0 – 20% (Figure 4.1(d)), signifying the ongoing acclimatization process in the vials and the microbial communities had not been well-established.


Hydrogen
Methane
Carbon dioxide
37 °C
39 °C
41 °C
43 °C
45 °C
Yield from cumulative biogas (ml/g CODfed)
Hydrogen (ml/CODfed)
Time (days)

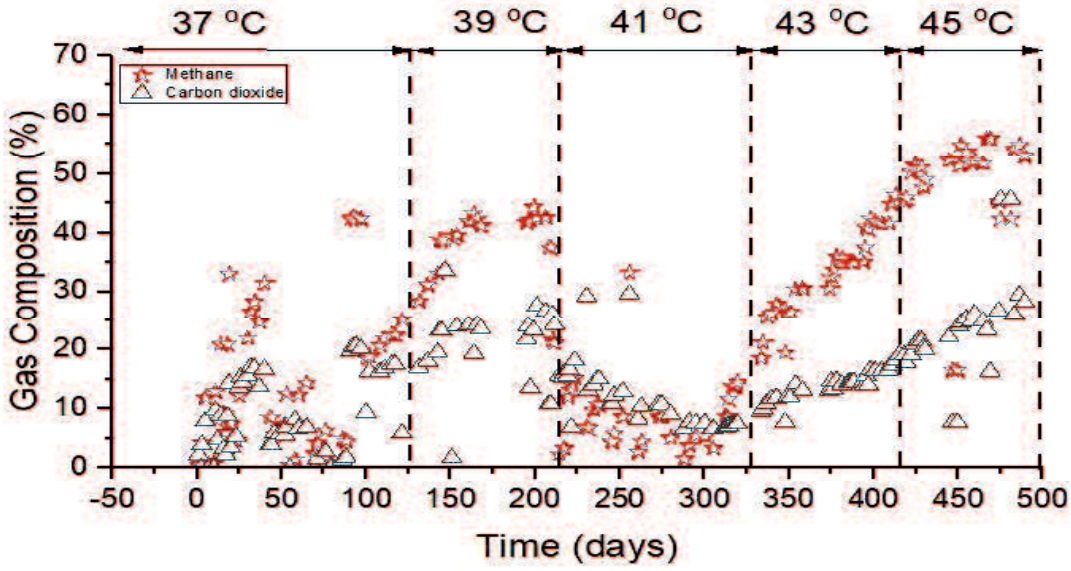
(a)


37 °C
39 °C
41 °C
43 °C
45 °C
Cumulative biogas production (ml)
Hydrogen production (ml)
Time (days)

(b)



(c)



(d)

Figure 4. 1 Biogas yield from cumulative biogas production (a), cumulative biogas production (b), daily volumetric biogas production (c), and gas composition (d) under different temperature shift conditions.

Upon the temperature being upshifted to 39 ºC, the CH_4 yield increased slightly at the end of incubation compared to 37 ºC (309 vs 298 ml CH_4/g COD_{fed} respectively) with a substantial decrease in CO_2 production (211 vs 165 ml CO_2/g COD_{fed} respectively) following to the increase in daily biogas production and stable CH_4 content at 28 – 45%. This finding

may indicate the involvement of a hydrogenotrophic methanogenesis pathway that converts CO_2/H_2 to produce CH_4. This was also confirmed by a lower level of CO_2 (3 – 27%) in the vials. Nevertheless, a further upshift in temperature to 41 °C caused the CH_4 content to deplete significantly from 33% to below 10% while CO_2 production fluctuated in the range of 7 – 29% throughout the incubation period. This resulted in the slump of CH_4 yield to 147 ml CH_4/g COD_{fed} and a surging CO_2 yield of 181 ml CO_2/g COD_{fed}. The decrease in CH_4 at 41 °C was supported by previous studies that also discovered immense perturbation when the AD operating temperature was adjusted at the range of 40–44 °C (Nie et al., 2021; Sudiartha et al., 2023, 2022; Sudiartha and Imai, 2022; Westerholm et al., 2018). The sole rationale for this phenomenon is that the temperature range may be greater than the range at which mesophiles can grow but not sufficiently high for thermophiles The switching predominant biogas content between CH_4 and CO_2 production indicates potential increasing activity of fermentative bacteria, acetogens, and syntrophic acetate oxidizing bacteria (SAOB) while hydrogenotrophic bacteria communities were inhibited at this temperature.

Interestingly, at 43 °C, the CH_4 production started to recover with a lower CO_2 production by a factor of 2.5. The CH_4 yield increased substantially to 259 ml/g COD_{fed} while the CO_2 yield eased at 106.77 ml/g COD_{fed}. The wide discrepancy between CH_4 and CO_2 production continues after the incubation temperature is upshifted to 45 °C with a thriving CH_4 production to 344 ml CH_4/g COD_{fed} after 80 days of incubation. This phenomenon signifies the activation and establishment of hydrogenotrophic communities due to long incubation and high temperature. The hydrogenotrophic pathway requires a longer incubation time to take place than the acetoclastic methanogen (Khanal, 2008). This finding is also supported by a previous study that found that hydrogenotrophic methanogen communities flourished in the long AD periods with higher temperatures and helped stabilize the reactor upon total degradation of propionate (Chen et al., 2019; Dyksma et al., 2020; Procópio, 2023; Tsapekos et al., 2022). Production rate in Figure 4.2 reveals that hydrogenotrophic pathway thrived at the end of 39 °C, yet the biogas production rate decreased significantly when the temperature decreased to 41 °C. Biogas production rate recovered at 43 - 45 °C prevailed by hydrogenotrophic pathway due to the wide discrepancy between CH_4 and CO_2 production.

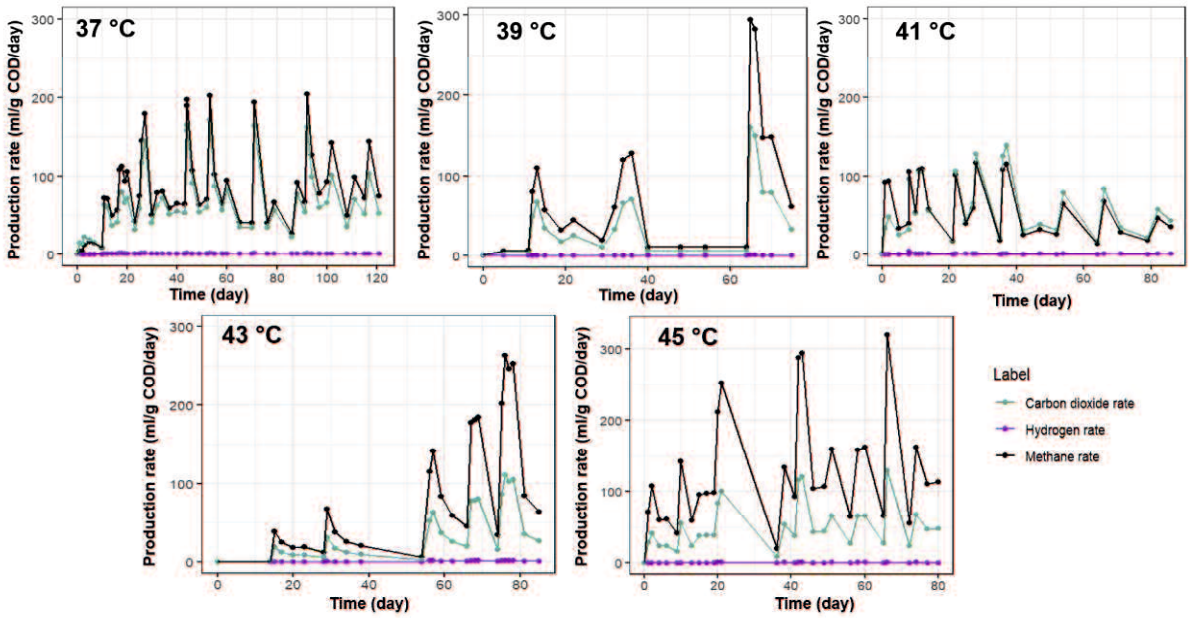
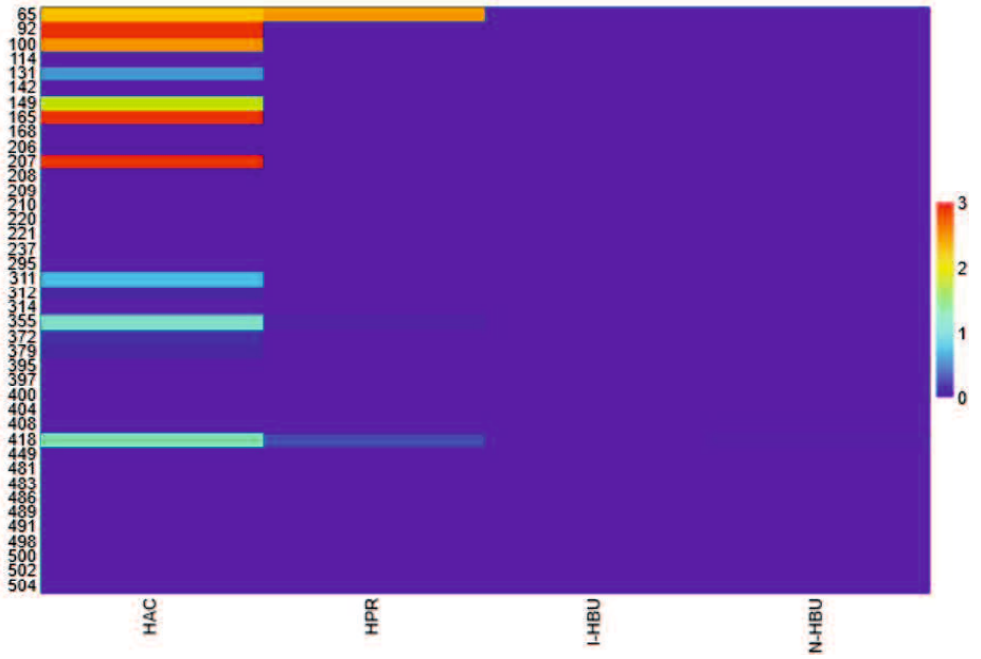


Figure 4. 2 Production rate in upshifted temperature scenario with 1.5 g/L glucose level

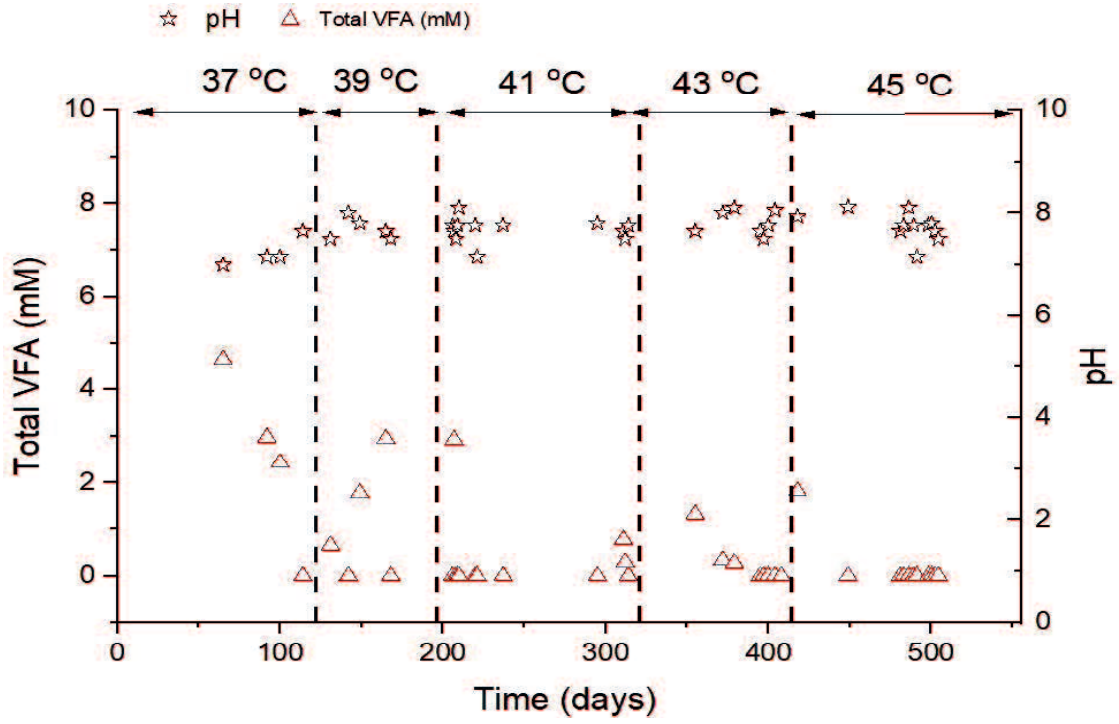
Inhibition in the methanogenesis process occurred when the propionate levels were in the range of 10 – 287 mM or 0.8 – 21 g HPr/l (Alavi-Borazjani et al., 2020; Franke-Whittle et al., 2014). As seen in Figure 4.3 (a), the maximum level of propionate in the present study was far below inhibition levels (2.4 mM, detected in the early 60 days of incubation). Propionate was completely scavenged in the next 30 days and barely found in the reactor in higher amounts until the end of 45 °C incubation. This indicates an uninhibited digestion process by fermentative bacteria to convert propionate to acetate and the ongoing syntrophic relationship with hydrogenotrophic bacteria that quickly devour the available H_2 to keep the H_2 partial pressure low. Hydrogenotrophic methanogenic bacteria efficiently consume hydrogen, maintaining an exceptionally low level of hydrogen partial pressure (Harirchi et al., 2022; Khanal, 2008; O-Thong et al., 2020). This creates a thermodynamically favorable environment for hydrogen-producing acetogenic bacteria, enabling them to break down the propionate into acetate, H_2, and CO_2.

The complete digestion of propionate helps to maintain the pH around the range of 7 – 8.1 over temperature change (Figure 4.3 (b)) which was the ideal pH for both acetoclastic and hydrogenotrophic methanogenesis that favors a pH range of 7 – 12 (L. Chen et al., 2021; Wormald and Humphreys, 2019). Propionate was digested to acetate via a syntrophic propionate oxidizing process and the abundance of acetate decreased over temperature shift incubation (Figure 4.3 (c)), this finding signify increasing activity of acetate consumption

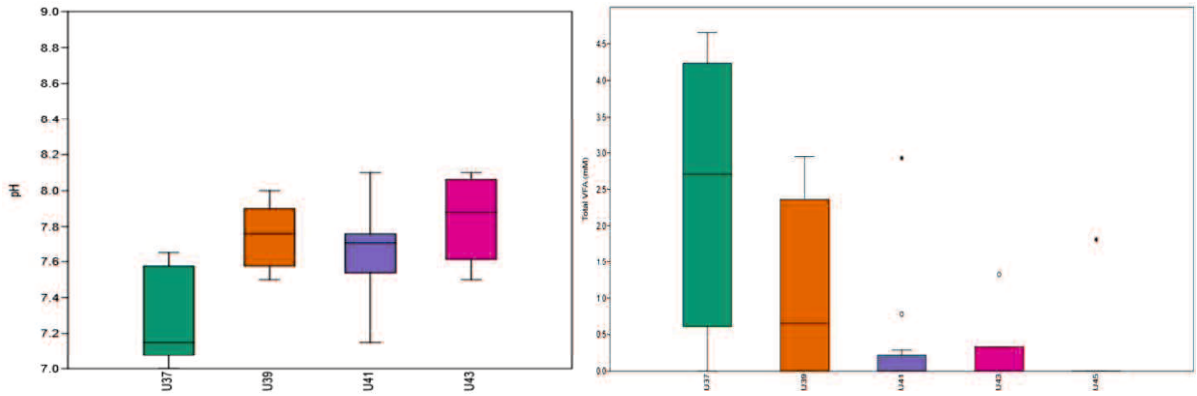
either from the acetoclastic methanogens or SAOB in syntropy with hydrogenotrophic methanogens.



(a)



(b)



(c) **(d)**

Figure 4. 3 Individual VFA profile in mM (a), total VFA, and pH (b) of digested sewage sludge under temperature upshift conditions over time and the boxplot of pH (c) and total VFA (d) difference among each temperature condition.

4.3.2 Microbial alpha diversity analysis

In this research, alpha diversity analysis, illustrated by Shannon, Simpson, Brillouin, Evenness and Chao1 indices (Figure 4.4), offered insights into the microbial community's responses to different temperature conditions. The application of these diversity indices parallels their use in prior studies examining the impact of environmental factors such as temperature (Sudiartha et al., 2023; Sudiartha and Imai, 2022; Xiang et al., 2021; Zhang et al., 2016).

Among the three diversity indices, both the Shannon and Brillouin indices displayed a similar trend, indicating higher microbial diversity at 37 °C compared to other temperature conditions. This finding is consistent with existing research, which shows that mesophilic temperatures, especially around 37 °C, promote greater microbial diversity than higher-temperature environments (H. Chen et al., 2021; Liu et al., 2022; Steiniger et al., 2023). The decline in microbial diversity with increasing temperature suggests the elimination of several microbial communities that were unable to withstand the changing thermal environment.

Interestingly, a slight increase in diversity was observed at 43 °C confirmed by Simpson's and Chao1 index indicating possible adaptation of several microbial communities following prolonged incubation and continuous temperature rise. Meanwhile, Evenness index showed relatively different trend at 43 °C and 45 °C. Spiking Evenness index followed by fluctuating diversity index like Chao1, Simpson, Shannon and Brillouin, signify that there

was a changing domination and abundance distribution among reigning microbial communities. High evenness with low diversity demonstrates an evenly distributed communities with more predominant species.

Despite these variations, our study did not observe a significant downfall trend in overall microbial community diversity, since it ranges in a narrow interval. This finding is intriguing, as microbial diversity is often regarded as an indicator of stability in the anaerobic digestion (AD) process. The observed stability in microbial diversity, even under varying temperature conditions, underscores the robustness of microbial communities in the AD process. This resilience may result from the adaptability of specific microbial groups to changing thermal environments, highlighting the complex relationship between microbial diversity and process stability in AD systems. Further investigation into the specific microbial taxa thriving under different temperature regimes could yield valuable insights for optimizing AD processes and enhancing their overall stability.

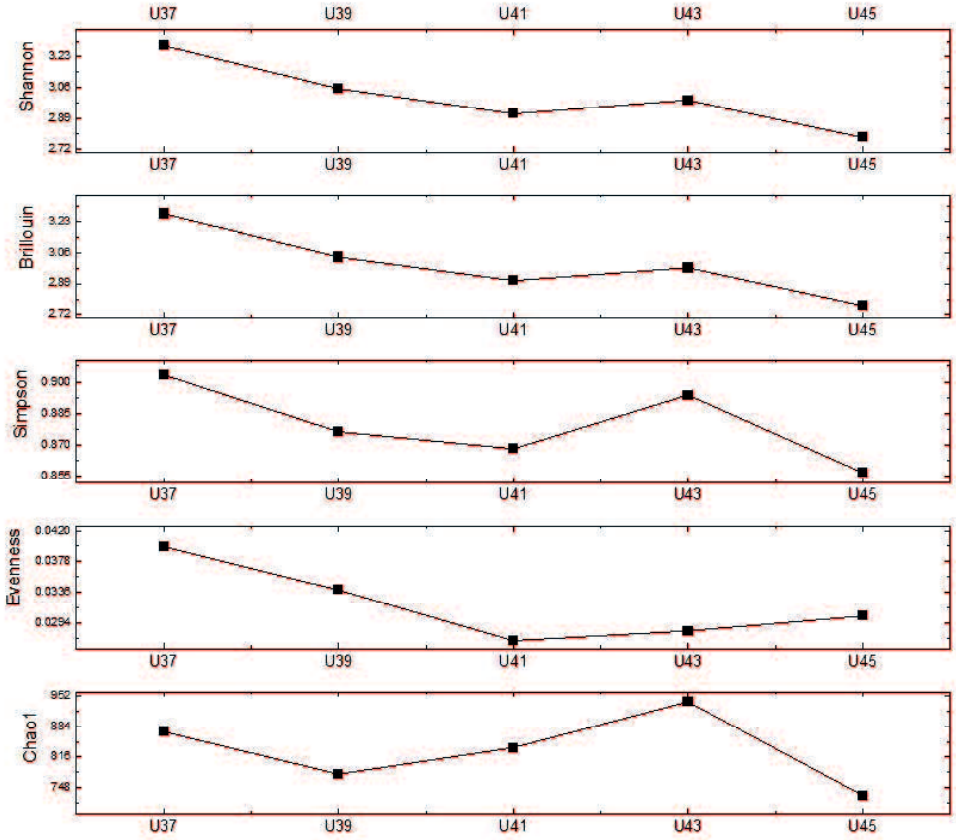


Figure 4. 4 Alpha-diversity analysis of microbial communities during incubation at the temperature shift scenario.

4.3.3 Microbial response toward temperature shift

The production process of CH_4 involves a wide array of microbial communities, from fermentative bacteria to methanogens, and the complex syntrophic relationship between those communities. As seen in Figure 4.5, the microbial communities are dominated by *Coprothermobacter* genus which comprises 19 – 30% of total relative abundance in all temperature conditions. *Coprothermobacter,* are fermentative bacteria who efficiently digest complex substrates into more biodegradable substrates, such as H_2, CO_2 and acetate, particularly in higher temperature conditions (Sudiartha et al., 2024). This microorganism primarily found in sewage sludge and cattle manure, engages in a syntrophic relationship with hydrogenotrophic methanogens (Luo et al., 2013; Palatsi et al., 2011; Tandishabo et al., 2012).

Acetomicrobium genus is the second most abundant microorganism found in the present study. *Acetomicrobium* is mainly found in sewage sludge and is responsible for the production of acetate, H_2, and CO_2 from the digestion of complex substrates (Li et al., 2022; Soutschek et al., 1984). Furthermore, it has a thermotolerant characteristic due to the wide range of growth temperatures (35 – 65 °C) with a high optimum temperature of 55 – 60 °C (Hania et al., 2016; Mavromatis et al., 2013). In the current study, at 37 - 41 °C, the abundance of *Acetomicrobium* predominates 9 – 12% of the total microbial communities. The abundance continues to rise to the level of 16% and 14% after the temperature upshifted to 43 and 45 °C, respectively, which marks possible syntrophic relationships with both acetoclastic and hydrogenotrophic methanogens at the higher temperature conditions. The other bacteria that were also found abundant in the current study are *Corynebacterium* (5% - 9%) and *Brachyspira* (4% - 9%). Both *Corynebacterium* and *Brachyspira* involved in the degradation of complex substrates, such as cellulose, polysaccharides, and hemicellulose, into digestible substrates such as VFA, H_2, and CO_2 (Dyksma et al., 2020; J.-Y. Kim et al., 2023; Westerholm et al., 2022).

The wide discrepancy between CH_4 and CO_2 levels at 43 and 45 °C may be a result of the increasing activity of hydrogenotrophic methanogens that consume CO_2 to produce CH_4, in syntropy with acetogens and SAOB. *Methanobacterium* as the predominant hydrogenotrophic methanogen, demonstrated a gradual decrease in abundance along with the temperature upshift. This creates a negative correlation between *Methanobacterium* abundance and temperature, and a low correlation to CH_4 production. Interestingly, previous studies also showed that the CH_4 production is not directly correlated to the methanogen's

abundance, as every methanogen has diverse affinity and efficiency toward substrate conversion (H. H. Kim et al., 2023; Kong et al., 2019). Despite the decreasing abundance, *Methanobacterium* still prevailed at > 1% of the total microbial communities. Compared to the other hydrogenotrophic methanogens, the *Methanobacterium* genus was discovered as the most efficient CH_4 producer since it can convert CO_2 to CH_4 despite very low hydrogen levels with a CH_4 production rate of 108 fmol CH_4/cell/day for CO_2 consumption of 69.62 fmol CO_2/cell/day (Chen et al., 2019). The support from syntrophic bacteria also enhanced CH_4 production by facilitating the swift degradation of complex organic compounds into more preferable substrates to the methanogens, despite lower methanogens abundance (Khanal, 2008).

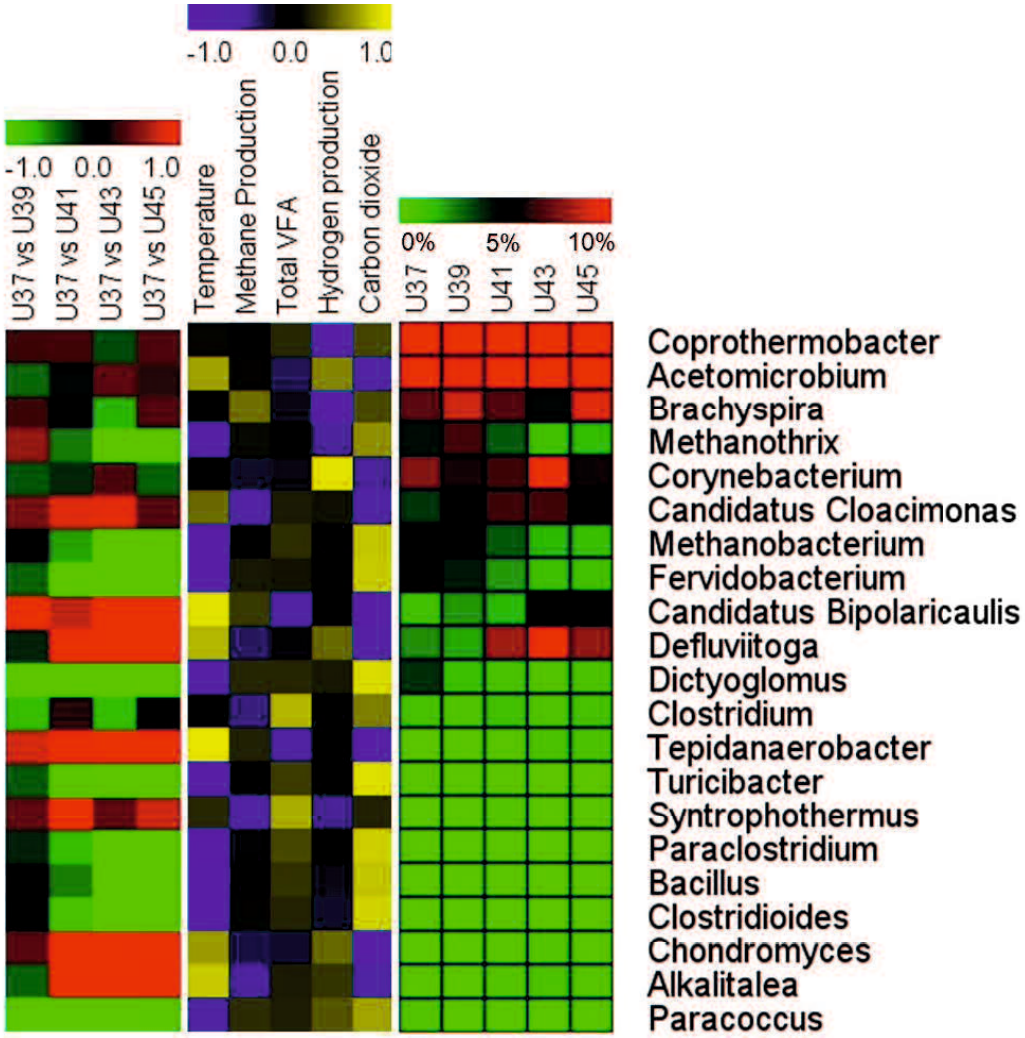


Figure 4. 5 Heatmap representing the log2 abundance changes of microbial communities compared to initial temperature incubation at 37 ºC (left side), (middle side), and Pearson correlation of OTUs relative abundance against AD parameters, relative abundance of the top 1% most abundant OTUs (right side).

A noticeable spike in abundance was observed in the *Candidatus Bipolaricaulis* and *Candidatus Cloacimonas* genus, as the prominent SAOB via Wood-Ljungdahl Pathway (WLP) (Coskun et al., 2023; Solli et al., 2014; Youssef et al., 2019), exhibited a 40 – 80% rise in population along with the increase in temperature. The flourishing abundance of *Candidatus Cloacimonas* and *Candidatus Bipolaricaulis* at 43 and 45 °C, further strengthened the conviction that there was an increasing activity of hydrogenotrophic pathway in the CH_4 production as these microorganisms formed a syntrophic relationship with hydrogenotrophic methanogens, to make the hydrogenotrophic pathway becomes thermodynamically possible through conversion of acetate, propionate and other organic acids chain to CO_2 and H_2 (Harirchi et al., 2022; Khanal, 2008; Li and Khanal, 2016).

Despite the potential CH_4 production through the syntrophic relationship between fermentative bacteria and hydrogenotrophic methanogen, methanogen communities were still dominated by the acetoclastic *Methanothrix* (formerly *Methanosaeta*). The domination of *Methanothrix* remains unchanged until the shift of temperature to 45 °C. The relative abundance of *Methanothrix* fluctuated in the range of 4% - 7% at 37 – 41 °C and gradually decreases to below 4% following the stepwise temperature shift to 45 °C. *Methanothrix* exhibited a significant drop in the abundance after the temperature entered the thermotolerant zone of 41 - 45 °C. The previous study also found that immense perturbation occurred in the interval of 40–44 °C, signifying that the 40–44 °C temperature range had a significant impact on both mesophilic and thermophilic microbial populations (Westerholm et al., 2018).

Nevertheless, despite harsh changing temperature conditions, higher CH_4 production with low acetate levels and the absence of propionate and butyrate at 43 and 45 °C may prove that *Methanothrix* can efficiently produce CH_4 despite the decrease in abundance. Previous studies also reported the resiliency of *Methanothrix* in CH_4 synthesis due to its vast array of operating temperatures, high acetate affinity, and low substrate requirement (Kurade et al., 2019; Sudiartha et al., 2023; Sudiartha and Imai, 2022; Zhang et al., 2023). As an acetoclastic methanogen that requires acetate to produce CH_4, *Methanothrix* establishes a syntrophic relationship with acetogenic bacteria such as those from the genus *Acetomicrobium*, *Defluviitoga*, and *Clostridium* (Dyksma et al., 2020).

The existence of two major CH_4 production, acetoclastic and hydrogenotrophic pathways, has created a more stable AD process with low CO_2 production and better CH_4

yield during temperature shift conditions compared to our previous research that experienced an 83% decline in CH_4 production during temperature shift conditions (Sudiartha et al., 2023) and stable CH_4 production yet with high CO_2 contents with existence of only acetoclastic pathway (Sudiartha et al., 2024).

A chi-square test was performed to determine which microbial communities exhibit the most significant changes in abundance during temperature shift conditions. These changes can be used as statistical measures to assess the activity of bacteria according to the changes in abundance. Figure 4.6 shows the high activity of *Acetomicrobium* during the incubation at a temperature of 37 – 41 °C ($p < 0.05$). The other prominent fermentative bacteria such as *Corynebacterium, Fervidobacterium,* and *Defluviitoga* also demonstrated high abundance changes at the similar temperature changes ($p < 0.005$). Meanwhile, upon the shift in temperature to the range of 43 – 45 °C, the dominance of *Acetomicrobium* in term of abundance-based activity was outstripped by *Defluviitoga* strains. The increasing activity of *Defluviitoga* was expected as this strain usually thrives in thermophilic temperature (Fontana et al., 2018a; Giuliano et al., 2014). In methanogens communities, *Methanobacterium* activity gradually increases along with the increase in temperature, and outpace the *Methanothrix* activity at 43 °C and 45 °C. This finding signifies potential shift in CH_4 production pathway from acetoclastic (promotes by *Methanothrix*) to hydrogenotrophic (promotes by *Methanobacterium*) which further explains the wide discrepancy between CH_4 and CO_2 production.

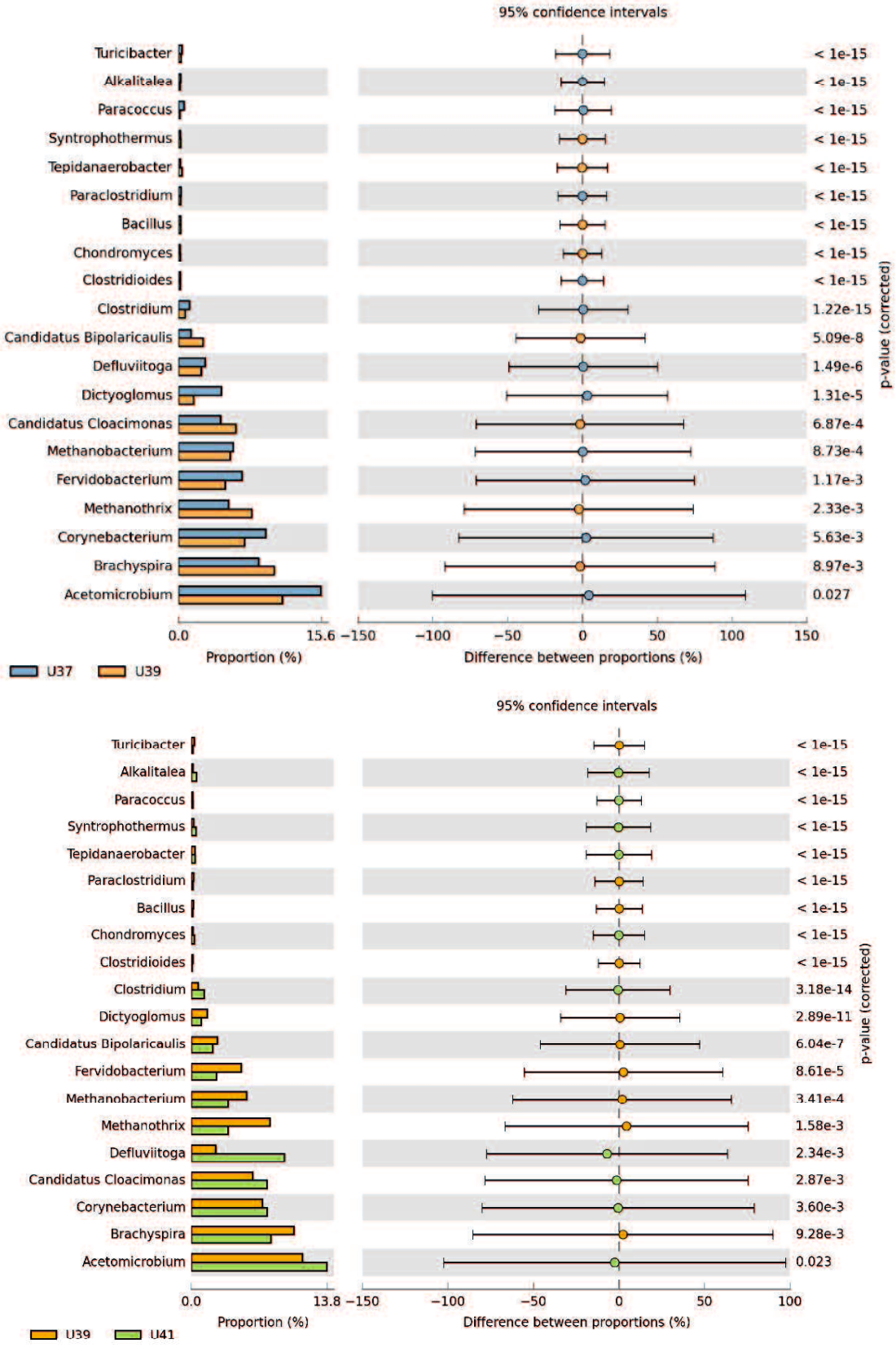


Figure 4. 6 *cont.*

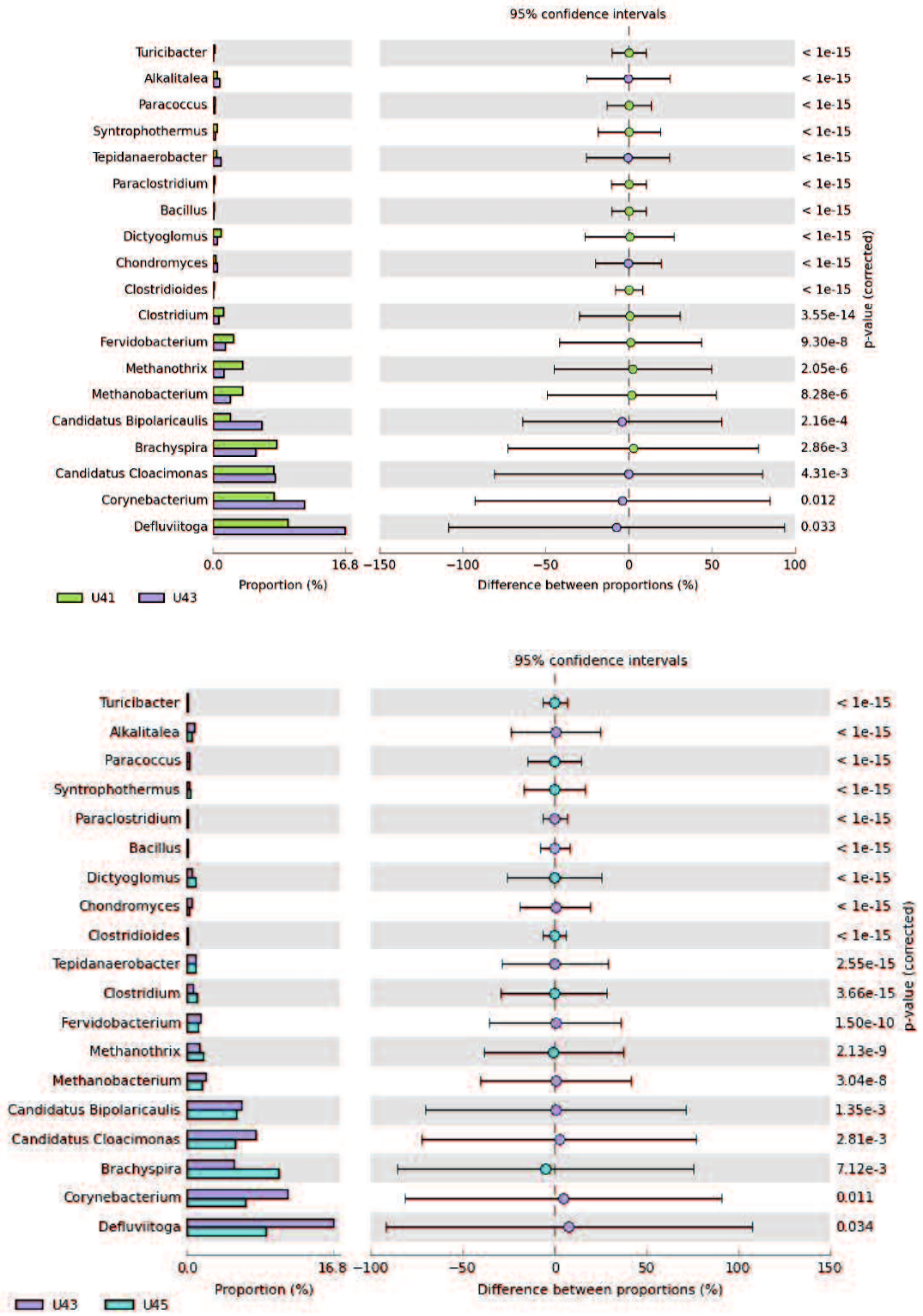


Figure 4. 6 Statistical comparison between sample for each temperature scenario. The left part of the panel represents the proportions of microbial communities (>0.5%) while the right part shows the difference between proportions with 95% confidence interval and the p-value estimation for each differences.

4.3.4 Temperature shift affects the critical metabolic functions and syntrophic relationship between several primary methanogenesis microbial communities

To comprehend the influence of temperature shifts on microbial metabolic pathways, a comprehensive KEGG analysis using KEGG database was conducted across five temperature conditions. In Figure 4.7(a), we observe that 44% - 87% of the genes were involved in metabolism pathways, followed by biosynthesis pathway (19 – 32%) and RNA degradation (9 % - 16%). Within the metabolism pathway, galactose metabolism and sugar metabolism dominated the metabolic functions in all temperature conditions which was supported by the abundance of fermentative bacteria. Galactose metabolism, amino sugar and nucleotide sugar metabolism (classified as carbohydrate metabolism) both have similar trends as both metabolisms are carried out by prevailing fermentative bacteria. These metabolisms showed an increasing abundance of gene from 37 – 41 °C. However, the gene abundance exhibited a 33% decline after the temperature was shifted to 43 °C, also followed by a significant drop in methane metabolism.

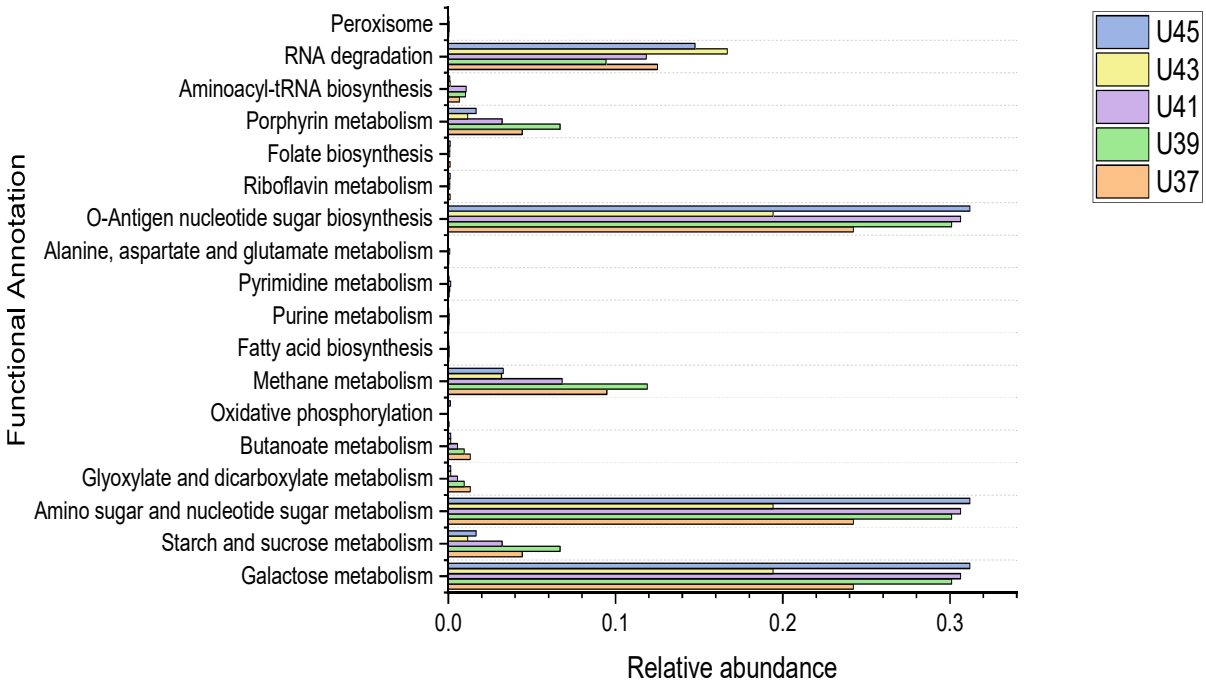
As seen in Figure 4.7(b), the acetoclastic pathway in methane metabolism annotated by the KEGG mapper was 62-77% dominated by acetate kinase (ackA), a pivotal enzyme in acetogenesis catalyzing the conversion of acetate to acetyl phosphate and adenosine diphosphate (ADP) (Barnhart et al., 2015). This gene showed a resiliency toward temperature shift conditions as this process is mainly performed by fermentative bacteria. However, the other genes that regulate the formation of acetyl-CoA as a main substrate for CH_4 production, such as those encoded phosphate acetyltransferase (pta) and acetyl-CoA synthetase (ACSS1_2), showed a mixed response towards temperature shift. Acetyl-CoA synthetase (ACSS1_2, EC: 6.2.1.1) is a crucial component of the acetyl-CoA decarboxylase/synthase (ACDS) responsible for acetate breakdown (Grahame, 2011; Zhang et al., 2022). The ACSS1_2 genes showed a 40% drop after the temperature was set to 41 °C and fluctuated in 8% range. Meanwhile, pta showed a continuous 20% increase in abundance along the way to 41 °C and slightly decreased at 45 °C.

In contrast, the major functional enzymes for hydrogenotrophic pathway such as Formylmethanofuran dehydrogenase (fwd, EC: 1.2.7.12), formyltransferase (FTR, EC: 2.3.1.101) and methenyltetrahydromethanopterin cyclohydrolase (mch, EC: 3.5.4.27) showed a considerable decrease after the temperature was shifted to 41 and 43 °C regime while slightly recover at 45 °C. The three enzymes are specifically catabolized by the genus

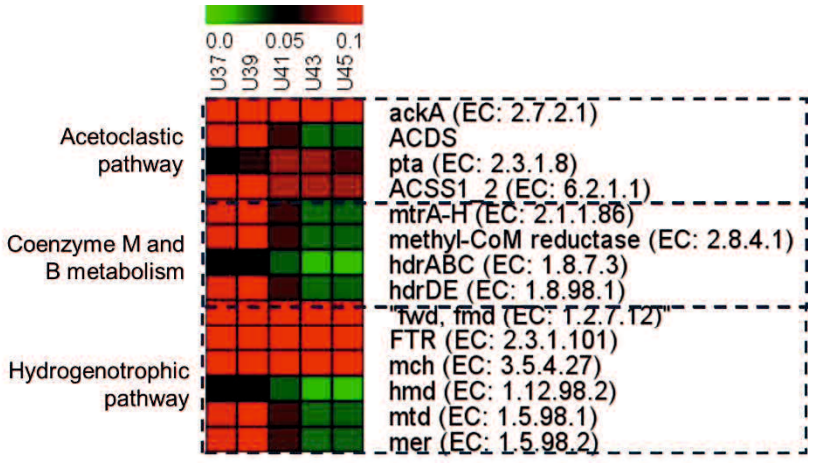
Methanobacterium and are involved in CH_4 formation from CO_2 (Welte and Deppenmeier, 2014). Despite the slow recovery at 45 °C, the syntrophic relationship between acetogenic bacteria, SAOB, and hydrogenotrophic methanogens may expedite the production of CH_4 via the hydrogenotrophic pathway. As seen in Figure 4.7 (c), the hydrogenotrophic *Methanobacterium* potentially obtained support from the growing abundance of SAOB that ensured the availability of CO_2/H_2 in the digester via reductive WLP pathway. Despite being a support for acetoclastic methanogens, *Defluviitoga sp.* may also enhanced the conversion of CO_2 to CH_4 as these strains catalyses the reduction of CO_2 to n-formyl-methanofuran which was primary metabolite for CH_4 production via hydrogenotrophic methanogenesis (Maus et al., 2016). The same properties also found in *Tepidanaerobacter sp.* beside supporting hydrogenotrophic methanogenesis with CO_2 and H_2 via acetate oxidizing pathway.

The domination of *Acetomicrobium sp.* at 43 and 45 °C may also lead to lower CO_2 level as this strain was capable to use CO_2 as their carbon source via reductive glycine pathway (RGP) and the glycine synthase-reductase pathway (GSRP) (De Bernardini et al., 2022). Previous study also found that *Anaerobaculum sp.* (basionym of *Acetomicrobium sp.*) and *Defluviitoga sp.* enhanced the electron transport chains genes, which aids H_2 scavenger during interspecies hydrogen transfer (IHT) mechanism (Fontana et al., 2018b). The rising abundance of *Coprothermobacter sp.* boosts more pyruvate production as this strain was previously identified to upregulate ferredoxin oxidoreductase (PFOR) enzyme that converts excess acetate into pyruvate (Fontana et al., 2018b). Pyruvate is an essential metabolite for acetyl-CoA synthesis for CH_4 production via acetoclastic methanogenesis.

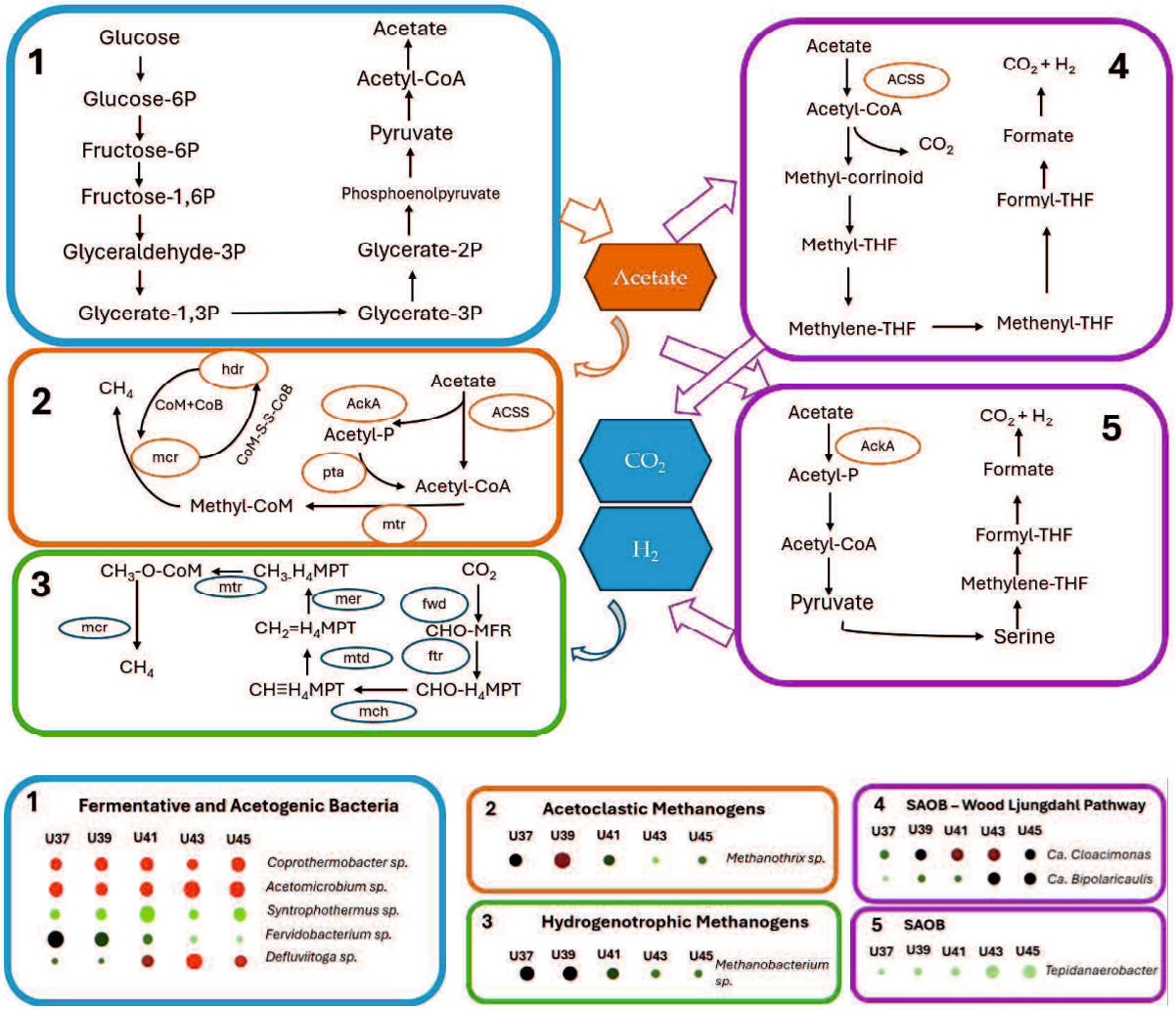
In the concluding stages of the methanogenesis pathway, the production of CH_4 was directly influenced by the synthesis of coenzyme M (CoM) and B (CoB). Nonetheless, the genes responsible for CoM and CoB synthesis exhibited relatively low abundance which decreased with rising temperatures. CoM and CoB enzymes are mainly catalysed by methanogens which are involved in the export of sodium ions and are also associated with the energy-conserving process performed by methanogens, mainly *Methanobacterium* (Hippler and Thauer, 1999; Upadhyay et al., 2016). Although the abundance of methanogens decreased, the observed increase in CH_4 production and the subsequent reduction in CO_2 indicate that CH_4 production was not exclusively reliant on methanogen presence. Rather, it was likely enhanced through a syntrophic relationship between methanogens, fermentative and acetogenic bacteria. These bacteria upregulate several enzymes, supplying essential metabolites that support methanogens to efficiently enhance CH_4 productivity.


Functional Annotation
Peroxisome
RNA degradation
Aminoacyl-tRNA biosynthesis
Porphyrin metabolism
Folate biosynthesis
Riboflavin metabolism
O-Antigen nucleotide sugar biosynthesis
Alanine, aspartate and glutamate metabolism
Pyrimidine metabolism
Purine metabolism
Fatty acid biosynthesis
Methane metabolism
Oxidative phosphorylation
Butanoate metabolism
Glyoxylate and dicarboxylate metabolism
Amino sugar and nucleotide sugar metabolism
Starch and sucrose metabolism
Galactose metabolism
0.0
0.1
0.2
0.3
Relative abundance
U45
U43
U41
U39
U37

(a)


0.0
0.05
0.1
U37
U39
U41
U43
U45
Acetoclastic pathway
Coenzyme M and B metabolism
Hydrogenotrophic pathway
ackA (EC: 2.7.2.1)
ACDS
pta (EC: 2.3.1.8)
ACSS1_2 (EC: 6.2.1.1)
mtrA-H (EC: 2.1.1.86)
methyl-CoM reductase (EC: 2.8.4.1)
hdrABC (EC: 1.8.7.3)
hdrDE (EC: 1.8.98.1)
fwd, fmd (EC: 1.2.7.12)
FTR (EC: 2.3.1.101)
mch (EC: 3.5.4.27)
hmd (EC: 1.12.98.2)
mtd (EC: 1.5.98.1)
mer (EC: 1.5.98.2)

(b)



(c)

Figure 4. 7 Pathway dominance over the metabolism found in each temperature conditions sample (a); methanogenesis metabolic pathway maps for acetoclastic and hydrogenotrophic built using KEGG database (red arrow: hydrogenotrophic methanogenesis, blue arrow: acetoclastic methanogenesis, green arrow: acetogenesis, black arrow: common pathway for methanogenesis) (b); relative abundance of genes involved in the formation of essential enzymes for methanogenesis (c).

4.4 Conclusion

This comprehensive investigation delved into the intricate interplay between temperature shifts, AD processes, microbial diversity, and methanogenesis pathways. Commencing incubation at 37 °C, our study unravelled nuanced responses across multiple facets, encompassing biogas production, microbial community structures, and metabolic pathways.

A standout observation was the potential enhancement of hydrogenotrophic methanogenesis, notably at 43 °C and 45 °C, due to the advance CH_4 production with lower CO_2 production. This finding was supported by the increasing activity of *Methanobacterium* over *Methanothrix* at higher temperature, in syntrophic relationship with fermentative bacteria such as *Acetomicrobium*, *Defluviitoga, Candidatus Cloacimonas, Candidatus Biploaricaulis,* and *Tepidanaerobacter* as notable acetogenic bacteria. Despite the decrease in methanogen abundance, increased CH_4 production and reduced CO_2 levels suggests that CH_4 production is not solely dependent on methanogen presence. Instead, it is likely augmented by a syntrophic relationship with fermentative and acetogenic bacteria, which upregulate enzymes to supply critical metabolites, thereby enhancing CH_4 productivity. These findings illuminate the potential shift in methanogenesis pathway in AD processes, as an effort from microbial communities to survive amidst temperature shifts, providing valuable insights for the optimization of biogas production. Further, higher CH_4 production with lower methanogens abundance may provide a clue that methanogens have a potential of increasing capabilities and efficiency in CH_4 production after given a temperature pressure. In the future, incorporating temperature variation scenarios into continuous reactors could greatly enhance research directions. This method offers the potential to uncover more profound understandings of microbial community behaviors and biogas generation under various environmental circumstances, thereby enriching our comprehension of anaerobic digestion processes.

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CHAPTER V
HIGHER GLUCOSE LEVEL PRESERVE STABLE DIGESTION PROCESS DURING STEPWISE TEMPERATURE UPSHIFT THROUGH ACETOCLASTIC PATHWAY

5.1 Introduction

Concerns regarding the production of sludge from wastewater treatment plants (WWTPs), originating from processes like primary sedimentation (PS) and activated sludge (AS), have become significant on a global scale. This issue is particularly pronounced in various countries, including EU nations, the United States, China, Iran, Turkey, Canada, and Brazil, which collectively contribute to an annual cumulative output of sewage sludge. Specifically, these nations produce approximately 8910, 6510, 2960, 650, 580, and 370 thousand metric tons, respectively (Mateo-Sagasta et al., 2015). Given the substantial volume of sludge generated, the handling and disposal of sewage sludge have evolved into critical international issues (Nazari et al., 2018).

To address the challenges associated with WWTPs sludge, a viable solution lies in anaerobic digestion (AD) technology. AD has the capability to produce bioenergy and other byproducts by processing diverse residual biomass materials, including sewage sludge, primary sludge, digested sludge, and waste-activated sludge (Campo et al., 2018; Hanum et al., 2019; Rühl et al., 2022). The products recovered from the AD process, including volatile fatty acids (VFAs) and biogas (mostly composed of methane (CH_4) (50-70%), carbon dioxide (CO_2) (30-50%), and other chemicals like ammonia (NH_3) and hydrogen sulphide (H_2S) (Porté et al., 2019)), make AD a promising approach. CH_4 production in AD depends on the engagement of various microorganisms with unique physiological characteristics encompassing four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Wu et al., 2022). The conversion of substrates into CH_4 depends on key components such as acetate (HAc), hydrogen (H_2), and CO_2, occurring through two primary methanogenic pathways: acetoclastic and hydrogenotrophic pathways (Tian et al., 2018b). *Methanosarcinaceae spp.* and *Methanosaetaceae spp.* are common in the acetoclastic pathway, while *Methanomicrobiales spp.* and *Methanobacteriales spp.* primarily influenced the hydrogenotrophic pathway (Kurade et al., 2019).

Throughout the four stages of CH_4 production, temperature plays a significant role in maintaining metabolic stability and biogas production, as microbial communities involved in AD are susceptible to perturbations caused by changing thermal conditions (Nie et al., 2021). Numerous studies have demonstrated how temperature conditions affect overall AD performance, causing imbalances in microbial communities, especially for acetogenic and methanogenic microorganisms (Hupfauf et al., 2018; Kovalovszki et al., 2020; Madigou et al., 2019). Another researchers, demonstrated that hydrolytic bacteria tend to be more receptive toward temperature conditions (Chen and Chang, 2020). Nevertheless, despite the established impact of temperature and extensive research in this area, precise effects of continuous temperature changes on the AD process remain less understood. Notably, there is a lack of understanding concerning the influence of dynamic temperature variations on the temperature adaptability (thermotolerance) of microorganisms participating in the AD process.

AD is commonly conducted within three distinct temperature ranges: psychrophilic (20 °C) (Fernández-Rodríguez et al., 2016), mesophilic (20–43 °C) with optimum temperatures ranging from 35–37°C (Wang et al., 2018), and thermophilic (50–60°C) (Madigou et al., 2019). Commercial-scale AD systems predominantly operate at mesophilic and thermophilic temperatures, with CH_4 production rates averaging between 0.03–0.65 L/g volatile solid (VS)/day and 0.04–0.70 L/g-VS/day, respectively (Kasinski, 2020; Nie et al., 2021). Despite extensive research conducted in these conventional temperature ranges, several challenges persist in the operational practices of AD processes, including the limited consideration given to the intermediate temperature range between mesophilic and thermophilic (40–45 °C) and the corresponding lack of understanding regarding the microorganisms inhabiting this range and their ability to adapt to both mesophilic and thermophilic conditions.

Recent studies in AD primarily focused on stable temperature conditions within individual reactors, aiming to reduce potential instability in microbial communities (Hidaka et al., 2022; Roopnarain et al., 2021). Consequently, limited attention has been devoted to temperature-adjusted AD processes, and the specific impact of temperature variations on biogas production and the dynamics of methanogenic bacteria remains poorly elucidated. Furthermore, comprehensive exploration of microbial populations and their intricate interactions in AD has been hampered by the complexity of microbial networks. Understanding the essential ecological niches within the AD process and the metabolic characteristics of microbial communities inhabiting these niches could pave the way for

innovative process designs and operational techniques, enhancing CH_4 recovery from organic waste. This study was motivated by the aforementioned challenges and aimed to investigate the fluctuations in biogas production and the interconnections among microbial communities under temperature shifts conditions.

In Chapter III, we investigated temperature shift scenarios: upshifted and downshifted (Sudiartha et al., 2023, 2022). However, in the upshifted scenario we encountered substantial disturbances within the temperature range of 40 – 44 °C, which other researchers classified as 'too low for thermophilic and too high for mesophilic' temperatures (Tezel et al., 2014; Westerholm et al., 2015). These conditions led to a significant decline in microbial communities and the absence of several essential methanogenic pathways. In Chapter IV, we performed AD at the optimum mesophilic temperature for all bacteria involved in methanogenesis and subsequently raising the operating temperature to thermotolerant temperature ranges with low glucose level (1.5 g/L) as representation of “food starvation”. In this Chapter V, we mimic the approach in Chapter IV with higher glucose level (10 g/L) as representation of “food abundance” and investigated the potential microbial dynamics and biogas production. The outcomes of this investigation contributed to a broader understanding of microbial behavior during temperature fluctuations in the context of methanogenesis.

5.2 Materials and methods

5.2.1 Inoculum and substrates

The anaerobically digested sludge used as the inoculum was obtained from the Eastern Ube Wastewater Treatment Plant in Ube City, Yamaguchi Prefecture, Japan. Table 5.1 provides detailed characteristics of the inoculum source. In the biogas production process, a glucose-based substrate with a concentration of 10 g/L of glucose was employed as the carbon source. Before initiating the AD process, the inoculum sample was mixed with the substrate at an inoculum-to-substrate ratio of 2:1 (w/v). The substrate solution was created by combining glucose with essential nutrients crucial for bacterial growth. These nutrients comprised (all in g/L): 4 $NaHCO_3$, 4 K_2HPO_4, 0.1 yeast extract, 85 NH_4Cl, 81 $MgCl_2{\cdot}6H_2O$, 75 KCl, 350 $(NH_4)_2HPO_4$, 42 $FeCl_3{\cdot}6H_2O$, 25 $MgSO_4{\cdot}7H_2O$, 1.8 $CoCl_2{\cdot}6H_2O$, and 150 $CaCl_2{\cdot}2H_2O$ (Zhou et al., 2006).

Table 5. 1 Characteristics of anaerobically digested sludge obtained from Eastern Ube Wastewater Treatment Plant, Yamaguchi Prefecture, Japan

Parameter	**Anaerobic digested sludge**
pH	6.09
Total Solid (TS) (mg/L)	5,714
Volatile Solid (VS) (mg/L)	3,809
Fixed Solid (FS) (mg/L)	1,904
Chemical Oxygen Demand (COD) (mg/L)	1,520
Acetate concentration (mg/L)	80.29
Propionate concentration (mg/L)	495.29

5.2.2 Experimental setup

The experiment utilized 500 mL serum vials with working volume of 300 mL. A fed-batch fermentation method was employed implementing a 20-days hydraulic retention time (HRT) estimated using the first-order kinetic equation, as detailed in previous studies (Caillet and Adelard, 2021; Wongarmat et al., 2022). During the initial incubation phase, 100 mL of substrate was introduced into the serum bottles. The vials were then tightly sealed with aluminium covers and butyl rubber stoppers, undergoing thorough nitrogen gas flushing to eliminate any residual oxygen, ensuring the establishment of anaerobic conditions. Incubation commenced at a temperature of 37 °C (referred to as U37) and continued for a duration of 80 days. The incubation setup was agitated at 100 rpm utilizing the EYELA SLI-700 incubator with a shaker. The selection of 37 °C as the initial acclimatization temperature was based on the prevalence of a diverse range of microorganisms with optimal growth temperatures in this vicinity, particularly favouring methanogens (Arikan et al., 2015; Song et al., 2023). While previous studies suggested incubation times of 21 days (Ángeles et al., 2021; Tetteh and

Rathilal, 2022), a longer operating period was chosen in the context of temperature variations. This extended period allows microorganisms to adapt to challenging conditions, promoting more stable operation (Sudiartha et al., 2023; Sun et al., 2015; Westerholm et al., 2015). In the current study, the temperature was shifted in the span of 80 days, the temperature was incrementally increased from 37 °C to 39 °C (U39), 39 °C to 41 °C (U41), 41 °C to 43 °C (U43), and finally to 45 °C (U45).

5.2.3 Data collection and analysis

The gas chromatography method was employed in the present study to observe the biogas production and volatile fatty acid (VFA). The biogas volume generated from AD was measured using the glass syringe method. Analysis of gas samples, encompassing H_2, N_2, CH_4, and CO_2, was performed using a GC-8APT/TCD instrument (Shimadzu Corp., Kyoto, Japan) equipped with a 60/80 activated charcoal mesh column measuring 1.5 mm × 3.0 mm internal diameter, with argon serving as the carrier gas. Throughout the analytical procedure, the injector, column, and detector temperatures were maintained at 50 °C, 60 °C, and 50 °C, respectively. Meanwhile, the concentration of VFA was determined using GC-8APF with an FID detector (Shimadzu Corp., Kyoto, Japan). TS, VS, FS, and COD were determined according to the Standard Methods for the Examination of Water and Wastewater (Eaton et al., 2005).

5.2.4 DNA extraction and next-generation sequencing (NGS)

DNA samples (1.5 mL each) from different temperature conditions were collected and stored at -22 °C. Subsequent DNA extraction followed the NucleoSpin® kit protocol as outlined in the NucleoSpin® Soil Manual. The concentration of the extracted DNA was determined using the Qubit® dsDNA Assay Kit on the Qubit® 4.0 Fluorometer (Life Technologies, CA, USA). PCR amplification occurred in two stages. The first PCR targeted the V4 region of the 16S rRNA gene, employing 515F/806RC primers and KAPA HiFi Hot Start ready mix (TaKaRa Bio Inc., Shiga, Japan). Post-denaturation, a cleanup step with NucleoSpin® Gel and PCR Clean-up removed free primers and primer dimer species. The second PCR attached dual indices and Illumina Sequencing Adapters using the Nextera XT V2 Index Kit. Similar to the first PCR, denaturation was performed, but with fewer cycles (30x cycles for the first PCR and 12x cycles for the second PCR).

To eliminate unincorporated indices and prevent cluster formation during sequencing, a cleanup step was executed using AMPure XP Beads, 10 mM Tris pH 8.5, and 80% EtOH. High throughput sequencing took place on the Illumina MiSeq platform with the iSeq 100 cartridge at the Department of Environmental Engineering, Yamaguchi University, Japan. Taxonomic classification was performed through the Dragen Metagenomics Pipeline using the Kraken database. For alpha diversity analysis, the Shannon Diversity Index, faith_pd, and chao1 were employed using QIIME2 2023.7. These metrics provided insights into changes in microbial diversity and richness during anaerobic digestion under varying temperature conditions. Significance of differences in microbial communities across temperature conditions was assessed through extended error bar analysis using STAMP. The microbial heatmap was generated analyzed using Multiple Experiment Viewer (MeV) 4.9.0, while OriginPro 2022 was utilized for other data visualization purposes. This comprehensive approach allowed for a thorough examination of microbial dynamics in the context of AD under different temperature regimes.

5.2.5 Functional annotations

For the functional analysis purposes, raw sequencing data were processed using QIIME2 version 2023.7, denoised, filtered, and clustered using DADA2 plugin to obtain the representative sequences in FASTA file format. The obtained nucleotide sequences were annotated using the KEGG Automatic Annotation Server (KAAS) with the BBH (bi-directional best hit) method. The KAAS annotation was performed against the KEGG GENES database to assign KEGG Orthology (KO) numbers to the sequences, which represent functional genes in KEGG pathways (Kanehisa et al., 2016; Takami et al., 2012).

5.3 Results and discussion

5.3.1 Effects of temperature shifts on AD performance

The impact of temperature variations on the AD performance of digested sewage sludge was assessed, examining biogas production yield, biogas composition, and daily CH_4 production (Figure 5.1 (a),(b),(c), respectively). Each vial underwent an 80-day incubation to allow microbial communities sufficient time to adapt to temperature changes and establish stable metabolic pathways, including hydrolysis, acetogenesis, acidogenesis, and methanogenesis, essential for sewage sludge conversion into biogas. Extending the AD incubation period has

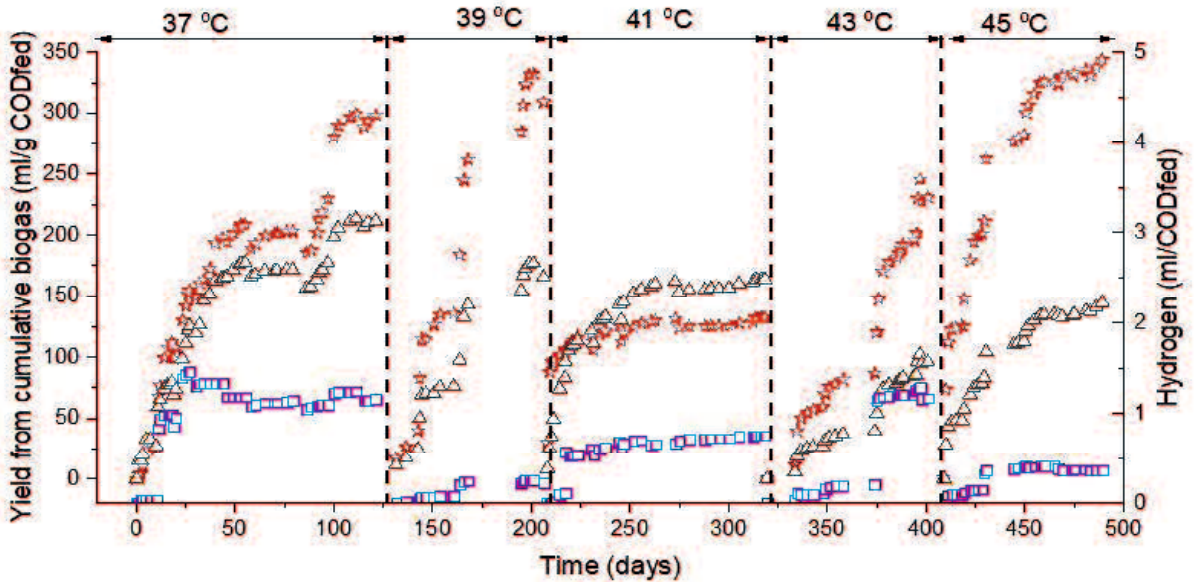
been advocated in challenging circumstances to facilitate microbial adaptation (Moestedt et al., 2014; Sun et al., 2015; Westerholm et al., 2015).

In the initial 10 days at 37 ºC, the vial exhibited high CO_2 production (66 mL CO_2/g COD) and a low CH_4 yield (15 mL CH_4/g COD). This outcome was expected indicating the onset of the acidogenesis process, with elevated CO_2 levels favouring acetogens. This facilitated the transformation of CO_2 into acetate, supporting subsequent methanogenesis. The present study observed a consistent acetogenesis process, evidenced by complete propionate digestion throughout the AD process (Figure 5.3). Syntrophic oxidation of propionate is crucial for the formation of CH_4 as 30% of the electrons produced during the breakdown of complex substrates pass via propionate during AD. Syntrophic oxidation of propionate is unfavourable unless the acetogenic bacteria are not inhibited and the hydrogen partial pressure is maintained at a low level (Lim et al., 2020).

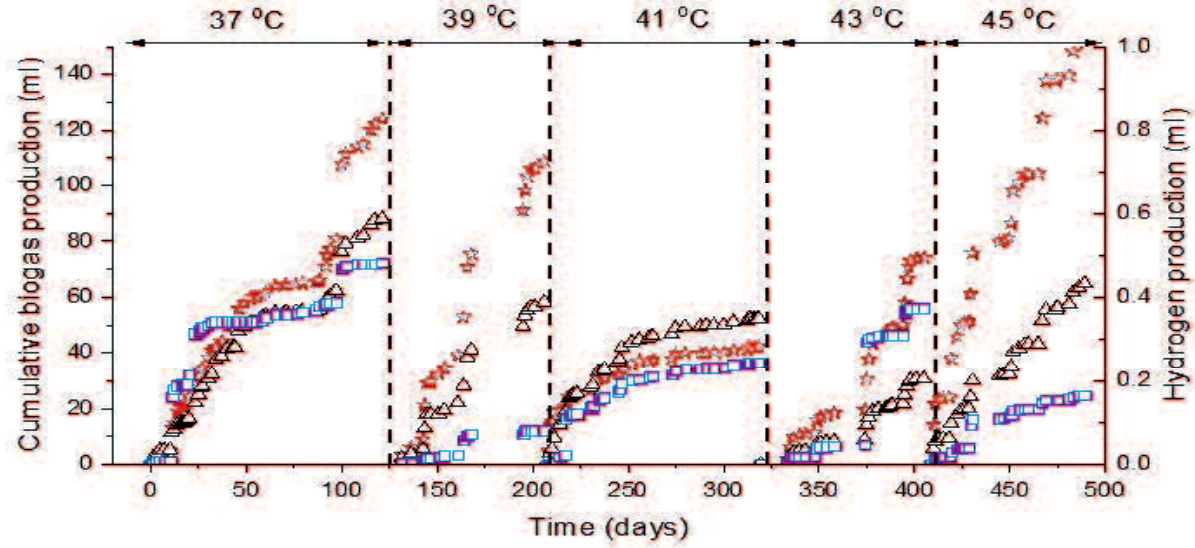
By the 14th day, CH_4 production surpassed CO_2, reaching a ratio of 1.2:1. At the end of the 37 ºC incubation period, CH_4 yield reached 245 mL CH_4/g COD, achieving an approximate of 70% conversion of COD into CH_4 if referring to the theoretical CH_4 yield achieved when COD is fully digested (350 mL CH_4/g COD) (Rühl et al., 2022). However, a temperature increase to 39 ºC resulted in a 25% reduction in CH_4 production (184 mL CH_4/g COD), consistent with previous studies indicating the sensitivity of methanogenic communities to temperature fluctuations (Beale et al., 2016; Sudiartha et al., 2023, 2022; Westerholm et al., 2018). Despite the decrease in CH_4 production, incubation at 39 °C generated a more stable CH_4 content (43-50%) with a lower peak compared to 37 °C (57% vs. 50%). Figure 5.1c confirmed this, showing a lower peak of daily CH_4 production at 39 °C (0.18 L_{CH4}/L_{vial}/day) compared to 37 °C (0.10 L_{CH4}/L_{vial}/day). Interestingly, the decrease in CH_4 production was not accompanied by a substantial increase in VFA concentrations. At 39 °C, only acetate level increased, while propionate levels remain below the limit of detection.

A further temperature increase to 41 °C caused an 11% decrease in CH_4 production, from 184.22 mL CH_4/g COD at 39 °C to 163.92 mL CH_4/g COD. This reduction was associated with a lower conversion ratio (below 38%) during the methanogenesis process. Additionally, CH_4 content predominantly fluctuated in the range of 20-30% which was considered low (Dai et al., 2016). The reduction in CH_4 generation at 41 °C aligns with earlier research findings showing significant disruption in AD processes when the operating temperature was varied within the 40–45 °C range (Nie et al., 2021; Sudiartha et al., 2023, 2022; Sudiartha and Imai,

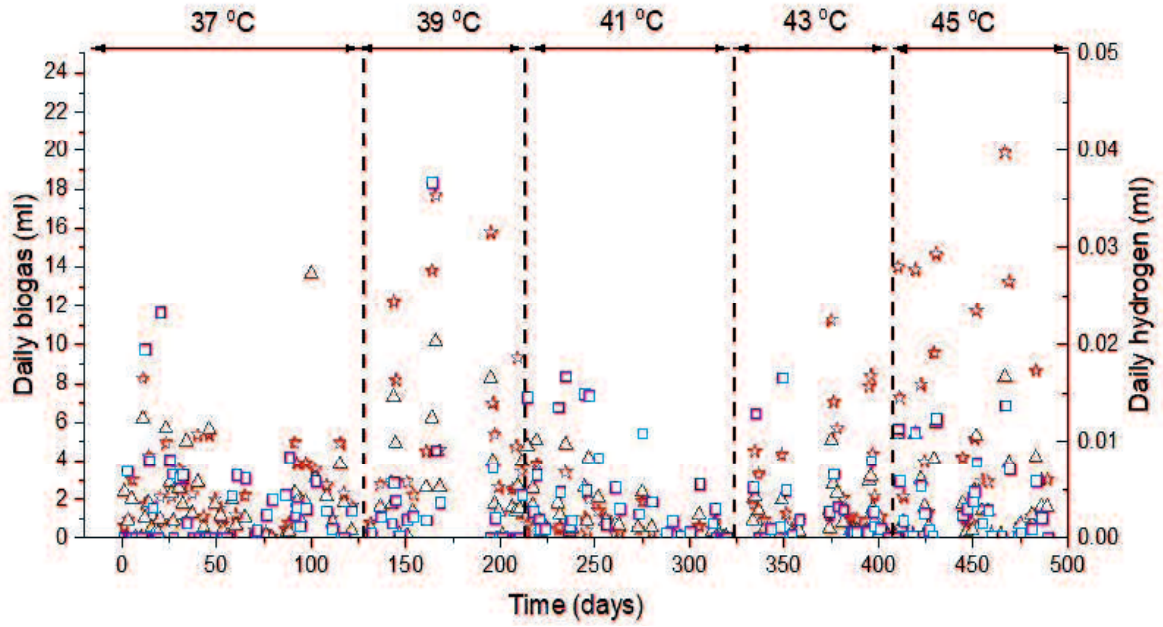
2022; Westerholm et al., 2018). However, a further temperature increase to 43 °C proved advantageous for the AD process, simultaneously recovering CH_4 production to 209 mL CH_4/g COD, with an increasing methanogenesis conversion rate. This suggests that microbial communities had successfully acclimatized to the changing temperature conditions, developing strains capable of tolerating temperatures too high for mesophilic strains and too low for thermophilic strains.



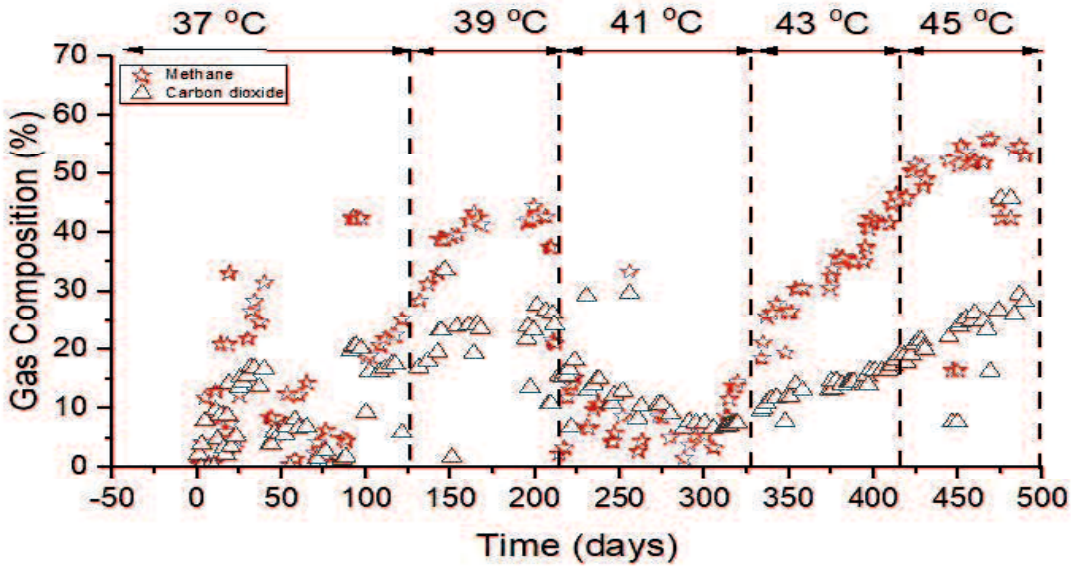
(a)



(b)



(c)



(d)

Figure 5. 1 Biogas yield from cumulative biogas production (a), cumulative biogas production (b), daily volumetric biogas production (c), and gas composition (d) under different temperature shift conditions.

Despite a slight reduction in CH_4 production at 45 °C, the CH_4 content remained stable at 40-50%, resulting in a CH_4 yield of 197 mL CH_4/g COD. This represents a noteworthy enhancement in stability compared to our previous study, where the AD process initiated directly at 42 °C (within the thermotolerant zone) and underwent a temperature shift of 3 °C

every 60 days. In that study, CH_4 production experienced a substantial 70% decline (Sudiartha et al., 2023, 2022). The findings suggest that initiating the AD process at an optimal temperature for acetogens and methanogens before any temperature shift may contribute to a more stable AD performance. This observation underscores the importance of early acclimatization at an optimal temperature, providing microbial communities the opportunity to establish robust metabolic pathways. Such pre-conditioning appears to enhance the overall resilience of the AD process, ensuring a more consistent and efficient CH_4 production even under subsequent temperature variations.

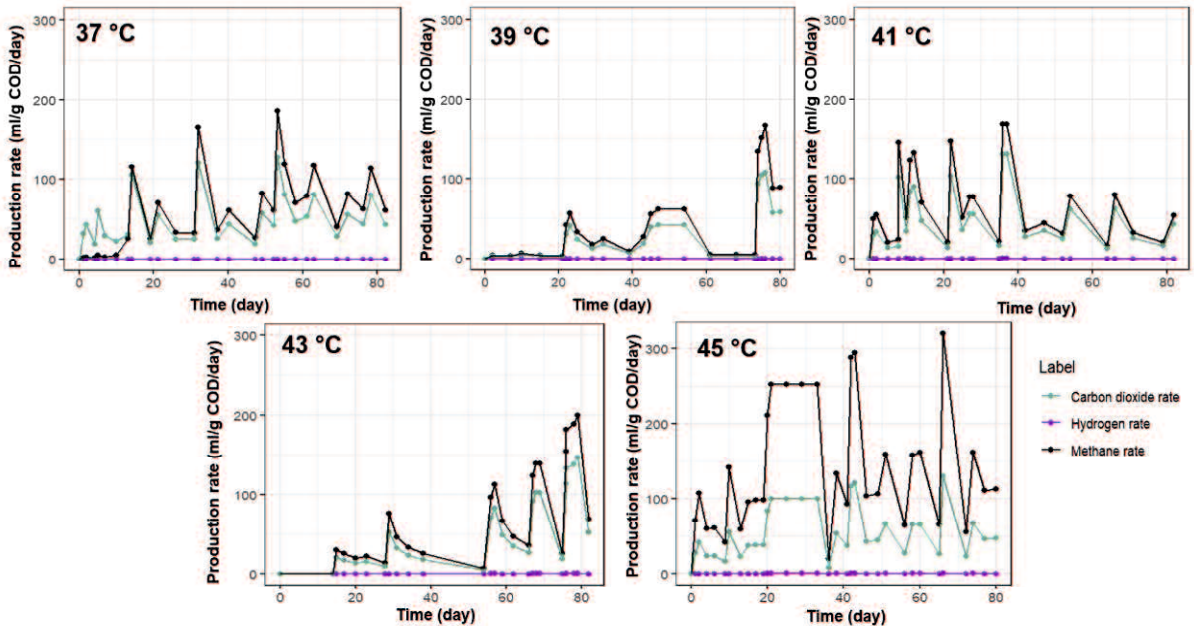


Figure 5. 2 Production rate in the upshifted temperature scenario in 10 g/L glucose level.

Figure 5.2 exhibited the relatively stable biogas production rate along with the shift in the temperature, from optimum mesophilic temperature to thermotolerant temperature range, in 10 g/L glucose level. This insight is valuable for optimizing AD strategies, emphasizing the significance of the initial temperature conditions on the subsequent performance of the AD process. The stable CH_4 content observed at 45 °C, despite a slight reduction in production, highlights the potential benefits of early acclimatization, offering a promising avenue for improving the stability and reliability of AD under changing temperature regimes.

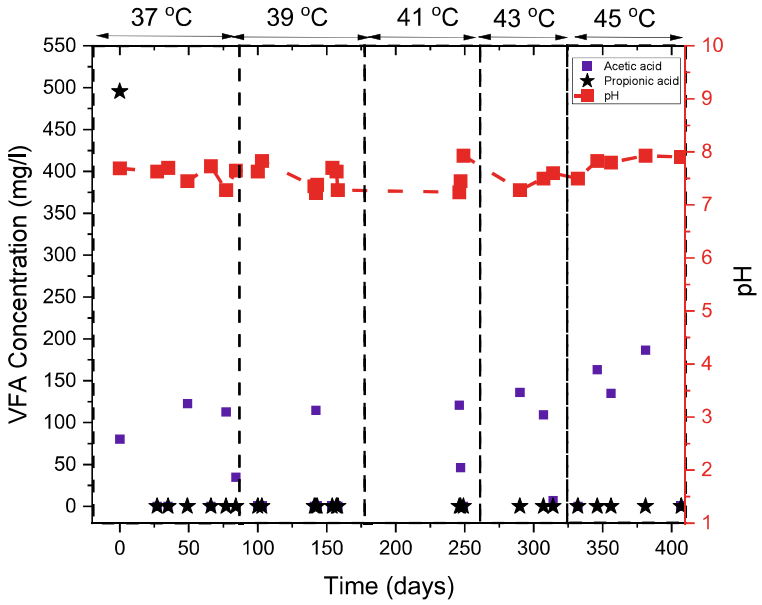


Figure 5. 3 VFA concentration in comparison to pH trends during incubation at the increasing temperature conditions.

5.3.2 Microbial diversity analysis

In this study, alpha diversity analysis, represented by chao1, faith_pd, and Shannon diversity (Figure 5.4), provided insights into the overall microbial community responses to varying temperature conditions. The utilization of diversity indices, such as those employed in previous studies focusing on environmental factors like temperature (Sudiartha et al., 2023; Sudiartha and Imai, 2022; Xiang et al., 2021; Zhang et al., 2016).

Among the three diversity indices, both the Shannon diversity index and faith_pd exhibited a similar pattern, revealing higher microbial diversity at 37 °C compared to the other temperature conditions. This outcome aligns with existing research demonstrating that mesophilic temperatures, particularly around 37 °C, foster greater microbial diversity than higher temperature environments (Chen et al., 2021; Liu et al., 2022; Steiniger et al., 2023). The decline in microbial diversity with increasing temperature suggests the elimination of several microbial communities unable to withstand the changing thermal environment.

Interestingly, a slight increase in diversity was observed at 43° C, potentially attributed to heightened activity and abundance of acetogens and methanogens in this temperature range. The increased conversion ratio of acetogenesis and methanogenesis at 43° C may have contributed to the observed rise in diversity. Despite these fluctuations, there was no

significant downward trend in microbial community diversity in our study. This is intriguing as microbial diversity is often considered and indicator of stability in the AD process. Previous research has suggested that diverse microbial communities exhibit greater resistance to disruptions, such as temperature fluctuations (Battin et al., 2016; Gunnigle et al., 2015; Steiniger et al., 2023).

The stability in microbial diversity, even under varying temperature conditions, emphasizes the robustness of microbial communities in the AD process. This resilience may be attributed to the adaptability of certain microbial groups to changing thermal environments, highlighting the intricate interplay between microbial diversity and process stability in AD systems. Further exploration into the specific microbial taxa thriving under diverse temperature regimes could provide valuable insights for optimizing AD processes and enhancing their overall stability.

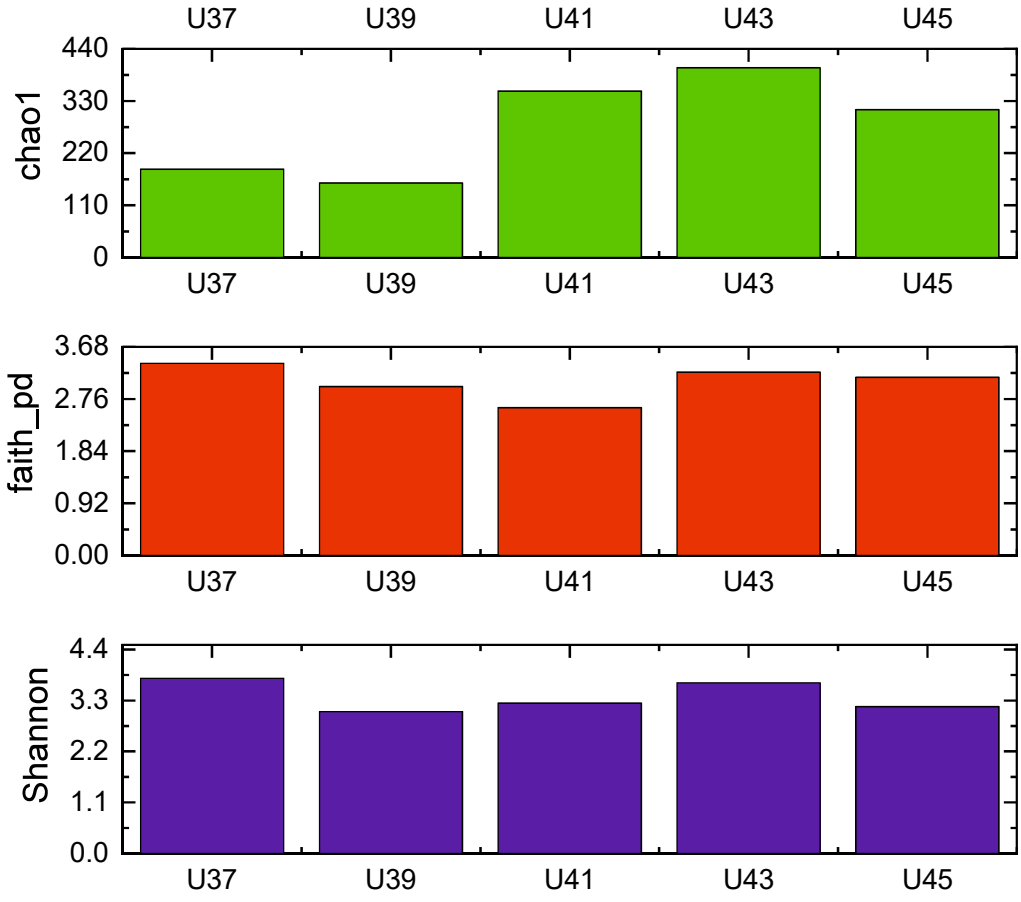


Figure 5. 4 Alpha-diversity analysis of microbial communities during incubation at the temperature shift scenario.

5.3.3 Microbial community dynamics under multiple temperature shifts

In our investigation, microbial communities exhibited dominance in the *Coprothermobacter* genus, constituting approximately 17 – 22% of relative abundance during temperature shifts. *Coprothermobacter,* known for its proteolytic capabilities, efficiently breaks down protein-rich waste, particularly in elevated temperature conditions (Dyksma et al., 2020). This bacterium, commonly found in sewage sludge, slaughterhouse waste, and cattle manure, engages in a syntrophic relationship with hydrogenotrophic methanogens (Luo et al., 2013; Palatsi et al., 2011; Tandishabo et al., 2012). In our study, *Coprothermobacter* displayed a positive correlation with hydrogen production (Figure 5.5), suggesting its involvement in supporting hydrogenotrophic methanogens for CH_4 production.

The role of methanogens in biogas production, especially through diverse methanogenesis pathways, likely explains variations in biogas production yields. As depicted in Figure 5.5, the dominant methanogen communities among the top 1% included *Methanothrix* (formerly *Methanosaeta*), *Methanoculleus*, and *Methanobacterium*, emphasizing their significant contributions to the methanogenesis process. *Methanothrix* predominated the methanogen communities in our study, comprising 2.1% of the total microbial communities at 37 °C , followed by *Methanoculleus* (1.2% of the total microbial communities) and *Methanobacterium* (1%). This dominance indicates that the acetoclastic pathway primarily drove CH_4 production despite temperature shifts. This finding aligns with a previous study reporting up to 70% of CH_4 produced via the acetoclastic pathway under normal AD conditions, while the hydrogenotrophic pathway requires a longer incubation time to take place (Khanal, 2008).

However, *Methanothrix* exhibited a noticeable decrease in abundance following temperature increments to 39 °C (1.6%) and 41 °C (1.7%). This decline was associated with low acetate concentration within these temperature ranges. Specifically, at 39°C, acetate levels were detected above the detection limit of the GC on day 77 (112.95 mg HAc/L) and day 84 (34.8 mg HAc/L), while at 41°C, acetate was found only on day 246 (121 mg HAc/L). The abundance of *Methanothrix* decreased sharply at 43 °C (1% abundance) after the acetate level drop further to 109 mg HAc/L. At this temperature level, a temporary shift in the methanogenesis pathway from acetoclastic to hydrogenotrophic occurred, with a 298% upsurge in the abundance of *Methanoculleus* replacing the dominance of *Methanothrix.* The rising population of *Methanoculleus* enhanced CH_4 production up to 22% at 43 °C. Changes in the methanogenesis pathway were likely influenced by factors such as ammonia stress and

the rising abundance of syntrophic-acetate oxidizing bacteria (SAOB) (Dyksma et al., 2020; H. Zhang et al., 2022). In the current study, *Tepidanaerobacter* was found as the most abundant SAOB and there was a significant increase in its abundance at 43 °C. However, the correlation between *Methanothrix* abundance to *Tepidanaerobacter* was insignificant.

At 45 °C, *Methanothrix* abundance spike to 2.3% following increase in acetate level to 163 mg HAc/L. Utilizing the Pearson correlation method (Fig. 5.5), *Methanothrix* abundance demonstrated a correlation coefficient of 0.74 with acetate concentration, emphasizing that *Methanothrix* growth primarily depends on acetate concentration and remains unaltered by temperature changes, as long as there is enough acetate in the AD vials. This finding further support previous studies that demonstrated the resilience of *Methanothrix* towards harsh changing temperature conditions and its reliance on acetate concentration due to its high acetate affinity and low substrates requirement, as long as there were around 100 – 150 mg HAc/L available in the environment (Kurade et al., 2019; Sudiartha et al., 2023; Sudiartha and Imai, 2022; Zhang et al., 2023).

Being an acetoclastic methanogen, *Methanothrix* reliant on acetate for CH_4 production, forms syntrophic relationships with acetogenic bacteria. The dominant acetogenic genera in our study were *Acetomicrobium*, *Corynebacterium*, and *Clostridium* (Dyksma et al., 2020; Kim et al., 2023; Westerholm et al., 2022). Despite consistently dominating the top 1% of the total microbial communities, *Acetomicrobium* exhibited a significant downturn in abundance ($p < 0.001$) during temperature shift treatment (Figure 5.6), indicating sensitivity to temperature changes. *Corynebacterium* proportion also decreased with the temperature increase, despite slight uptick at 39 °C ($p < 0.001$). In contrast to *Corynebacterium*, *Clostridium,* due to its thermotolerance, showed consistent growth after surpassing 39 °C.

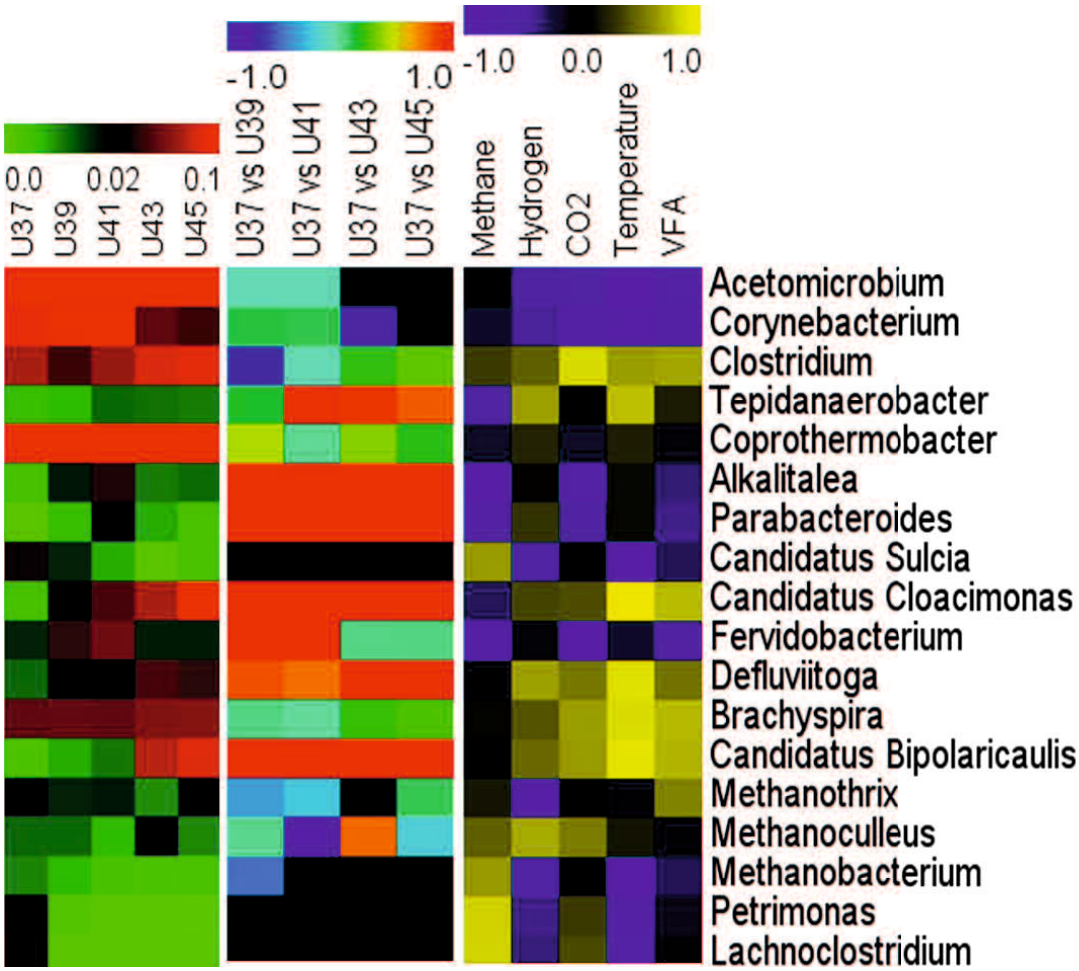
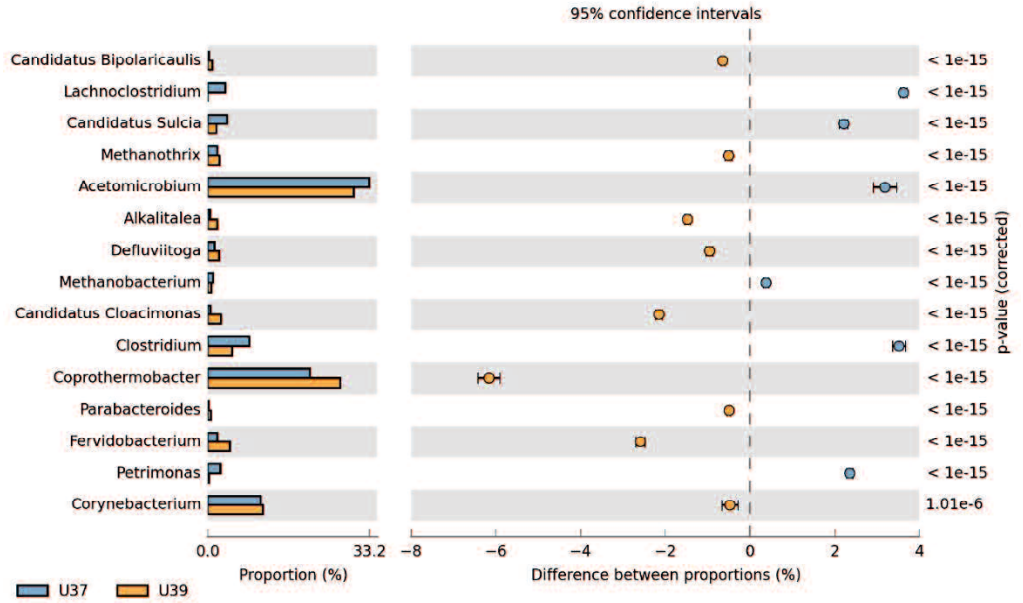
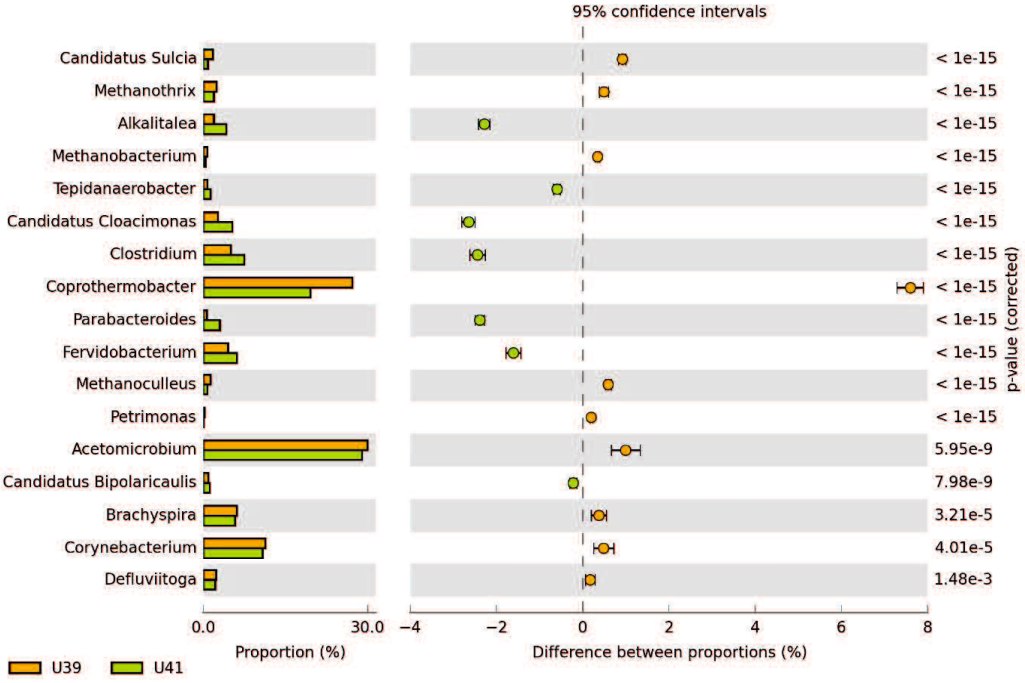


Figure 5. 5 Heatmap representing the relative abundance of the top 1% most abundant OTUs (left side), log2 changes of microbial communities compared to initial temperature incubation at 37 ºC (middle side), and Pearson correlation of OTUs relative abundance against AD parameters.

From the microbial communities analysis, the present study was able to identify how the stepwise increase in temperature affects the microbial dynamics that also lead to the fluctuations in CH_4 production. The temperature variations disrupted the overall abundance of microorganisms engaged in acetogenesis and methanogenesis, causing temporary shifts in CH_4 production pathways. However, since there were no radical changes in both CH_4 production and microbial composition suggests that the subtle approach, starting incubation at 37 ºC, may promote a more stable AD performance.


95% confidence intervals
Candidatus Bipolaricaulis
Lachnoclostridium
Candidatus Sulcia
Methanothrix
Acetomicrobium
Alkalitalea
Defluviitoga
Methanobacterium
Candidatus Cloacimonas
Clostridium
Coprothermobacter
Parabacteroides
Fervidobacterium
Petrimonas
Corynebacterium
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
1.01e-6
p-value (corrected)
0.0
33.2
Proportion (%)
−8
−6
−4
−2
0
2
4
Difference between proportions (%)
U37
U39


95% confidence intervals
Candidatus Sulcia
Methanothrix
Alkalitalea
Methanobacterium
Tepidanaerobacter
Candidatus Cloacimonas
Clostridium
Coprothermobacter
Parabacteroides
Fervidobacterium
Methanoculleus
Petrimonas
Acetomicrobium
Candidatus Bipolaricaulis
Brachyspira
Corynebacterium
Defluviitoga
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
5.95e-9
7.98e-9
3.21e-5
4.01e-5
1.48e-3
p-value (corrected)
0.0
30.0
Proportion (%)
−4
−2
0
2
4
6
8
Difference between proportions (%)
U39
U41

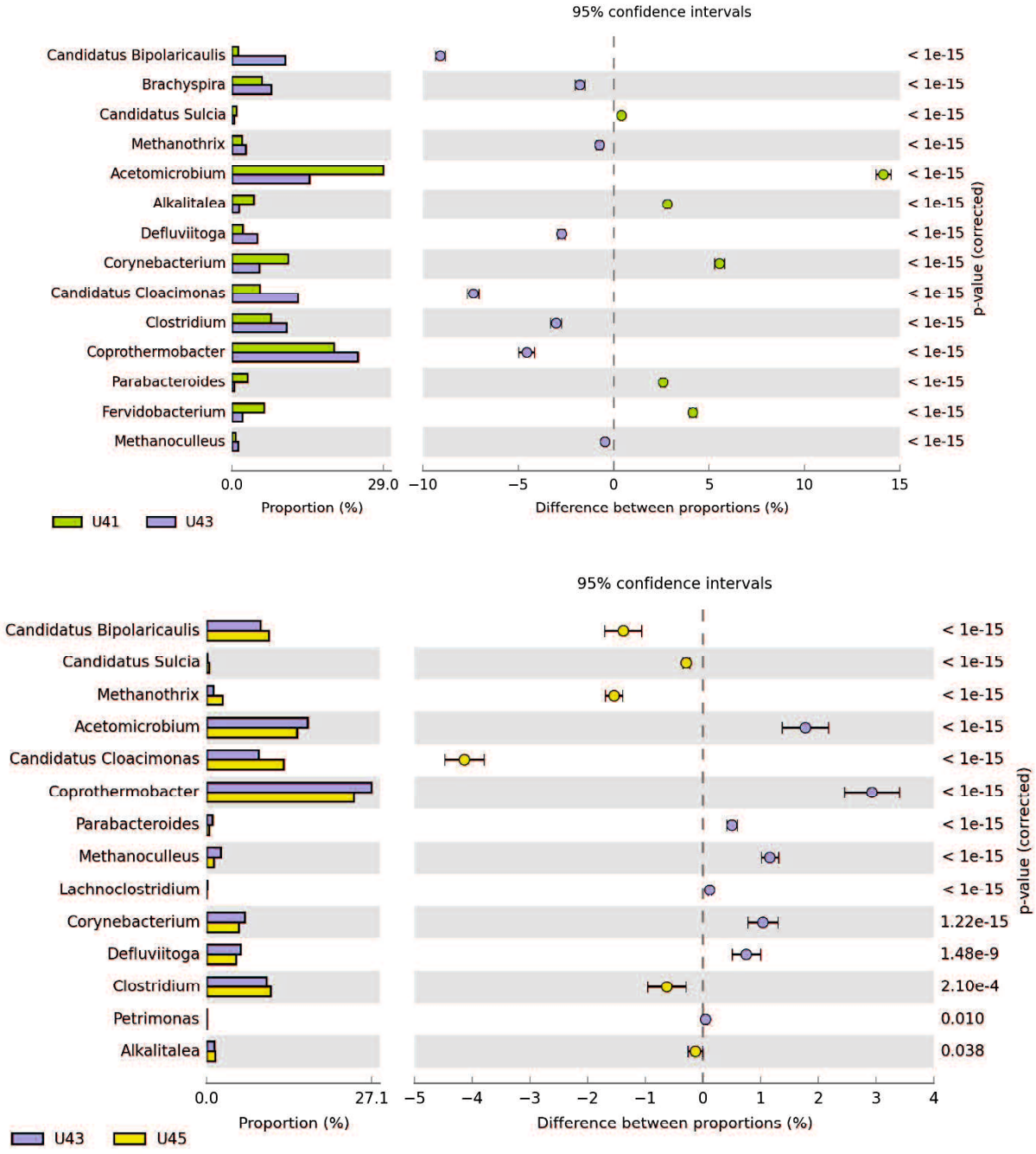


Figure 5. 6 Statistical comparison between sample for each temperature scenario. The left part of the panel represents the proportions of microbial communities (>0.5%) while the right part shows the difference between proportions with 95% confidence interval and the p-value estimation for each differences.

5.3.4 Influence of temperature shift AD on critical metabolic functions

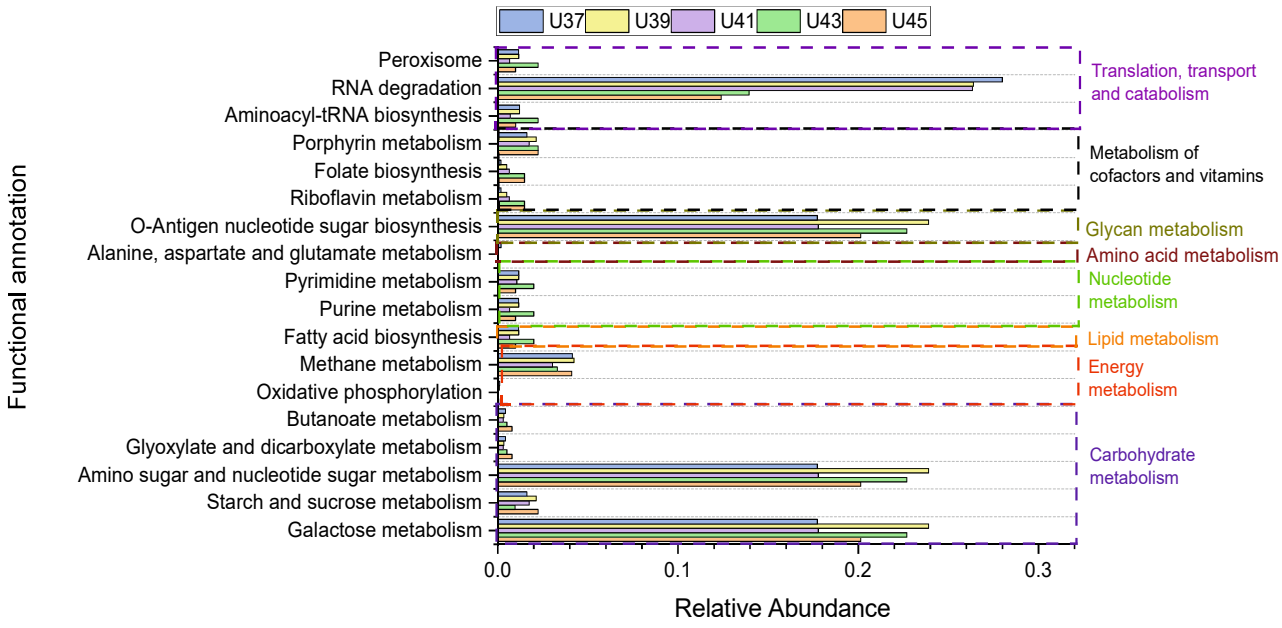
To comprehend the influence of temperature shifts on microbial metabolic pathways, a comprehensive KEGG analysis using KEGG database was conducted across five temperature

conditions. In Figure 5.7a, we observe that 64% - 85% of the genes were involved in metabolism pathways, followed by RNA degradation (12% - 28%) and biosynthesis pathways (20% - 28%). Within the metabolism pathway, carbohydrate metabolism, glycan metabolism, and energy metabolism dominated the metabolic functions with relative abundances of 37%, 17%, and 4% at 37 °C, respectively. Both carbohydrate and glycan metabolism exhibited parallel trends, peaking at 39 °C (12% increase) and decreasing up to 15% at 41 °C, before recovering at 43 °C and 45 °C. Similarly, energy metabolism (mainly methane metabolism) remained relatively stable at 37 °C and 39 °C (41% - 42%), but decreased by 12% at 41 °C. Notably, the gene abundance of methane metabolism recovered upon the temperature shift to 43 °C and 45 °C, suggesting a swift recovery of these essential pathways that likely contributed to the observed stable CH_4 production despite temperature fluctuations.

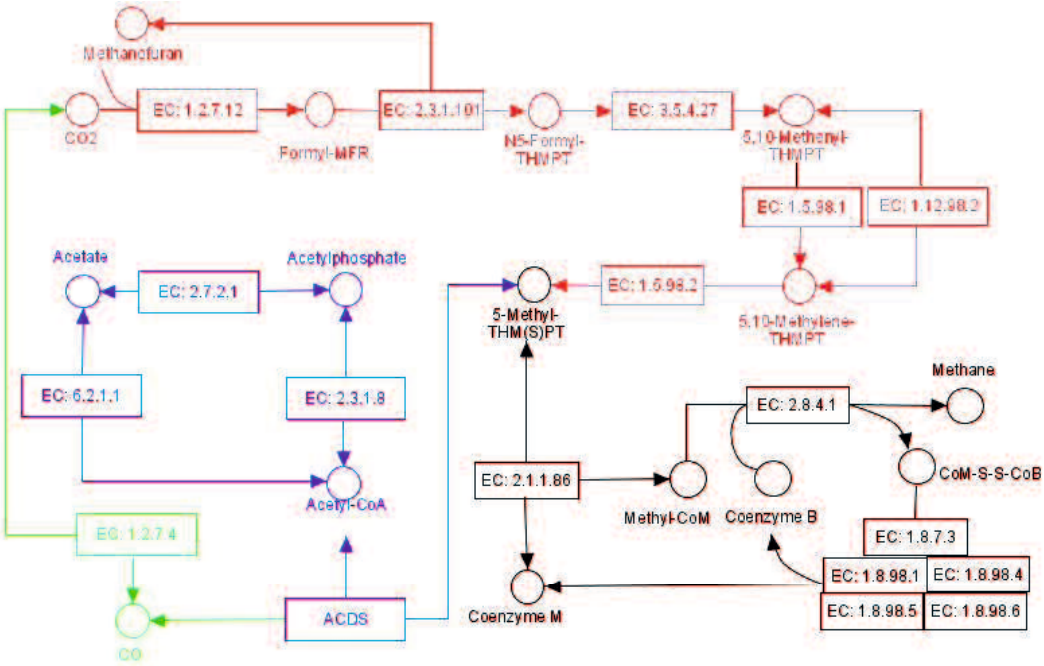
To delve deeper into the impact of temperature shifts on the methanogenesis process, a detailed analysis of microbial genes associated with CH_4 is crucial. The key enzymes involved in methane metabolism are depicted in Figure 5.7b. Acetate decarboxylation and CO_2 reduction emerged as dominant methanogenic pathways, with *Methanothrix* and *Methanobacterium* being the primary methanogens. This indicates that CH_4 production primarily occurred through acetoclastic and hydrogenotrophic methanogenesis, aligning with previous research (B. Wang et al., 2023; Wang et al., 2022). In Figure 5.7(c), approximately 77% of the microbial communities at 37 °C were associated with the acetate kinase (ackA), a pivotal enzyme in acetogenesis catalysing the conversion of acetate to acetyl phosphate and adenosine diphosphate (ADP) (Barnhart et al., 2015). Acetyl-CoA synthetase (ACSS1_2, EC: 6.2.1.1), a crucial component of the acetyl-CoA decarboxylase/synthase (ACDS) responsible for acetate breakdown, also showed significance in energy metabolism (Grahame, 2011; S. Zhang et al., 2022). The gradual decrease in the relative abundance of the ackA following temperature increase suggests potential adverse effects on this enzyme, in line with previous findings indicating declining ackA activity at incubation temperatures over 35 °C (Kushkevych, 2014). In contrast, ACSS and ACDS enzymes experienced a slight decline in gene abundance at 39 °C but quickly recovered at 43 °C. Notably, ACSS genes showed recovery even at 41 °C. *Methanothrix* abundance mirrored the trend ACSS genes abundance, indicating its dependency on the ACSS pathway rather than ackA. Within *Methanothrix*, acetate undergoes activation by ACSS enzymes, resulting in the formation of acetyl-CoA, accompanied by the hydrolysis of one ATP molecule to AMP and PPi (V_{max} = 55 μmol/mg/min and K_m acetate = 0.86 mM) (Barnhart et al., 2015; Jetten et al., 1992; Zhang et

al., 2020). These kinetic parameters are lower than those observed in other acetoclastic methanogens that convert acetate via ackA (Jetten et al., 1992). As the result, these distinctive kinetic properties elucidate the prevalence of *Methanothrix* in the current study, particularly in environments characterized by low acetate concentrations.

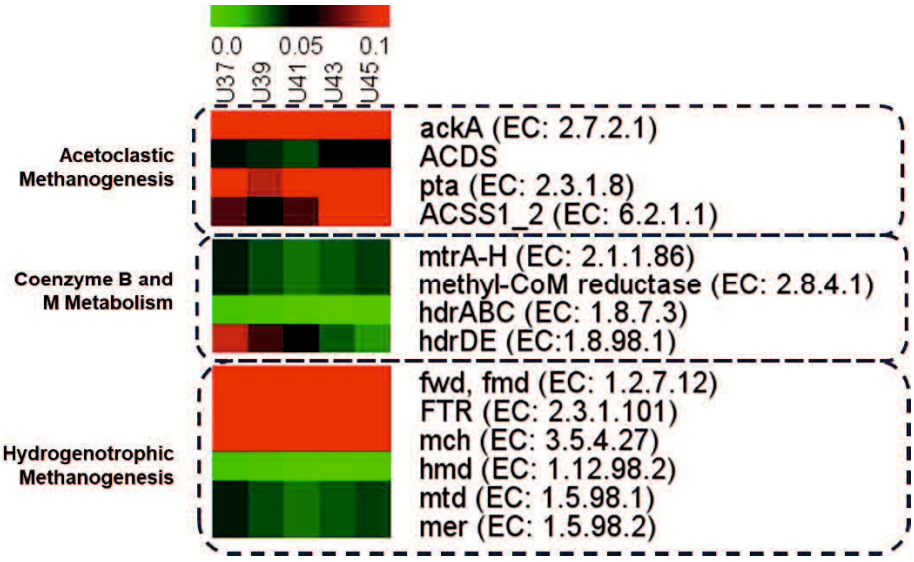
In hydrogenotrophic pathway, major functional enzymes—Formylmethanofuran dehydrogenase (fwd, EC: 1.2.7.12), formyltransferase (FTR, EC: 2.3.1.101) and methenyltetrahydromethanopterin cyclohydrolase (mch, EC: 3.5.4.27)—ranging from 17% - 39% in relative abundance. This finding was in line with the previous study that also found fwd enzyme domination in hydrogenotrophic pathway as this enzyme was the primary enzyme that catalysed the initial step of methanogenesis (B. Wang et al., 2023; Welte and Deppenmeier, 2014). However, although consistent with previous studies, the significant decrease in fwd genes (1% - 3%) with each temperature shift in our study suggests an adverse effect on the hydrogenotrophic pathway. FTR and mch genes also deteriorated after surpassing 39 °C. These two enzymes are specifically catabolized by genus *Methanobacterium* and involved in CH_4 formation from CO_2 (Welte and Deppenmeier, 2014). In the concluding stages of the methanogenesis pathway, the production of CH_4 was directly influenced by the synthesis of coenzyme M (CoM) and B (CoB). Nonetheless, the genes responsible for CoM and CoB synthesis exhibited relatively low abundance (0.9% to 8%), notably decreasing with rising temperatures.



(a)



(b)



(c)

Figure 5. 7 Pathway dominance over the metabolism found in each temperature conditions sample (a); methanogenesis metabolic pathway maps for acetoclastic and hydrogenotrophic built using KEGG database (red arrow: hydrogenotrophic methanogenesis, blue arrow: acetoclastic methanogenesis, green arrow: acetogenesis, black arrow: common pathway for methanogenesis) (b); relative abundance of genes involved in the formation of essential enzymes for methanogenesis (c).

Despite fluctuations in functional genes, the acetoclastic pathway displayed a remarkable stability in biogas production and metabolic resilience to temperature shifts, particularly evident in the swift recovery observed at of 43 °C and 45 °C. In contrast, the hydrogenotrophic pathway exhibited a comparatively less stable response. This aligns with previous research indicating that hydrogenotrophic methanogenesis is more susceptible to temperature variations compared to the robust CH_4 production demonstrated by acetoclastic methanogenesis (Krohn et al., 2022; Lavergne et al., 2021).

Prior studies have emphasized that, contrary to temperature influences, ammonia nitrogen, long-chain fatty acids, and high salt levels are more likely to inhibit acetoclastic methanogenesis. Our findings provide further support for the resilience of acetoclastic pathways to temperature fluctuations, reinforcing its potential for consistent CH_4 production even in the face of changing temperatures (Kurade et al., 2019; Tian et al., 2018a; H. Wang et al., 2023). The present study introduces a noteworthy insight into incubation approaches, revealing that initiating the process at a mesophilic temperature of 37 °C promotes more stable CH_4 production. This contrasts with previous studies that commenced incubation at higher temperatures, ranging from 40 °C to 45 °C (Sudiartha et al., 2023, 2022). The choice of the mesophilic temperature for early incubation appears to be a strategic approach in fostering a stable microbial community and ensuring sustained CH_4 production over time.

Our study contributes to a comprehensive understanding of microbial responses and biogas production dynamics during temperature shifts, particularly from mesophilic to thermotolerant conditions. To advance our knowledge further, incorporating temperature shift scenarios into continuous reactors could unveil deeper insights into how microbial communities adapt and influence biogas production. Additionally, future studies should consider exploring the impact of various environmental factors, such as VFAs, increased loading rates, pH alterations, and the introduction of free ammonia, under diverse temperature fluctuations. These comprehensive approaches will not only enrich our understanding in this domain but also find applications in broader research areas investigating the dynamic shifts in microbial populations across different environmental conditions.

5.4 Conclusions

This comprehensive investigation delved into the intricate interplay between temperature shifts, AD processes, microbial diversity, and methanogenesis pathways. Commencing incubation at 37 °C, our study unravelled nuanced responses across multiple facets,

encompassing biogas production, microbial community structures, and metabolic pathways. A standout observation was the robustness exhibited by the acetoclastic pathway, notably evident at 43 °C and 45 °C, in stark contrast to the heightened vulnerability observed in hydrogenotrophic pathways under temperature fluctuations. Microbial diversity analyses portrayed shifting community compositions, with a decline in diversity correlating with temperature increments. *Methanothrix*, a pivotal acetoclastic methanogen, emerged as a resilient player, showcasing an intriguing correlation with acetate concentration rather than temperature variations. These findings illuminate the steadfast nature of acetoclastic methanogenesis in AD processes, even amidst temperature shifts, providing valuable insights for the optimization of biogas production. Looking ahead, future research trajectories could benefit from integrating temperature shift scenarios into continuous reactors. This approach holds the promise of unravelling deeper insights into microbial community dynamics and biogas production across diverse environmental conditions, further enhancing our understanding of AD processes.

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CHAPTER VI
CONCLUSION

6.1 Conclusion

The current study can be concluded as follows:

- Starting the incubation at thermotolerant temperature ranges (41 – 45 ºC) and upshifting the temperature with an interval of 3 ºC leads to deterioration in biogas production as well as the abundance of methanogens and fermentative bacteria. In this scenario, the growth of *Methanosaeta* (also known as *Methanothrix*), the primary acetoclastic methanogen, was inhibited while the sulfate-reducing bacteria strains (SRB) as a major competitor in craving acetate, increased significantly.
- Downshifting the temperature from the optimum thermophilic temperature to thermotolerant temperature ranges demonstrated a more stable AD performance. No SRB strains were found in considerable amounts in this scenario. This result then inspires the possibility of achieving better biogas production in an upshifted temperature scenario by starting the incubation at optimum mesophilic temperatures.
- Commencing at 37 ºC followed by gradual upshift temperatures to 41 ºC, with 2 ºC intervals while being fed using 1.5 g/l glucose, the digestion process consistently yields 169 – 214 ml CH_4/g COD with lower CO_2 level of 100 - 186 ml CO_2/g COD.
- Further shift to 43 ºC generated more CH_4 than CO_2 with the factor of 2.5. The wide discrepancy between CH_4 and CO_2 production continues after the incubation temperature upshifted to 45 °C with a slight decrease in CH_4 production. This phenomenon signifies the activation of hydrogenotrophic communities and potential pathway shifts from the acetoclastic pathway due to long incubation and high temperature.
- The shift in the pathway was supported by the increasing activity of *Methanobacterium* over *Methanothrix* at higher temperatures, in syntrophic relationship with fermentative bacteria such as *Acetomicrobium*, *Defluviitoga, Corynebacterium*, and *Fervidobacterium*, and *Candidatus Cloacimonas* as hydrogen producer. Furthermore, there was also an increase in several primary enzymes related to hydrogenotrophic methanogenesis at 43 °C and 45 °C.

- Higher glucose levels (10 g/l) produced more CH_4 in volume yet lower CH_4 yield and high CO_2 production in comparison to 1.5 g/l glucose levels, especially at 43 °C and 45 °C.
- Longer incubation periods at 1.5 g/l glucose levels (80 – 120 days), compared to 10 g/l glucose level (80 days) may potentially influenced the thriving hydrogenotrophic methanogenesis leading to higher CH_4 production over CO_2.
- High CO_2 production alongside high CH_4 production is the main characteristic of acetoclastic methanogenesis and a sign of inactivation of hydrogenotrophic methanogenesis. This indicates that contrary to 1.5 g/l glucose levels, higher glucose treatment achieves stable AD performance with the sole dependence on glucose addition and conversion of substrate through acetoclastic pathway.
- *Methanothrix*, a pivotal acetoclastic methanogen, emerged as a resilient player at 10 g/l glucose treatment, showcasing an intriguing correlation with acetate concentration rather than temperature variations. These findings illuminate the steadfast nature of acetoclastic methanogenesis in AD processes, even amidst temperature shifts, providing valuable insights for the optimization of biogas production.

6.2 Engineering implications

In anaerobic digestion (AD), an increase in operating temperature during high-temperature season and failure in air conditioning are inevitable events. Our work highlighted potential biogas production generated from a temperature upshift scenario from mesophilic to thermotolerant temperature regime (imitating temperature increase), and how it impacted microbial communities as the black box of anaerobic digestion. The current study presented information regarding the range of temperatures that are possibly safe for the growth of microorganisms associated with AD and at what temperature it deteriorates. This insight is valuable for optimizing AD strategies, emphasizing the significance of the initial temperature conditions on the subsequent performance of the AD process. The stable and remarkable CH_4 production observed at 43 and 45 °C, highlights the potential benefits of early acclimatization at optimum temperature, offering a promising avenue for improving the stability and reliability of AD under changing temperature regimes.

6.3 Research limitations and possible future works

Although the current study provides an in-depth metagenomic analysis of upshifted temperature scenarios of digested sewage sludge anaerobic digestion, several parameters still can be improved to advance the result and help understand the phenomena thoroughly. The metagenomic analysis is only one part of a broad 'omics' study of microorganisms that have been developed to deduce the whole anaerobic digestion process. The other analyses are metatranscriptomics (the study of enzymes through RNA sequence), metaproteomics (the study of proteins of microorganisms), and metabolomics (the study of the metabolite of anaerobic digestion). These analyses are necessary to untangle the complex microbial networks, and like jigsaw puzzles that are related to each other, one analysis can explain the other analysis results. The application of the latest sequencing technology will facilitate those analyses and present a wide array of perspectives to understand the interrelationships of microbial communities in the anaerobic digestion process.

The other omics analysis possibly helps to comprehend the interesting findings in the current research, for example, the potential of increased methanogen capabilities in producing CH_4 after surviving temperature shifts, despite the decreasing abundance. Metatranscriptomics and metaproteomics analysis may show the working enzymes in the reactor and validate whether the increasing CH_4 was caused by the efficient capabilities of methanogen in converting substrates or it was due to some help from syntrophic fermentative bacteria that released a particular enzyme to aid the formation of CH_4. Then, metabolomics analysis confirms the findings by giving information regarding the metabolite resulting from the enzymatic process.

The current study is also limited in terms of reactor size. Taking too many samples for examination of parameters may affect the overall digestion process. Hence, upgrading the size of the reactor is necessary for future research. The comprehensive strategies for understanding the impact of the other environmental factors such as VFA, higher loading rate, pH changes, and addition of free ammonia in multiple temperature shift conditions can also be applied to future research in this field or the other research field that also works to explore the dynamic transitions in a microbial population under several circumstances.

APPENDIX

LIST OF PUBLICATIONS

1. Sudiartha, G.A.W., Imai, T., Hung, Y.-T., 2022. Effects of Stepwise Temperature Shifts in Anaerobic Digestion for Treating Municipal Wastewater Sludge: A Genomic Study. *International Journal of Environment Research and Public Health* 19, 5728. https://doi.org/10.3390/ijerph19095728
2. Sudiartha, G.A.W., Imai, T., 2022. An Investigation of Temperature Downshift Influences on Anaerobic Digestion in the Treatment of Municipal Wastewater Sludge. *Journal of Water and Environment Technology* 20, 154–167. https://doi.org/10.2965/jwet.22-056
3. Sudiartha, G.A.W. and Imai, T. 2023. Investigation of Methane Production and Microbial Communities of Digested Sewage Sludge on Thermotolerant Anaerobic Digestion. *Proceedings of the International Conference on Sustainable Engineering, Infrastructure and Development by European Union Digital Library*. http://dx.doi.org/10.4108/eai.23-11-2022.2341542
4. Sudiartha, G.A.W., Imai, T., Mamimin, C., Reungsang, A., 2023. Effects of Temperature Shifts on Microbial Communities and Biogas Production: An In-Depth Comparison. *Fermentation*. https://doi.org/10.3390/fermentation9070642
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LIST OF PRESENTATIONS

1. Sudiartha, G.A.W., and Imai, T. 2022. Investigation of Methane Production and Microbial Communities of Digested Sewage Sludge on Thermotolerant Anaerobic Digestion. *International Conference on Sustainable Engineering, Infrastructure and Development, ICO-SEID Universitas Pertamina 2022*, 23-24 November 2022, Jakarta, Indonesia. (Oral Presentation).
2. Sudiartha, G.A.W., and Imai, T. 2022. Methane Production and Microbial Communities of the Stepwise Temperature Shifts Anaerobic Digestion Treating Digested Wastewater Sludge. *The 19th Young Scientist Seminar,* 26th – 27th November 2022 online. (Oral Presentation)
3. Sudiartha, G.A.W., and Imai, T. 2023. Unraveling the Effects of Temperature Shifts on Microbial Communities and Biogas Production of Digested Sewage Sludge Anaerobic Digestion. *The 7th Environmental Technology and Management Conference*, 1st – 3rd November 2023, Bali, Indonesia. (Oral Presentation)
4. Sudiartha, G.A.W., and Imai, T. 2024. Temperature-Driven Dynamics of Methane Production Pathways in Anaerobic Digestion: Insights from Incremental Temperature Shifts. *Water and Environment Technology Conference*, 20th – 21st July 2024, Okayama, Japan. (Oral and Poster Presentation)