

Article

Performance of Anaerobic Digestion of Acidified Palm Oil Mill Effluent under Various Organic Loading Rates and Temperatures

Muhammad Arif Fikri Hamzah ¹, Peer Mohamed Abdul ^{1,2,*} , Safa Senan Mahmod ¹, Azratul Madihah Azahar ¹  and Jamaliah Md. Jahim ^{1,2}

¹ Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia (UKM), UKM, Bangi 436000, Selangor, Malaysia; arifxok@gmail.com (M.A.F.H.); safa@ukm.edu.my (S.S.M.); azratul.madiah@gmail.com (A.M.A.); jamal@ukm.edu.my (J.M.J.)

² Research Centre for Sustainable Process Technology (CESPRO), Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia (UKM), UKM, Bangi 436000, Selangor, Malaysia

* Correspondence: peer@ukm.edu.my

Received: 8 May 2020; Accepted: 17 July 2020; Published: 29 August 2020



Abstract: This study compared the performance of thermophilic and mesophilic digesters of an anaerobic digestion system from palm oil mill effluent (POME), in which temperature is a key parameter that can greatly affect the performance of anaerobic digestion. The digesters were incubated at two distinct temperatures of 55 and 37 °C, and operated with varying organic loading rates (OLRs) of 2.4, 3.2, and 4.0 g COD/L.d by altering the chemical oxygen demand (COD) of acidified POME during feeding. The results indicated that the performance of anaerobic digestion increased as the OLR increased from 2.4 to 4.0 g COD/L.d. At the OLR of 4.0 g COD/L.d, the thermophilic condition showed the highest methane yield of 0.31 ± 0.01 L/g COD, accompanied by the highest COD removal and volatile solid reduction, which were found to be higher than the mesophilic condition. Microbial community analysis via denaturing gradient gel electrophoresis (DGGE) revealed that *Methanothermobacter sp.* emerges as the dominant microbe, which is known to utilize the carbon dioxide pathway with hydrogen acting as an electron donor for methane formation

Keywords: anaerobic digestion; temperature; palm oil mill effluent; organic loading rate

1. Introduction

Nowadays, researchers have successfully produced biogas from various resources including, but not limited to, food waste, wastewater, lignocellulosic waste, municipal waste, and agricultural by-products such as palm oil mill effluent (POME). POME is a wastewater that is produced from palm oil processing and contains a high amount of suspended solids, has a brownish color, and is discharged at 80–90 °C with a mild acidic condition [1]. Additionally, POME is rich in soluble organic content and has a high concentration of chemical oxygen demand (COD) that varies greatly between 50–100 g/L, depending on the palm oil mill processing technology [2]. Major constituents in raw POME include fermentable sugars, volatile fatty acids (VFA), oil and grease, fibers, and organic materials. The discharge of untreated POME can be detrimental to the environment as it is associated with an unpleasant smell with other dissolved organic materials that can contaminate water bodies. However, POME has tremendous potential in bioprocessing and fermentation industries as POME can be utilized as low-cost feedstock that can be converted to various biochemical building blocks [3]. As Malaysia and Indonesia produce the highest volume of palm oil in the world, with the correct technologies, both countries can positively benefit from its massive production of POME.

Some of the current technologies that have been developed to treat POME include anaerobic digestion, aerobic process, membrane separation, water evaporation, and solid removal via coagulation-flocculation [4]. Traditionally, most palm oil mills have adopted an open ponding system to treat POME due to its simplicity, easy maintenance, and cost-effectiveness, but this system has disadvantages in terms of no energy recovery, ungoverned greenhouse gas emissions, and long retention time with a larger footprint [2]. Conversely, anaerobic digestion coupled with systematic biogas capture offers advantages for treating POME because of its suitability for treating high-strength wastewater, has less power requirements than aerobic systems, less solid generation, and stability against environmental changes while simultaneously improving bioenergy harvesting from POME [5]. The production of biogas, therefore, is beneficial as a renewable source of energy for the palm oil mill itself and potentially adding new streams of income. Various efforts have been carried out in order to ensure a stable and high biogas output from anaerobic digestion. One of the efforts includes the separation of traditional single tank anaerobic digestion into a two-stage system where the first system is involved in the production of biohydrogen while simultaneously increasing the VFA content in POME. The production of hydrogen will reduce the overall POME's COD and is always accompanied by the production of VFA such as acetate, butyrate, and propionate, and solvents such as ethanol, which are excellent carbon sources for methane production [6]. The effluent from hydrogen production is colloquially known as acidified POME, and is highly apt for the methanogens to be converted to methane. Continuation of anaerobic digestion of this acidified POME effluent into biogas will further increase overall COD reduction and result in higher energy recovery compared to a single stage anaerobic digestion using raw POME [7].

The government of Malaysia has planned a few strategies to maximize methane production by mandating all mills capture the emitted biogas and use it as an energy source by 2020 in order to mitigate environmental pollution [4]. For now, about half of the palm oil mills in Malaysia are still using the conventional pond system to treat POME without trapping methane gas [4], probably due to the availability of land that can be used for treating waste and the constraints in modern digester development funds [8].

Furthermore, the rate of methane generation and total biogas produced are a function of the organic fraction and biodegradability of POME [9], hence, in order to obtain a high production of biogas, the operational conditions (biotic and abiotic) of anaerobic digestion such as pH, organic loading rate (OLR), hydraulic retention time (HRT), and temperature must be maintained at optimal conditions. Temperature and OLR are the two vital parameters that critically affect the overall performance of anaerobic digestion. OLR refers to the amount of organic content available for microorganisms and the parameter can be estimated by measuring the amount of volatile solids (VS) present in a digester [10]. Uncontrolled high OLR could easily dampen the biogas productivity due to the inhibition of methanogen growth at extreme food to microbe ratio (F/M). Moreover, a thermophilic digester was claimed to be superior to its mesophilic counterpart as it could be operated at higher OLRs and afforded favorable biogas production [11]. However, mesophilic digesters are still widely in use due to their cost effectiveness and simplicity in daily operations [12].

In the past, several studies have investigated the effect of temperature on anaerobic digestion with raw POME as the source of feed. However, they only focused on the performance in terms of methane yield, COD removal, and VS reduction without much consideration of other factors such as VFAs and total alkalinity, which also affect the stability of anaerobic digestion. Therefore, the aim of this study was to compare the performance and condition (operating parameters) of anaerobic digestion between mesophilic and thermophilic conditions at three different OLRs (2.4, 3.2 and 4.0 g COD/L.d) using acidified POME, and to evaluate the VFA consumption and total alkalinity, as both parameters are important for monitoring the performance of anaerobic digestion. Additionally, physical observation and the change of bacterial communities were carried out to elucidate their performance under different conditions.

2. Material and Methods

2.1. Acidified Palm Oil Mill Effluent (POME) and Inoculum of methanogens

The substrate in this study, acidified POME was collected from the hydrogen fermentation effluent in our laboratory as performed by Maaroff et al. [13], which operated the digester at thermophilic conditions with raw POME as a feedstock. The characteristics of acidified POME are summarized in Table 1. Acidified POME has an advantage of high concentration of VFAs compared to raw POME, which allows more substrates to be degraded into methane [6]. It was stored in a 20 L container and kept in a chiller at 4 °C to reduce microbial activities. Upon usage, the sample was heated to ambient temperature and stirred until homogenized before being fed to the digester. The inoculums for thermophilic and mesophilic methanogens were collected from a POME treatment pond at the Sime Darby Plantation Tennamaram palm oil mill, Selangor, Malaysia and had a dark brownish color with volatile suspended solids (VSSs) of 22.3 g/L and pH of 7.30. The inoculums were acclimatized before the OLR was altered to compare the performance between mesophilic and thermophilic temperatures.

Table 1. Characteristics of the substrate.

ID	Parameter	Concentration Range
		Acidified POME
1	pH	5.08 ± 0.1
2	Chemical oxygen demand (g/L)	35.5 ± 3.3
3	Total solid (g/L)	31.0 ± 3.6
4	Total suspended solid (g/L)	24.1 ± 4.2
5	Volatile suspended solid (g/L)	19.0 ± 5.0
6	Volatile fatty acid (mg/L as CH ₃ COOH)	6703.6 ± 1515.1
7	Total nitrogen (mg/L)	263 ± 28

2.2. Anaerobic Digestion Operation

The performance of anaerobic digestion using acidified POME as the substrate was investigated at two distinct temperatures. Figure 1 shows a schematic diagram of the experimental setup that included Schott Duran bottles with a working volume of 500 mL in a water bath shaker (80 rpm). The temperature of the thermophilic condition was maintained at 55 °C, whereas the mesophilic condition was at 37 °C. The top of the bottle was inserted with two rubber tubes for substrate feeding, sample collection at pre-determined intervals, and nitrogen purging to create anaerobic conditions, whereas the second tube was used for channeling the biogas into the inverted collection cylinder. The experiments for both thermophilic and mesophilic conditions were carried out in sequencing batch mode at HRT of eight days by adding acidified POME shortly after removing the effluent. Meanwhile, the OLRs in both bottles were set at 2.4, 3.2, and 4.0 g COD/L.d by diluting the initial COD of influent (substrate feed) between 29.2 and 37.3 g/L, as suggested by Badiei et al. [14]. The experiment started with a low OLR of 2.4 g COD/L.d and then the OLR was changed incrementally, as suggested by Jeong et al. [15]. Each OLR run was maintained for at least 20 days, then shifted to the next OLR run. pH and biogas production were monitored daily to ensure that the anaerobic digestion operated in a good condition. Approximately 60 days were needed to complete the trials for both mesophilic and thermophilic temperatures.

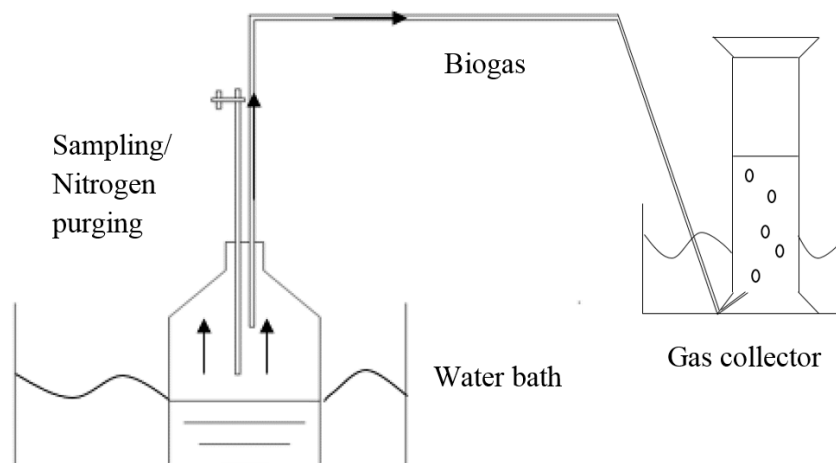


Figure 1. Schematic diagram of the anaerobic digestion process.

2.3. Start-Up Operation

The digesters were first acclimatized at a constant HRT of 30 days with an OLR of 1.1 ± 0.2 g COD/L.d, and operated in a sequencing batch mode where the substrate (acidified POME) was fed every two days. The acclimatization was considered completed when the biogas production reached a steady state with stable biogas volume production at the variation of 10%. At steady state, the biogas production kinetics and methanogenic microbial community were presumed to achieve a stability that would allow impartial comparison between the two digesters. The OLR of the feed was then adjusted to 2.4, 3.2, and 4.0 g COD/L.d, with each OLR maintained for at least 20 days consecutively. Furthermore, anaerobic digestion between mesophilic and thermophilic temperatures was carried out at low OLR (2.4, 3.2 and 4.0 g COD/L.d) compared to other studies that carried out anaerobic digestion using raw POME. For example, Krishnan et al. [16] operated their system at an OLR of 12.0 g COD/L.d using raw POME as a substrate. In contrast, the acidified POME used in this study contained higher VFA proportions than raw POME. Compared to the same study by Krishnan et al. [16], the concentration of the acidified POME's VFA in this study was about 10 times higher than that of the raw POME's VFA. Therefore, in order to avoid the possibility of VFA accumulation at a high OLR, both mesophilic and thermophilic digesters were operated at low OLR to compare their performances. The stability of anaerobic digestion was monitored, especially in terms of the concentration of VFAs and biogas production to avoid failure during the experiment.

2.4. Analytical Method

The volume of biogas produced was measured by the water displacement technique as described in previous studies [6,17], whereas the percentage of methane was calculated by gas chromatography (Shimadzu GC-2014, Kyoto, Japan) equipped with a thermal conductivity detector. Helium gas was used as a carrier gas and operated at a flow rate of 25 mL/min. The calibration method was done by using pure methane and carbon dioxide gas (approximately 99.99% purity). The initial temperature of the column was set at 43 °C and maintained for 6 min, and then the temperature was ramped until 220 °C with a ramping rate of 20 °C/min and maintained for 3 min. The measured biogas composition involved two gases, namely carbon dioxide and methane. The liquid sample was analyzed for the pH, VSS, and total alkalinity using the method from the American Public Health Association standard [18]. COD analysis was done according to the dichromate method by using a DR 2800 COD analyzer (HACH, Loveland, CO, USA). Volatile fatty acids (propionic acid, butyric acid, and acetic acid) were analyzed using Agilent 1100 series HPLC system (Agilent, Santa Clara, CA, USA) with a diode array detector at 210 nm UV wavelength. The system was equipped with REZEX ROA column (Phenomenex, Torrance, CA, USA) and 2.5 mM sulfuric acid was used as the mobile phase at a flow rate of 0.6 mL/min with isocratic elution at room temperature.

2.5. Microbial Community Analysis

The samples for DGGE analysis were collected from the effluent of mesophilic and thermophilic digesters before the digesters were fed. The samples were stored in 50 mL centrifuge tubes, and kept in a freezer at $-20\text{ }^{\circ}\text{C}$ before the analysis was conducted. The modified DNA extraction was done by utilizing the FavorPrep Soil DNA Isolation Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan). The extracted DNA was amplified using the nested polymerase chain reaction (PCR) technique, which involved the archaeal 16S rRNA gene primer set 21F (5'-TTCCGGTTGATCC YGCCGGA-3') and 958R (5'-YCCGGCGTTGAMTCCAATT-3') [19] and primer set GC-341F (5' CGCCCGCCGCGCGGCGGGCGGGGCGGGGACGCGGGGGCCTACGGGAG GCAGCAG-3') and 518R (5'-A TTACCGCG GCTGCTGG-3') [20]. The PCR component (PCR Master Mix, Promega, Madison, WI, USA) was carried out based on the manufacturer's instructions. The PCR program consisted of an initial 3 min denaturation step at $94\text{ }^{\circ}\text{C}$, followed by 35 cycles of repeated denaturation at $94\text{ }^{\circ}\text{C}$ for 30 s, annealing at $60\text{ }^{\circ}\text{C}$ for 1 min, and extension at $72\text{ }^{\circ}\text{C}$ for 1.5 min, followed by a final extension at $72\text{ }^{\circ}\text{C}$ for 10 min. The samples were amplified using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany).

The PCR products were separated using VS20WAVE—DGGE (Cleaver Scientific, Warwickshire, UK) on a vertical gel containing 10% (w/v) acrylamide with a denaturant concentration of 30% to 60% from the top to the bottom of the polyacrylamide gel. Electrophoresis was carried out in $1\times$ Tris-acetate-EDTA (TAE) buffer at 145 V for 4 h at $60\text{ }^{\circ}\text{C}$. After that, the gel was stained with SYBR Green nucleic acid gel stain for 40 min and then visualized and sliced out on Gel Imaging (FireReader V10, Uvitec, Cambridge, UK). The PCR program and DNA sequencing for the eluted DGGE bands were conducted as prescribed by Yasin et al. [21]. The closest matches for partial 16S rRNA gene sequences were identified by the ribosomal database project [22].

2.6. Heat Requirement and Heat Loss during Anaerobic Digestion

The heat energy requirement to maintain the temperature at mesophilic and thermophilic conditions was calculated according to Equation (1) [23], while calculation for heat loss was according to Equation (2) [24]. The total energy needed for heating the substrate can be obtained from the sum of the heat required (Equation (1)) and heat loss (Equation (2)).

$$Q_s = V_s \rho_s C_s (T - T_o) \quad (1)$$

where Q_s indicates the heat required, J/d; V_s indicates the volume of sludge, m^3/d ; ρ_s indicates the density of sludge, kg/m^3 ; C_s indicates the specific heat of sludge, (assumed to be same as water [25]), $4200\text{ J}/\text{kg}\cdot^{\circ}\text{C}$; T indicates the operating temperature, $^{\circ}\text{C}$; and T_o indicates the temperature feed sludge, $^{\circ}\text{C}$

$$Q = UA(T_1 - T_2) \quad (2)$$

where Q indicates the heat loss, J/s; U indicates the heat transfer coefficient, $\text{J}/\text{m}^2\cdot\text{s}\cdot^{\circ}\text{C}$; A indicates the surface area of digester through which heat losses occur, m^2 ; T_1 indicates the temperature of sludge in the digester, $^{\circ}\text{C}$; T_2 indicates the temperature outside the digester, $^{\circ}\text{C}$.

3. Results and Discussion

3.1. Methane Yield

At a glance, the methane yield for thermophilic and mesophilic conditions was elevated in both mesophilic and thermophilic conditions with increasing OLR. As shown in Figure 2, the methane yield of the thermophilic condition was consistently higher than that of the mesophilic condition. At the highest tested OLR of $4.0\text{ g COD}/\text{L}\cdot\text{d}$, both mesophilic and thermophilic conditions registered the highest methane yield of 0.23 ± 0.01 and $0.31 \pm 0.01\text{ L/g COD}$, respectively. A similar trend was

observed by Musa et al. [26], in which an increase of OLR from 7.0 to 10.0 g COD/L.d improved the methane yield from 0.36 to 0.38 L/g COD. The result from Musa et al. [26] and this study not only accomplished a high methane yield but also achieved high COD removal and VS reduction. Operating at the OLR of 4.0 g COD/L.d also resulted in a higher consumption rate of VFAs by the methanogen, which was translated into higher methane yield. Moreover, there was no overloading of organic matter during the digestion at the highest tested OLR as no significant reduction of methane yield could be observed during anaerobic digestion.

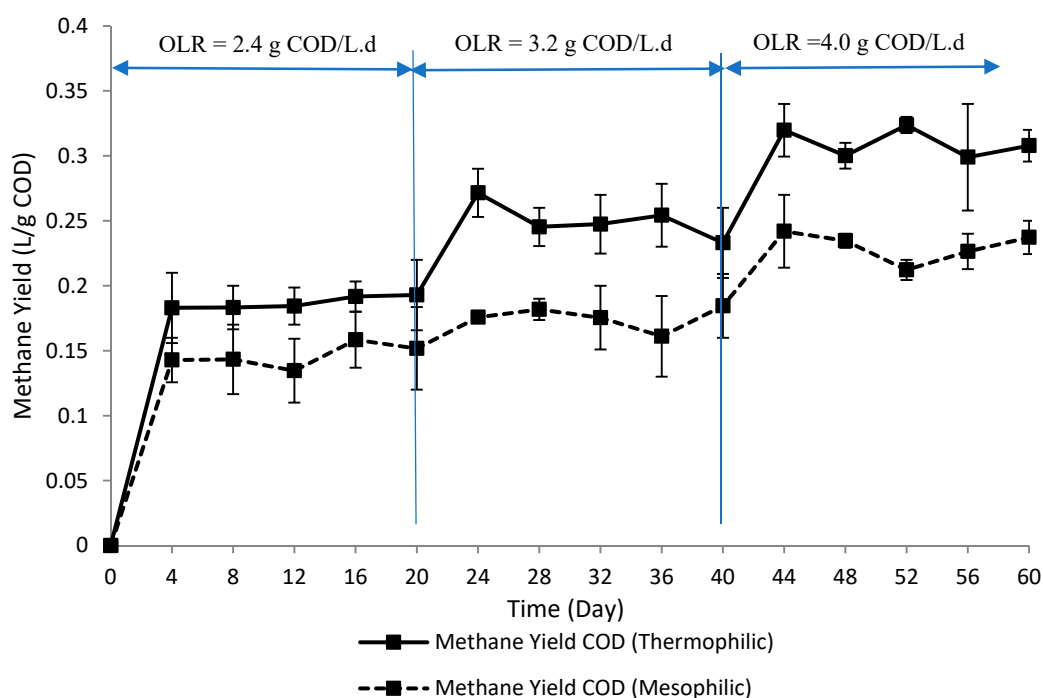


Figure 2. The methane yield at various organic loading rates (OLRs). Notes: error bars show standard deviation of means ($n = 3$).

At each tested OLR, the methane yield for the mesophilic condition was much lower than the thermophilic condition. For instance, at the OLR of 2.4 g COD/L.d, the methane yield for thermophilic temperature was 0.15 ± 0.07 L/g COD, whereas for mesophilic temperature, the methane yield was 0.12 ± 0.05 L/g COD. Meanwhile, at the OLR of 3.2 g COD/L.d, the methane yield for thermophilic and mesophilic temperatures was 0.25 ± 0.01 and 0.17 ± 0.01 L/g COD, respectively. The same conclusion was corroboratively reported by Choorit and Wisarnwan [2], who claimed that the methane yield in the thermophilic condition was higher than the mesophilic condition with a difference of 29%. Likewise, the anaerobic digestion of cyanobacterium *Spirulina maxima* resulted in an increase in the methane yield in the thermophilic condition that surpassed the yield in the mesophilic condition by 21% when operated at a HRT of 16 days [27]. Anaerobic digestion (hydrolytic, acidogenic, acetogenic, and methanogenic) reaction rate is typically higher under thermophilic conditions due to higher microbial activities. The high methane yield in the thermophilic condition suggested the higher degradation rate of the substrate by the methanogen; hence, it generated a favorable methane yield.

3.2. Substrate Removal

In the mesophilic digester, the lowest VS reduction and COD removal ($39.4\% \pm 3.5\%$ and $49.7\% \pm 1.7\%$, respectively) were observed at the OLR of 2.4 g COD/L.d, as shown in Figure 3. As the OLR increased from 2.4 to 4.0 g COD/L.d, the VS reduction and COD removal correspondingly rose by 34.9% and 26.3%, respectively. The same trend was noted in the thermophilic condition with the VS reduction and COD removal in the thermophilic condition, which increased by 26.4% and 24.1%,

respectively, when the OLR increased from 2.4 to 4.0 g COD/L.d. Furthermore, for both mesophilic and thermophilic conditions, the highest OLR tested (4.0 g COD/L.d) registered the highest performance in VS reduction and COD removal. A high COD removal and VS reduction in the discharge effluent implies a successful consumption of substrate to produce methane. Fang and Chui [28] reported that the elevation of OLR from 1.0 to 3.0 g COD/L.d led to 90% of COD removal in the system. The factor underlying the increase in COD removal with increased OLR is that the methanogen has already effectively acclimatized to the new environment and becomes more active in digesting organic matters [29]. The use of high OLR can result in the superior performance of anaerobic digestion. However, excessive OLR could contribute to the failure of anaerobic digestion as it leads to low COD removal and VFA accumulation. Both studies agreed that high COD removal comes with high VFA consumption. The increase in the substrate in the digester promotes methanogenic growth, which eventually improves substrate conversion to methane by the methanogen.

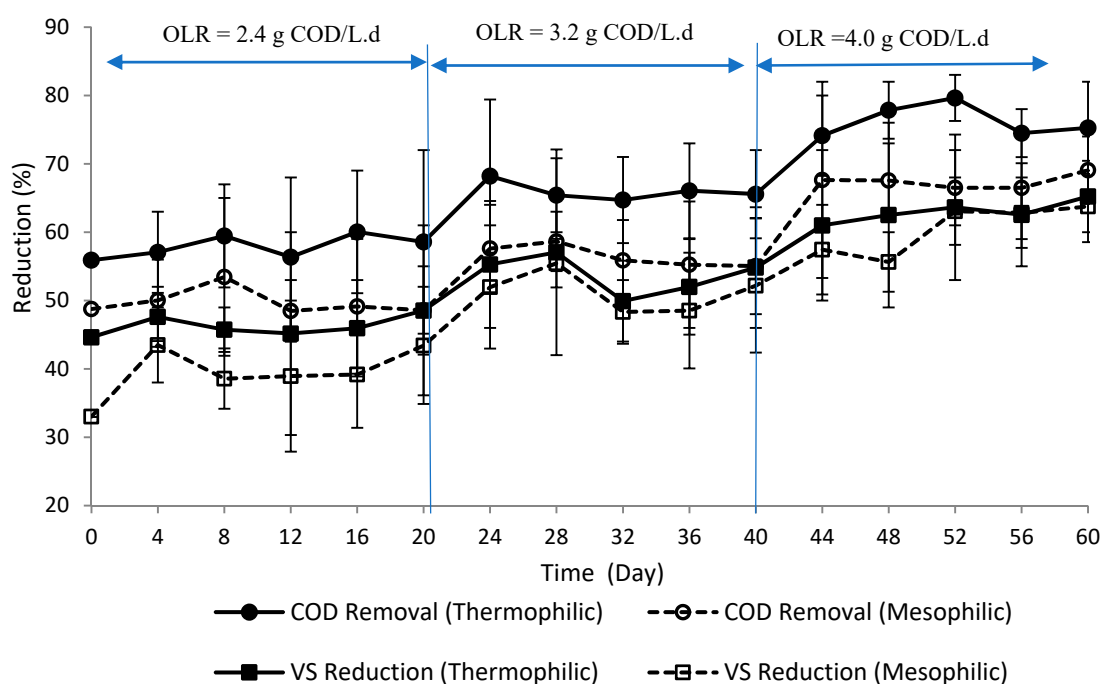


Figure 3. Chemical oxygen demand (COD) removal and volatile solids (VS) reduction at various loading rates. Notes: Error bars show standard deviation of means ($n = 3$).

The superior performance of thermophilic digestion in terms of COD removal and VS reduction is probably due to the higher acceleration of the biochemical reaction when functioning at higher temperature [30], which is directly linked to a greater reduction of VFA content in the effluent under the thermophilic condition, when compared to the mesophilic condition. At higher temperatures, the growth rate and microorganism activity are much higher than lower temperatures due to the increase of metabolism in bacteria. Thus, higher VS reduction typically signifies equally higher methane yield. This finding is in agreement with the study reported by Cavinato and co-workers [31], where VS reduction was much larger in the thermophilic condition than the mesophilic condition with 16.3% variation between these two conditions. Higher VS reduction in the thermophilic condition indicates a greater consumption of organic matters by the methanogen. The greater VS reduction achieved in our study is reflected by the higher resultant methane concentration of this study, which was 7% higher than the latter study [31].

3.3. Total Alkalinity and pH

Parameters such as pH and total alkalinity are critical to determine the stability of anaerobic digestion. Observation in the variations of pH enables the monitoring of the condition in anaerobic

digestion because pH fluctuation is a direct result due to changes in buffering capacity [3]. Methanogens are sensitive to pH changes; conducting anaerobic digestion outside of neutral condition is not favorable for methanogenic growth because it can attribute to the accumulation of VFAs in the system. Furthermore, the buffering capacity in the anaerobic system is the outcome of total alkalinity, in which high total alkalinity in the system will result in pH elevation.

In this study, the influent, acidified POME, was primarily in an acidic condition (5.1 ± 0.1) and its pH was quite similar to the previous study (5.2 ± 0.2) that used acidified POME [32]. The low pH in acidified POME is due to the presence of VFAs such as acetic acid and butyric acid. Operating anaerobic digestion at low pH would affect methanogenic activity and finally lead to failure in anaerobic digestion [33]. This is because low pH indicates that VFA was not efficiently converted into methane. However, this study proved that despite the low initial pH of the substrate, the pH levels of both thermophilic and mesophilic effluents remained close to neutrality at all OLRs, possibly due to the successful degradation of fatty acids into biogas, thereby elevating the pH reading. Inactive methanogens could not utilize fatty acid fully, resulting in low pH of the effluent, hence indicating a failed or unstable anaerobic digestion system.

At low OLR of 2.4 g COD/L.d, the methanogens appeared to slow down, as reflected by the low pH. This is probably due to the low consumption of VFAs, as shown in Figure 4. At the OLR of 3.2 g COD/L.d, the pH continued to rise for both mesophilic and thermophilic conditions and attained its peak at 4.0 g COD/L.d. The reason for the increase is that the total alkalinity for both mesophilic and thermophilic conditions rose proportionally with pH from 2.4 to 4.0 g COD/L.d. The thermophilic total alkalinity increased from 7857.7 ± 328 to 9748.0 ± 246 mg/L as CaCO_3 , whereas the mesophilic total alkalinity increased from 7322.7 ± 334 to 9344 ± 101 mg/L as CaCO_3 . High total alkalinity is crucial as it prevents the decrease in pH and ensures that the anaerobic digestion is stabilized. Operating at an acidic condition may be fatal to methanogens and lead to anaerobic digestion failure. At the OLR of 4.0 g COD/L.d, the total alkalinity of thermophilic and mesophilic conditions increased by 19% and 22%, respectively, compared to that at the OLR of 2.4 g COD/L.d. Similar findings were observed in the literature [34]. It has been suggested that the correspondingly high total alkalinity at a high OLR was probably because of the production of ammonia [35]. According to Najafpour et al. [36], anaerobic digestion has been demonstrated to retain operation at OLRs up to 23.2 g COD/L.d, as long as the VFAs were well-buffered by the alkalinity. Anaerobic digestion typically fails to operate at high OLRs because of the accumulation of VFAs. In this study, the high total alkalinity at 4.0 g COD/L.d indicated a desirable buffering capacity, which thus contributed to the favorable methane yield. The concentration of VFA in the system did not appear to have perturbed the anaerobic process at all tested OLRs as the pH was still at the optimum for methanogen growth.

3.4. Metabolite Production

The determination of VFAs present in this study (i.e., acetic acid, butyric acid, and propionic acid) is vital because it offers insights into the metabolic pathways involved in the digestion. The concentration of VFAs is related with the methane yield, in which a favorable methane yield is a result of high VFA consumption by the methanogen. At the OLR of 4.0 g COD/L.d, the thermophilic condition registered the highest VFA consumption ($69.2\% \pm 3.0\%$) whereas the mesophilic condition recorded the consumption of $51.3\% \pm 5.5\%$, which resulted in high methane yield, as can be seen from Figure 5. Conversely, at the OLR of 2.4 g COD/L.d, both mesophilic and thermophilic conditions registered the lowest VFA consumption, which declined by 67.3% and by 27.3%, respectively, compared to those at the OLR of 4.0 g COD/L.d. This study has verified that operating at the OLR of 4.0 g COD/L.d (i.e., the highest OLR tested) did not impair anaerobic digestion because the VFA consumption by the methanogen remained high; thereby its performance was unaffected.

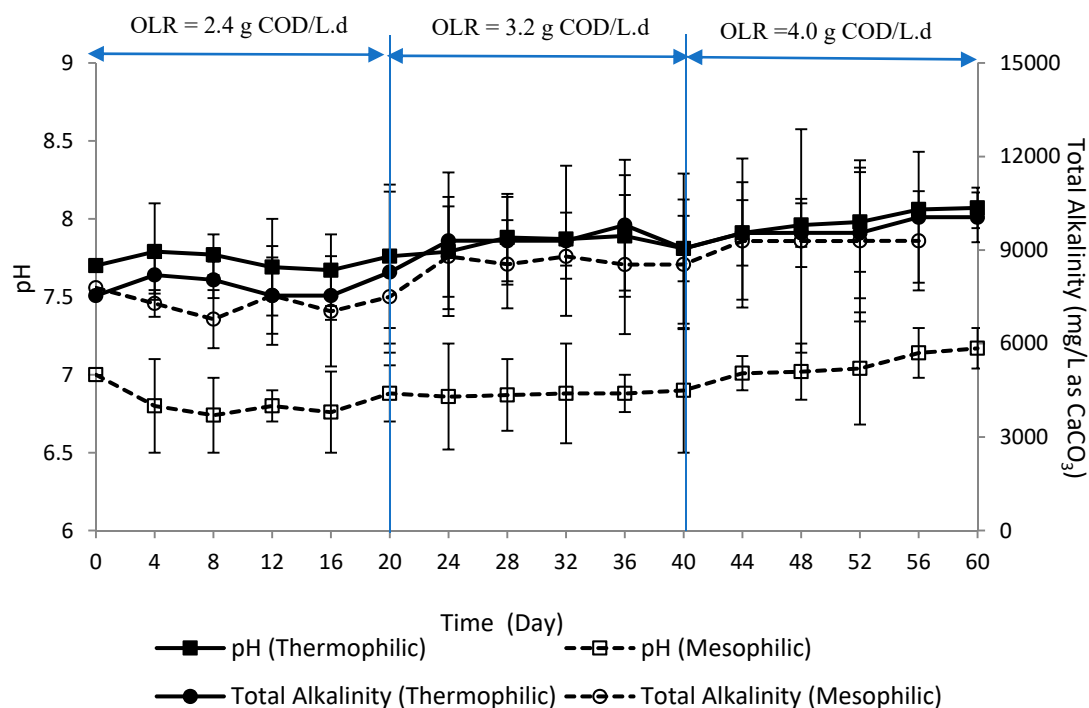


Figure 4. The pH and total alkalinity at various loading rates. Notes: Error bars show standard deviation of means ($n = 3$).

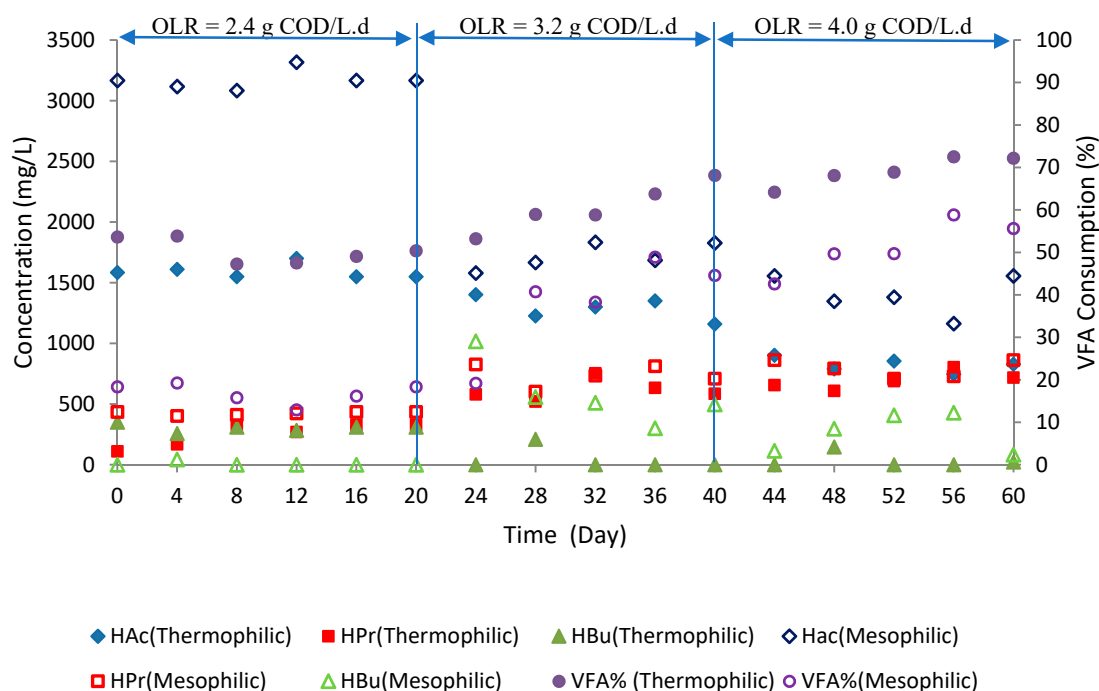


Figure 5. Volatile fatty acids (VFAs) concentration at various organic loading rates. Notes: HAc (Acetic acid); HPr (Propionic acid); HBu (Butyric acid); VFA% (Volatile fatty acid consumption).

Notably, at all tested OLR, the total VFA consumption at three different OLR (2.4, 3.2, and 4.0 g COD/L.d) was consistently much higher in the thermophilic condition than in the mesophilic condition with $42.2\% \pm 40.2\%$ difference, which might be attributed to the greater methanogenic activity. This resulted in higher degradation rate by methanogens as the temperature increased. As a result, lower residual VFAs in the thermophilic condition could be detected at each tested OLR. It has been reported that the concentration of residual VFAs in the mesophilic condition surpassed that in the

thermophilic condition. Yu and co-authors [37] reported that on the twentieth day of operation at pH 10, the thermophilic condition recorded half of the total VFAs than the mesophilic condition. Furthermore, this study has revealed a better stability for the thermophilic condition than in the mesophilic condition due to the low VFA effluent content of the former. The accumulation of VFAs can perturb anaerobic digestion and, in the worst-case scenario, culminate in systemic failure.

Moreover, the existence of propionic acid that may hinder anaerobic digestion was noteworthy in this study. For both thermophilic and mesophilic conditions, the average concentrations of propionic acid (522 ± 52.6 and 650 ± 76.6 mg/L, respectively) were below 951 mg/L. A previous study showed that the concentrations of propionic acid exceeding 951 mg/L may hinder methanogenesis [38], probably because of its low conversion rate for methane production, which could lead to accumulation [33]. A low rate of conversion to methane by propionate was probably due to the high hydrogen pressure inside the digester, which resulted in propionate-oxidizing bacterium performing other reactions that required sufficient energy for growth [39]. In terms of the conversion of VFAs into methane, the highest rate was associated with acetic acid, followed by ethanol, butyric acid, and, finally, propionic acid. According to Inanc et al. [40], methanogenic bacteria can tolerate 10 times a higher concentration of butyrate compared to propionate. A similar observation was made by Wang et al. [33], in which propionic acid at 5000 mg/L reduced methane production by 22–38%. Therefore, the concentration of propionic acid needs to be controlled during anaerobic digestion.

3.5. Microbial Community at Best Performance OLR

The analysis from the denaturing gradient gel electrophoresis (DGGE) (Figure 6 and Table 2) revealed the bacterial communities responsible for the mesophilic and thermophilic anaerobic digestion at the OLR of 4.0 g COD/L.d. In this study, two-step nested PCR was applied as an alternative of a single-step procedure in order to improve the sensitivity of PCR as well as to amplify the community structure of the methanogenic communities [41,42].

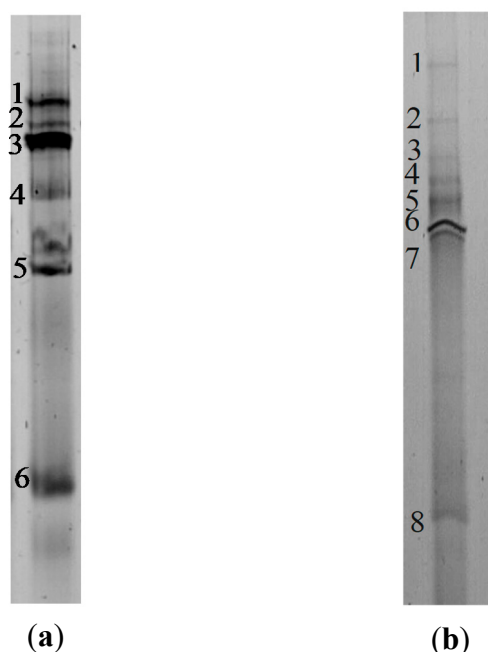


Figure 6. Denaturing gradient gel electrophoresis (DGGE) analysis of bacterial community structures from the methane fermentation systems under (a) mesophilic and (b) thermophilic temperatures.

Table 2. Classification of bacterial community taxa in methane producing communities at mesophilic and thermophilic conditions.

Mesophilic			Thermophilic		
Band	Nearest Relative	Accession	Band	Nearest Relative	Accession
1	<i>Methanosarcina thermophila</i>	CP009502	1	<i>Methanothermobacter crinale</i> sp.	HQ293273
2	Uncultured <i>Methanothermobacter</i> sp.	MF471083	2	<i>Methanothermobacter</i> sp.	JN983061
3	<i>Methanothermobacter</i> sp.	JN983061	3	<i>Methanothermobacter crinale</i> sp.	HQ293273
4	Uncultured <i>Methanosarcina</i> sp.	KU661854	4	<i>Methanothermobacter crinale</i> sp.	HQ293273
5	<i>Methanosarcina thermophila</i>	MH708238	5	<i>Methanothermobacter crinale</i> sp.	HQ293273
6	<i>Methanosarcina thermophila</i>	MG008505	6	<i>Methanothermobacter tenebrarum</i>	AB5237865
			7	<i>Methanothermobacter</i> sp.	JN983061
			8	<i>Methanothermobacter tenebrarum</i>	AB5237865

The assemblage of mesophilic methanogens was found to coexist mainly of those in the class *Methanosarcina thermophila* and the addition of few *Methanothermobacter* sp. *M. thermophila* is known to predominate with higher levels of VFAs, as observed in the mesophilic condition with a positive correlation ($p < 0.05$) between the VFA concentration and *Methanosarcina* [43]. Commonly, *M. thermophila* is present in feedstock such as food waste, wastewater, and sewage sludge [44]. Another characteristic of *M. thermophila* is its versatility of thriving in different substrates such as acetate, methanol, and methylamine. The slow growth rate of acetotrophic methanogen can upset anaerobic digestion since it can result in the accumulation of acetate. The most dominant archaea in a mesophilic anaerobic digester treating POME were *Methanosaeta* sp. and *Methanosarcina* [45]. The literature also suggests that in mesophilic conditions, syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis was a crucial pathway for biogas production. In addition, *Methanosarcina thermophila* was the dominant genus in mesophilic anaerobic digestion, which directed stable digestion [46].

In contrast, the thermophilic methanogen community was dominated by *Methanothermobacter* sp. The genus of *Methanothermobacter* sp. is known to utilize the carbon dioxide pathway whereas hydrogen acts as an electron donor. Patil and co-authors [47] found that *Methanothermobacter* sp. predominated in a thermophilic digester whereas *M. thermophila* predominated in a mesophilic reactor. Similarly, *Methanothermobacter* sp. was abundant in the thermophilic condition with food and lignin waste as a substrate [48]. Hydrogenotrophic methanogen could also be found in a digester with a low concentration of acetate [49]. In terms of the waste with a high concentration of organics, hydrogenotrophic methanogen is superior to acetotrophic methanogen [50]. In this study, the VFA concentration in the thermophilic condition was much lower than the mesophilic condition with a difference of 38%, hence resulted in a less favorable condition for the growth of acetotrophic methanogen in thermophilic conditions. Furthermore, the thermophilic digester showed an abundance of hydrogenotrophic methanogens due to its abilities to adjust to high temperature conditions as well as its capability to tolerate toxic conditions compared to acetotrophic methanogens [51].

3.6. Scanning Electronic Microscopy

Figure 7 depicts the granular structure of thermophilic and mesophilic methanogens via scanning electronic microscopy (SEM), which is suitable to determine the morphology of the microbial species in anaerobic digestion [52]. For each sample, the images are presented with 15,000× magnification that permits visualization of different types of sludge developed in the reactors and detailed morphological features of the microbial species. The examination of the sludge revealed that cocci- and rod-shaped microbes were dominant and appeared to be concentrated in the mesophilic sludge, whereas the rod-shaped microbes were found loosely in the thermophilic sludge. An identical result was reported by Zellner et al. [53], in which rod- and cocci-shaped microbes were discovered in the anaerobic digester. It is believed that the rod-shaped microbes are similar to *Methanothermobacter* sp. [54] while the cocci shape microbes are comparable to *Methanosarcina* sp. [55].

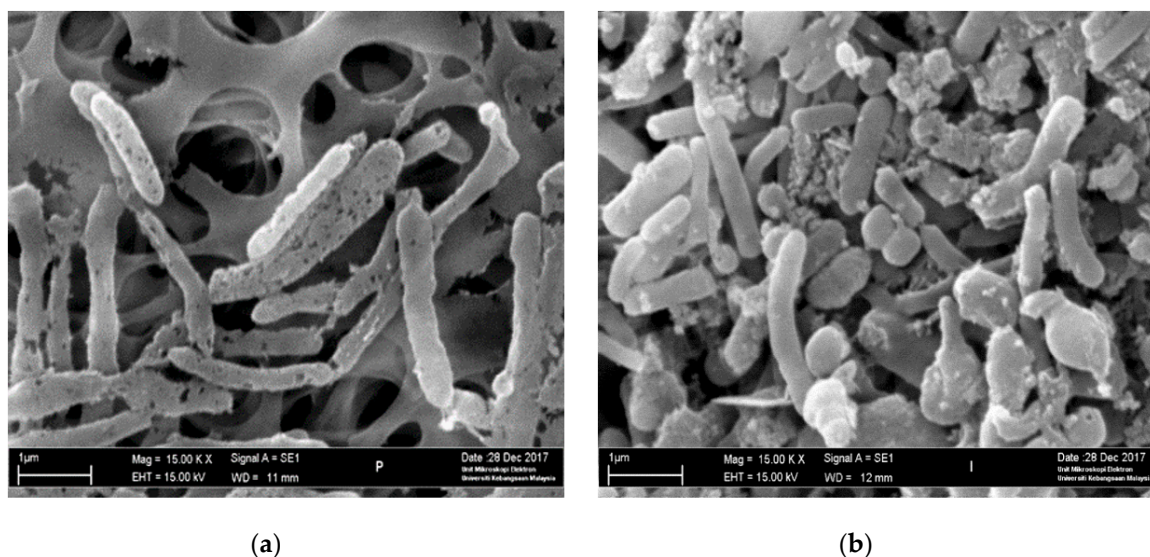


Figure 7. Photograph of scanning electron microscope (SEM) of the (a) thermophilic and (b) mesophilic conditions.

3.7. Overall Performance and Energy Requirement

As shown in Table 3, most previous studies were conducted in either the mesophilic or thermophilic condition, except for this study, which operated in both conditions. All studies were conducted at OLRs typically used in anaerobic digestion and succeeded in achieving the digestion without causing an overloading of organic matters in the digester. Borja and co-authors [56] operated at the highest OLR (12.9 g COD/L.d), which was more than three times higher than the OLR in the present study, which explains the highest methane yield compared to the other studies in Table 3. The selection of OLR has become a key role to produce notable methane yield. Interestingly, the shortest HRT of 3.5 days was recorded without resulting in VSS washout and nonetheless achieved high methane yield. The selection of the HRT of eight days in this study appears to have attained a favorable balance between the performance and stability of the system of anaerobic digestion because the methanogen had sufficient time to digest the substrates and efficiently performed anaerobic digestion. Furthermore, all studies shared the similarity that the high methane yield was verified by the high value of COD removal, which presented high utilization of substrate concentrations to produce high methane yield. However, Krishanan and co-authors [16] did not reach significant methane yield, although the COD reduction could be as considered high. High-energy requirement is one of the main problems when operating with thermophilic digestion. The temperature set for the thermophilic digester was 55 °C, which was 18 °C higher than the mesophilic digester. The thermophilic digester requires 1.1 W of energy input to maintain the temperature at 55 °C, which is twice the energy needed for the mesophilic condition. Despite this, it is more worthy to operate in the thermophilic condition than in the mesophilic condition due to such noticeable differences in the yield, despite the higher energy demand incurred by the thermophilic condition. The energy requirement of the thermophilic digester is similar to the mesophilic digester process, which consists of the heat required to elevate the temperature of sludge and to cancel out energy loss during digestion. The result is also comparable with the study by Amani et al. [57], which required 3 W of power to maintain the temperature of a 2 L digester in the thermophilic condition.

Table 3. Comparison of previous studies on the anaerobic digestion of methane from POME based on different OLR.

Substrate	Temperature	Best OLR Performance (g COD/L.d)	Methane Yield (LCH ₄ /g COD)	COD Removal (%)	Reference
Acidified POME	Mesophilic	4.0	0.23	67.5	Present study
Acidified POME	Thermophilic	4.0	0.31	76.3	
POME	Mesophilic	12.9	0.35	94.1	
POME	Thermophilic	12.0	0.16	85.0	
POME	Thermophilic	12.9	0.20	87.8	

3.8. Statistical Analysis

Table 4 shows the t-test results for methane yield with mesophilic and thermophilic temperatures by using IBM SPSS Statistics 23 (IBM Corporation, New York, NY, USA). In this analysis, $p < 0.05$ was considered significant. T-test analysis was carried out to determine whether methane yield was affected by temperature during anaerobic digestion. Theoretically, high temperature leads to high methane yield. This is because the degradation of the substrate is high at high temperatures. From the result, the sig. (2-tailed) for methane yield was 0.01, which is below 0.05. Therefore, there was a significant difference between the temperature and methane yield. From this study, for the three OLRs that were tested, the thermophilic digester showed a 42.2% higher methane yield when compared to the mesophilic digester.

Table 4. Summary of t-test for the measured methane yield between mesophilic and thermophilic digesters.

Independent Samples Test							
		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Productivity	Equal variances assumed	2.503	0.125	3.884	28	0.001	0.065
	Equal variances not assumed			3.884	25	0.001	0.065
Group Statistics							
	Condition	N	Mean	Std Deviation	Std Error Mean		
Methane yield	Thermophilic	15	0.31	0.05	0.01		
	Mesophilic	15	0.23	0.04	0.01		

where F denotes for the value for Levene's test of equality of variance, Sig. = an abbreviation for significance, t = value for t-test of equality of variance, df = degrees of freedom and N = number of valid observations used in calculating the t-test.

4. Conclusions

The results indicated that the thermophilic digester registered a higher methane yield than the mesophilic digester at every OLR tested. This finding was supported by the percentage of COD removal and VS reduction in the thermophilic condition, which was also higher than the mesophilic condition. This indicates that the reaction of methanogen was high at higher temperatures, as illustrated by the result of thermophilic digestion. Overall, the performance of anaerobic digestion increased with increasing OLR. The OLR of 4.0 g COD/L.d indicated the highest methane yield, COD removal, and VS reduction for both mesophilic and thermophilic conditions. Furthermore, the pH for the mesophilic and thermophilic conditions remained in the range of optimum methanogenic activities at 6.8–7.2. Therefore, the VFAs inside the digesters did not influence the performance of anaerobic digestion. High alkalinity in the system seemed to neutralize the VFAs and maintained the stability of anaerobic digestion. Furthermore, the mesophilic condition was associated with more diverse bacterial community than the thermophilic condition. The colony of the thermophilic digester was comprised of *Methanothermobacter* whereas the mesophilic digester contained an abundance of *Methanothermobacter* and *Methanosarcina* sp. It can be concluded that thermophilic digestion is recommended to operate in the

anaerobic digestion of POME compared to mesophilic digestion as it produced higher methane yield, although it requires higher heating energy to maintain thermophilic temperature during operation.

Author Contributions: Conceptualization, project supervision and validation, P.M.A., and J.M.J.; funding acquisition, J.M.J.; formal analysis and writing—original draft preparation, M.A.F.H.; microbial analysis, A.M.A.; writing—review and editing, S.S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Government of Malaysia and Universiti Kebangsaan Malaysia grant number (GUP-2018-105) and (DIP-2018-024) and the APC was funded by Universiti Kebangsaan Malaysia.

Acknowledgments: We gratefully acknowledge the financial and technical support provided by the Universiti Kebangsaan Malaysia-Yayasan Sime Darby (UKM-YSD) Chair for Sustainable Development: Zero Waste Technology. Thanks are also extended to the Makmal Berpusat i-CRIM of UKM for providing the fabrication and measurement facilities needed for this research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mahmud, S.S.; Jahim, J.M.; Abdul, P.M. Pretreatment Conditions of Palm Oil Mill Effluent (POME) for Thermophilic Biohydrogen Production by Mixed Culture. *Int. J. Hydrogen Energy* **2017**, *42*, 27512–27522. [\[CrossRef\]](#)
2. Choorit, W.; Wisarnwan, P. Effect of Temperature on the Anaerobic Digestion of Palm Oil Mill Effluent. *Electron. J. Biotechnol.* **2007**, *10*, 376–385. [\[CrossRef\]](#)
3. Hosseini, S.E.; Wahid, M.A. Feasibility Study of Biogas Production and Utilization as a Source of Renewable Energy in Malaysia. *Renew. Sustain. Energy Rev.* **2013**, *19*, 454–462. [\[CrossRef\]](#)
4. Chan, Y.J.; Chong, M.F. *Palm Oil Mill Effluent (POME) Treatment—Current Technologies, Biogas Capture and Challenges*; Springer: Singapore, 2018. [\[CrossRef\]](#)
5. Yanti, F.M.; Hastuti, Z.D.; Murti, S.D.S.; Valentino, N.; Sholihah, A.; Juwita, A.R. Utilization Palm Oil Mill Effluent for Biogas Using Continous-Stirred-Tank-Reactor: Production and Biogas Cleaning. In *AIP Conference Proceedings*; AIP Publishing LLC: Melville, NY, USA, 2019; Volume 2097. [\[CrossRef\]](#)
6. Hamzah, M.A.F.; Jahim, J.; Abdul, P.M.; Asis, A.J. Investigation of Temperature Effect on Start-Up Operation from Anaerobic Digestion of Acidified. *Energies* **2019**, *12*, 2473. [\[CrossRef\]](#)
7. Mamimin, C.; Singkhala, A.; Kongjan, P. Two-Stage Thermophilic Fermentation and Mesophilic Methanogen Process for Biohythane Production from Palm Oil Mill Effluent. *Int. J. Hydrogen Energy* **2015**, *40*, 6319–6328. [\[CrossRef\]](#)
8. Wu, T.Y.; Mohammad, A.W.; Jahim, J.M.; Anuar, N. Pollution Control Technologies for the Treatment of Palm Oil Mill Effluent (POME) through End-of-Pipe Processes. *J. Environ. Manag.* **2010**, *91*, 1467–1490. [\[CrossRef\]](#)
9. Kamarudin, S.K.; Muniandy, A.; Shamsul, N.S.; Kofli, H.T. Enhanced Biogas Production from Agro Wastes by Co-Digestion with Crude Glycerol. *J. Kejuruter.* **2018**, *1*, 47–57. [\[CrossRef\]](#)
10. Rabii, A.; Aldin, S.; Dahman, Y.; Elbeshbishy, E. A Review on Anaerobic Co-Digestion with a Focus on the Microbial Populations and the Effect of Multi-Stage Digester Configuration. *Energies* **2019**, *12*, 1106. [\[CrossRef\]](#)
11. Vindis, P.; Mursec, B.; Janzekovic, M.; Cus, F. The Impact of Mesophilic and Thermophilic Anaerobic Digestion on Biogas Production. *J. Achiev. Mater. Manuf. Eng.* **2009**, *36*, 192–198.
12. Meegoda, J.N.; Li, B.; Patel, K.; Wang, L.B. A Review of the Processes, Parameters, and Optimization of Anaerobic Digestion. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2224. [\[CrossRef\]](#)
13. Maaroff, R.M.; Md Jahim, J.; Azahar, A.M.; Abdul, P.M.; Masdar, M.S.; Nordin, D.; Nasir, M.A.A. Biohydrogen Production from Palm Oil Mill Effluent (POME) by Two Stage Anaerobic Sequencing Batch Reactor (ASBR) System for Better Utilization of Carbon Sources in POME. *Int. J. Hydrogen Energy* **2019**, *44*, 3395–3406. [\[CrossRef\]](#)
14. Badiei, M.; Jahim, J.; Anuar, N.; Rozaimah, S.; Abdullah, S. Effect of Hydraulic Retention Time on Biohydrogen Production from Palm Oil Mill Effluent in Anaerobic Sequencing Batch Reactor. *Int. J. Hydrogen Energy* **2011**, *36*, 5912–5919. [\[CrossRef\]](#)
15. Jeong, J.-Y.; Son, S.-M.; Pyon, J.-H.; Park, J.-Y. Performance Comparison between Mesophilic and Thermophilic Anaerobic Reactors for Treatment of Palm Oil Mill Effluent. *Bioresour. Technol.* **2014**, *165*, 122–128. [\[CrossRef\]](#)

16. Krishnan, S.; Singh, L.; Sakinah, M.; Thakur, S.; Wahid, Z.A.; Sohaili, J. Effect of Organic Loading Rate on Hydrogen (H₂) and Methane (CH₄) Production in Two-Stage Fermentation under Thermophilic Conditions Using Palm Oil Mill Effluent (POME). *Energy Sustain. Dev.* **2016**, *34*, 130–138. [\[CrossRef\]](#)
17. Abdullah, M.F.; Md Jahim, J.; Abdul, P.M.; Mahmud, S.S. Effect of Carbon/Nitrogen Ratio and Ferric Ion on the Production of Biohydrogen from Palm Oil Mill Effluent (POME). *Biocatal. Agric. Biotechnol.* **2020**, *23*, 101445. [\[CrossRef\]](#)
18. American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2005.
19. Roussel, E.G.; Sauvadet, A.L.; Chaduteau, C.; Fouquet, Y.; Charlou, J.L.; Prieur, D.; Cambon Bonavita, M.A. Archaeal Communities Associated with Shallow to Deep Subseafloor Sediments of the New Caledonia Basin. *Environ. Microbiol.* **2009**, *11*, 2446–2462. [\[CrossRef\]](#)
20. Nübel, U.; Engelen, B.; Felsre, A.; Snaidr, J.; Wieshuber, A.; Amann, R.I.; Ludwig, W.; Backhaus, H. Sequence Heterogeneities of Genes Encoding 16S rRNAs in *Paenibacillus Polymyxa* Detected by Temperature Gradient Gel Electrophoresis. *J. Bacteriol.* **1996**, *178*, 5636–5643. [\[CrossRef\]](#)
21. Mohd Yasin, N.H.; Rahman, N.A.; Man, H.C.; Mohd Yusoff, M.Z.; Hassan, M.A. Microbial Characterization of Hydrogen-Producing Bacteria in Fermented Food Waste at Different PH Values. *Int. J. Hydrogen Energy* **2011**, *36*, 9571–9580. [\[CrossRef\]](#)
22. Maidak, B.L.; Olsen, G.J.; Larsen, N.; Overbeek, R.; McCaughey, M.J.; Woese, C.R. The Ribosomal Database Project (RDP). *Nucleic Acids Res.* **1997**, *25*, 109–110. [\[CrossRef\]](#)
23. Nordlander, E.; Olsson, J.; Thorin, E.; Nehrenheim, E. Simulation of Energy Balance and Carbon Dioxide Emission for Microalgae Introduction in Wastewater Treatment Plants. *Algal Res.* **2017**, *24*, 251–260. [\[CrossRef\]](#)
24. Zupančič, G.D.; Roš, M. Heat and Energy Requirements in Thermophilic Anaerobic Sludge Digestion. *Renew. Energy* **2003**, *28*, 2255–2267. [\[CrossRef\]](#)
25. Davis, M.L. *Water and Wastewater Engineering: Design Principles and Practice*; McGraw-Hill Education: New York, NY, USA, 2010.
26. Musa, M.A.; Idrus, S.; Hasfalina, C.M.; Daud, N.N.N. Effect of Organic Loading Rate on Anaerobic Digestion Performance of Mesophilic (UASB) Reactor Using Cattle Slaughterhouse Wastewater as Substrate. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2220. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Varel, V.H.; Chen, T.H.; Hashimoto, A.G. Thermophilic and Mesophilic Methane Production from Anaerobic Degradation of the Cyanobacterium *Spirulina Maxima*. *Resour. Conserv. Recycl.* **1988**, *1*, 19–26. [\[CrossRef\]](#)
28. Fang, H.H.P.; Chui, H.K. Comparison of Startup Performance of Four Anaerobic Reactors for the Treatment of High-Strength Wastewater. *Resour. Conserv. Recycl.* **1994**, *11*, 123–138. [\[CrossRef\]](#)
29. Kanimozhi, R.; Vasudevan, N. An Overview of Wastewater Treatment in Distillery Industry. *Int. J. Environ. Eng.* **2010**, *2*, 159–184. [\[CrossRef\]](#)
30. Westerholm, M.; Schnürer, A. Microbial Responses to Different Operating Practices for Biogas Production Systems. In *Anaerobic Digestion*; In Tech: Vienna, Austria, 2019. [\[CrossRef\]](#)
31. Cavinato, C.; Bolzonella, D.; Pavan, P.; Fatone, F.; Cecchi, F. Mesophilic and Thermophilic Anaerobic Co-Digestion of Waste Activated Sludge and Source Sorted Biowaste in Pilot- and Full-Scale Reactors. *Renew. Energy* **2013**, *55*, 260–265. [\[CrossRef\]](#)
32. Nasir, M.A.A.; Jahim, J.; Mohamed, P.; Silvamany, H.; Muzhafar, R.; Yunus, M.F.M. The Use of Acidified Palm Oil Mill Effluent for Thermophilic Biomethane Production by Changing the Hydraulic Retention Time in Anaerobic Sequencing Batch Reactor. *Int. J. Hydrogen Energy* **2019**, *44*, 3373–3381. [\[CrossRef\]](#)
33. Wang, Y.; Zhang, Y.; Wang, J.; Meng, L. Effects of Volatile Fatty Acid Concentrations on Methane Yield and Methanogenic Bacteria. *Biomass Bioenergy* **2009**, *33*, 848–853. [\[CrossRef\]](#)
34. Babae, A.; Shayegan, J. Effect of Organic Loading Rates (OLR) on Production of Methane from Anaerobic Digestion of Vegetables Waste. In *Proceedings of the World Renewable Energy Congress-Sweden, Linköping, Sweden, 8–13 May 2011*; Volume 57, pp. 411–417. [\[CrossRef\]](#)
35. Khanal, S.K. *Anaerobic Biotechnology for Bioenergy Production: Principles and Applications*, 1st ed.; Wiley-Blackwell: Iowa, IA, USA, 2009.

36. Najafpour, G.D.; Zinatizadeh, A.A.L.; Mohamed, A.R.; Hasnain Isa, M.; Nasrollahzadeh, H. High-Rate Anaerobic Digestion of Palm Oil Mill Effluent in an Upflow Anaerobic Sludge-Fixed Film Bioreactor. *Process Biochem.* **2006**, *41*, 370–379. [[CrossRef](#)]
37. Yu, G.H.; He, P.J.; Shao, L.M.; He, P.P. Toward Understanding the Mechanism of Improving the Production of Volatile Fatty Acids from Activated Sludge at PH 10.0. *Water Res.* **2008**, *42*, 4637–4644. [[CrossRef](#)]
38. Demirel, B.; Yenigün, O. Two-Phase Anaerobic Digestion Processes: A Review. *J. Chem. Technol. Biotechnol.* **2002**, *77*, 743–755. [[CrossRef](#)]
39. Junicke, H.; van Loosdrecht, M.C.M.; Kleerebezem, R. Kinetic and Thermodynamic Control of Butyrate Conversion in Non-Defined Methanogenic Communities. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 915–925. [[CrossRef](#)] [[PubMed](#)]
40. Inanc, B.; Matsui, S.; Ide, S. Propionic Acid Accumulation in Anaerobic Digestion of Carbohydrates: An Investigation on the Role of Hydrogen Gas. *Water Sci. Technol.* **1999**, *40*, 93–100. [[CrossRef](#)]
41. Ziels, R.M.; Beck, D.A.C.; Martí, M.; Gough, H.L.; Stensel, H.D.; Svensson, B.H. Monitoring the Dynamics of Syntrophic β -Oxidizing Bacteria during Anaerobic Degradation of Oleic Acid by Quantitative PCR. *FEMS Microbiol. Ecol.* **2015**, *91*, 1–13. [[CrossRef](#)] [[PubMed](#)]
42. Mahmood, S.S.; Azahar, A.M.; Amru, A.; Luthfi, I.; Jahim, J.M.D. Potential Utilisation of Dark- Fermented Palm Oil Mill Effluent in Continuous Production of Biomethane by Self-Granulated Mixed Culture. *Sci. Rep.* **2020**, *10*, 1–12. [[CrossRef](#)]
43. Franke-Whittle, I.H.; Walter, A.; Ebner, C.; Insam, H. Investigation into the Effect of High Concentrations of Volatile Fatty Acids in Anaerobic Digestion on Methanogenic Communities. *Waste Manag.* **2014**, *34*, 2080–2089. [[CrossRef](#)]
44. Kim, Y.M.; Jang, H.M.; Lee, K.; Chantrasakdakul, P.; Kim, D.; Park, K.Y. Changes in Bacterial and Archaeal Communities in Anaerobic Digesters Treating Different Organic Wastes. *Chemosphere* **2015**, *141*, 134–137. [[CrossRef](#)]
45. Tabatabaei, M.; Zakaria, M.R.; Rahim, R.A.; Wright, A.D.G.; Shirai, Y.; Abdullah, N.; Sakai, K.; Ikeno, S.; Mori, M.; Kazunori, N.; et al. PCR-Based DGGE and FISH Analysis of Methanogens in an Anaerobic Closed Digester Tank for Treating Palm Oil Mill Effluent. *Electron. J. Biotechnol.* **2009**, *12*. [[CrossRef](#)]
46. Conklin, A.; Stensel, H.D.; Ferguson, J. Growth Kinetics and Competition Between Methanosarcina and Methanosaeta in Mesophilic Anaerobic Digestion. *Water Environ. Res.* **2006**, *78*, 486–496. [[CrossRef](#)]
47. Patil, S.S.; Kumar, M.S.; Ball, A.S. Microbial Community Dynamics in Anaerobic Bioreactors and Algal Tanks Treating Piggery Wastewater. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 353–363. [[CrossRef](#)]
48. David, A.; Govil, T.; Tripathi, A.K.; McGeary, J.; Farrar, K.; Sani, R.K. Thermophilic Anaerobic Digestion: Enhanced and Sustainable Methane Production from Co-Digestion of Food and Lignocellulosic Wastes. *Energies* **2018**, *11*, 2058. [[CrossRef](#)]
49. Kor-bicakci, G.; Ubay-cokgor, E.; Eskicioglu, C. Comparative Analysis of Bacterial and Archaeal Community Structure in Microwave Pretreated Thermophilic and Mesophilic Anaerobic Digesters Utilizing Mixed Sludge under Organic Overloading. *Water* **2020**, *12*, 887. [[CrossRef](#)]
50. Kouzuma, A.; Tsutsumi, M.; Ishii, S.; Ueno, Y.; Abe, T.; Watanabe, K. Non-Autotrophic Methanogens Dominate in Anaerobic Digesters. *Sci. Rep.* **2017**, *7*, 1–13. [[CrossRef](#)]
51. Krakat, N.; Westphal, A.; Schmidt, S.; Scherer, P. Anaerobic Digestion of Renewable Biomass: Thermophilic Temperature Governs Methanogen Population Dynamics. *Appl. Environ. Microbiol.* **2010**, *76*, 1842–1850. [[CrossRef](#)] [[PubMed](#)]
52. Enitan, A.M.; Kumari, S.; Swalaha, F.M.; Odiyo, J.O.; Bux, F. Microbiota of a Full-Scale UASB Reactor Treating Brewery Wastewater Using Illumina MiSeq Sequencing. *Open Microbiol. J.* **2019**, *13*, 1–9. [[CrossRef](#)]
53. Zellner, G.; Macario, A.J.L.; Conway De Macario, E. Microbial Subpopulations in the Biofilm Attached to the Substratum and in the Free Flocs of a Fixed-Bed Anaerobic Bioreactor. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 443–449. [[CrossRef](#)]
54. Boone, D.R.; Whitman, W.B.; Koga, Y. The Archaea and the Deeply Branching and Phototrophic Bacteria. In *Bergey's Manual of Systematic Bacteriology*; Springer-Verlag: New York, NY, USA, 2001; Volume 1, pp. 268–294.
55. Kern, T.; Fischer, M.A.; Deppenmeier, U.; Schmitz, R.A.; Rother, M. *Methanosarcina flavescens* sp. nov., a Methanogenic Archaeon Isolated from a Full-Scale Anaerobic Digester. *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 1533–1538. [[CrossRef](#)]

56. Borja, R.; Banks, C.J.; Khalfaoui, B.; Martín, A. Performance Evaluation of an Anaerobic Hybrid Digester Treating Palm Oil Mill Effluent. *J. Environ. Sci. Health Part A* **1996**, *31*, 1379–1393. [[CrossRef](#)]
57. Amani, T.; Nosrati, M.; Sreekrishnan, T.R. A Precise Experimental Study on Key Dissimilarities between Mesophilic and Thermophilic Anaerobic Digestion of Waste Activated Sludge. *Int. J. Environ. Res.* **2011**, *5*, 333–342. [[CrossRef](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).