Effect of *Hyphomicrobium* sp. in Biogas Formation from Organic Waste Treated by Batch Mode Anaerobic Digestion

Etih Hartati[1*], Nurul Aiman Musodiqoh[1], Elin Nurlina[2], and Didin Agustian Permadi[1]

[1]*Environmental Engineering, Institut Teknologi Nasional Bandung, Indonesia*
[2]*Electrical Engineering, Universitas Jendral Ahmad Yani, Cimahi, Indonesia*

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*** Corresponding author:**
E-mail: etih@itenas.ac.id

ABSTRACT

Huge consumption of fossil fuel energy creates environmental problems, while the amount of organic solid waste is in an increasing trend especially in developing countries. Biogas is known as a renewable energy that can be used as an alternative fuel which is promising to reduce dependency on fossil fuels. This study identified the concentration of methane gas that can be produced from organic waste treated in an anaerobic digestion with the addition of *Hyphomicrobium* sp. Two-stage batch anaerobic digestion was used with an operational volume of 6 L. Four different starting concentrations of bacteria *Hyphomicrobium* sp. added to the reactors: 0.29×10^9 cells/L in reactor A, 0.87×10^9 cells/L in reactor B, 1.75×10^9 cells/L in reactor C, and no *Hyphomicrobium* sp. in the control reactor. The results showed that the concentration of methane gas produced was 38.4% in the reactor A while in reactor B and C were 35.6% and 33%, respectively. In reactor K with no addition of bacteria, the amount of methane produced was 52%. In contrary to the hypothesis, the addition of *Hyphomicrobium* sp. to the anaerobic process was contra-productive to the yield of methane. Further research is required to investigate the role of *Hyphomicrobium* sp. on the methane gas formation in the reactor.

1. INTRODUCTION

Energy has an important and indispensable role in human life, especially nowadays since almost all human activities are highly dependent on fossil fuel energy. Therefore, using renewable energy is a potential option to reduce both greenhouse gas and air pollutant emissions. In Indonesia, the fourth most populous country in the world, the pressure on energy demand is high and the amount of organic waste has been increasing. Indonesia was ranked 2nd in the world as the major contributor to the total generated food waste and the amount reached 113 Tg annually (EIU, 2017). Therefore, it is an opportunity to utilize the huge amount of generated waste to produce an alternative energy such as biogas. Biogas is a combustible mix of gases produced by anaerobic digestion process to degrade biodegradable materials (Ilaboya et al., 2010). Biogas is produced with methanogen bacteria which exist naturally in organic waste and is able to produce methane and other gases under anaerobic condition (Utami, 2010).

Several factors that may affect the biogas yield from the anaerobic digestion process have been well studied such as temperature, pH, organic loading rate and retention time in the reactor (Noraini et al., 2017). In an anaerobic digestion process, due to the symbiotic effects of various anaerobic and relatively anaerobic bacteria, organic substances are decomposed into simple, chemically stabilized compounds of methane and CO_2 (Naik et al., 2010). A two-stage thermophilic fermentation process has been reported to effectively enhance biomethane production and at the same time reduce the amount of organic waste (Wongthanate and Mongkarothai, 2018). One of the common bacteria used for methane formation is *Hyphomicrobium* sp. which can be isolated from mixed bacteria (Wilkinson and Hamer, 1972). Its habitat is widespread in soil and water and

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it is considered as a facultative anaerobic bacterium with a gram-negative genus that uses various carbon compounds as a source of energy. It also acts as helper to increase the methane oxidation rate in the methanotrophic process (Jeong and Kim, 2019). However, its effect in the anaerobic digestion process to treat organic waste has not been widely investigated.

This research examined the amount of methane gas concentration resulting from the utilization of organic waste from food waste as substrate with variations of *Hyphomicrobium* sp. bacteria concentrations. We used *Hyphomicrobium* sp. as the inoculum source for methane production based on previous research and we found *Hyphomicrobium* sp. as a dominant microorganism existing locally in the collected food waste. The effects on methane gas production are particularly of most concern, especially its synergistic effect to increase biogas yield using a two-stage fermentation process. The results can serve as science-based evidence to promote a feasible and workable system to produce renewable energy.

2. METHODOLOGY

2.1 Source of substrates

Organic solid waste was sampled from a restaurant with a consideration of having high organic content. Grab sampling was done in the morning time at 08.00 AM in a working day to collect of about 5 kg with the average measured density of 0.798 kg/L. Characteristics of organic waste such as density, water content, volatile level, C-organic, Total Kjehdahl Nitrogen (TKN), and C/N ratio were measured once a day (duplicate) as presented in Table 1. Hard materials such as animal bones and shells in food waste were removed before chopping the waste into more homogenous sizes to increase the surface area. Further it was mixed with tap water to obtain the substrate.

The purpose of addition of water is to resolute organic matter in the waste. Tap water from the Environmental Engineering Research Laboratory, Environmental Engineering Department, National Institute of Technology Bandung (ITENAS), Indonesia was used for the purpose. Several water characteristics such as pH, temperature and COD were measured once a day (duplicate) as presented in Table 1.

Table 1. Measurement methods

Parameter	Method	Reference	Measurement frequency			
			Waste and water characteristics	Acid formation	Substrate characteristics	Methane formation
pH	Potentiometry	SNI 06-6989.11:2004	1× /day	1× / 2 day	1× /day	1×/ 3 day
Temperature	Potentiometry	SNI 06-6989.11:2004	1× /day	1× / 2 day	1× /day	1×/ 3 day
Density	Gravimetry	-	1× /day	-	-	-
Water content	Gravimetry	SNI 03-197-1990	1× /day	-	-	-
Volatile levels	Gravimetry	SMEWW 5220,2012	1× /day	-	-	-
TVA	Distillation, titrimetric	SMEWW 5570,2012	-	1× / 2 day	1× /day	1×/ 3 day
COD	Reflux	SNI 6989.72:2009	1× /day	1×/ 2 day	1× /day	1×/ 3 day
BOD	Winkler Titration	SNI 6989.72:2009	-	-	1× /day	1× /day
Alkalinity	Acid-base Titration	SNI 06-2422-1991	-	-	1× /day	1×/ 3 day
C-Organic	Reflux	SMEWW 5220,2012	1× /day	-	-	-
TKN	Kjeldahl Analyzer	SNI 2081:2010	1× /day	-	1× /day	-
Phosphate	Spektrophotometry	SNI 6246-2010	1× /day	-	1× /day	-
Biogas	Orsat Analyzer	SNI 0029:2008	-	-	-	1× /2 day

Note: 1× /day: samples were taken once per day (duplicate), 1× /2 day: samples were taken once in two days (duplicate), 1× /3 day: samples were taken once in three days (duplicate). SNI–National Standard of Indonesia, SMEWW: Standard Methods for the Examination of Water and Wastewater.

2.2 Type of reactor

This research used a two stage anaerobic digestion system using two types of reactor: a hydrolysis reactor and a methanogenesis reactor. This system was chosen because the pH adjustment in the hydrolysis reactor caused the methanogen bacteria to digest organic matter quickly and accelerated methane formation (Hayes et al., 1988).

The hydrolysis reactor used in this study has a capacity of 180 L, made of a plastic drum equipped

with lids and taps with a working volume of 5 L. The methanogenesis reactor consists of four tubular reactors with a height of 35 cm and 16 cm in diameter with an operating volume of 6 L each. The methanogenesis reactor was made of glass fiber and was equipped with a thermometer, stirrer, influent and effluent ports, pressure regulator, sample point, and gas holder. Schematic illustrations of the reactors are presented in Figure 1(a) (hydrolysis) and Figure 1(b) (methanogenesis). Hydraulic retention time (HRT) for this batch mode reactor was set to 111 days. The value was actually based on the observation during the reactor operation.

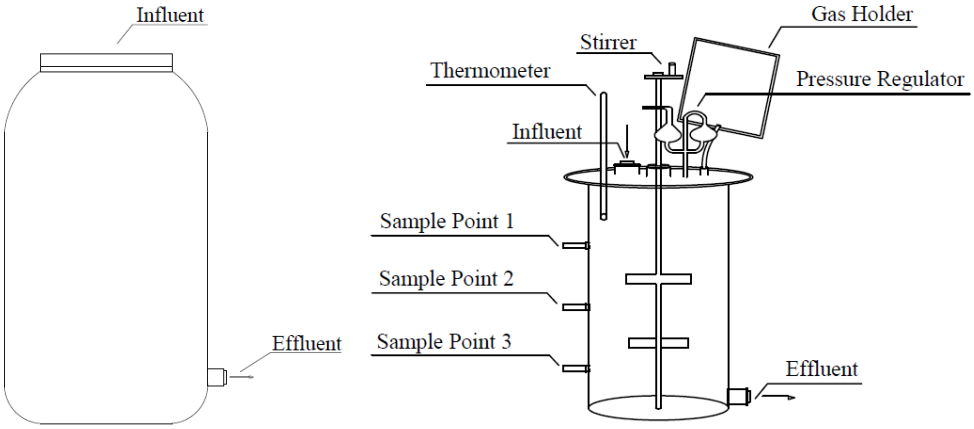

Figure 1. Scheme of hydrolysis (a); and methanogenesis (b) reactor

2.3 Research stages

2.3.1 Stages of acid formation

The ratio of organic waste and water used in this study was set to 1:1. This is because of the optimal C:N:P ratio of 400:5:1 in an anaerobic process was generally achieved using that ratio (Liberty and Prayatni, 2008). The density of organic waste in the hydrolysis reactor was 0.789 kg/L, while the amount of organic waste and water put in the hydrolysis reactor was set in the ratio of 25:20 (weight-based ratio). Parameters measured in this stage were pH, temperature, total volatile acid (TVA) and chemical oxygen demand (COD). The ratio of 1:1 was a volumetric ratio. Then, the density of organic waste and water were used to calculate the mass-based ratio of 1 kg/L: 0.789 kg/L. It yielded an actual weight ratio of 1.25 (25:20).

The measurement of substrate characteristics was performed to determine the initial conditions of the substrate used for methane formation. The substrate used was organic waste and water that have passed the stage of acid formation.

2.3.2 Stages of methane formation

This stage was done at the methanogenesis reactor with four variations of *Hyphomicrobium* sp. concentration (measured in cells per liter) bacteria added to the substrate. Different variations were done for different reactors (namely A, B, C, and K) with the different concentrations of bacteria as shown in Figure 2. *Hyphomicrobium* sp. bacteria was obtained from the identification of mixed bacteria used in methane formation and the concentration was calculated using the counting chamber method with a hemocytometer (Marin et al., 2014).

The concentrations of bacteria added to each reactor are as follows:

- Reactor A = substrate + 0.29×10^9 cells/L
- Reactor B = substrate + 0.87×10^9 cells/L
- Reactor C = substrate + 1.75×10^9 cells/L
- Reactor K = substrate without *Hyphomicrobium* sp.

Substrate amount was 5 L for each reactor.

3. RESULTS AND DISCUSSION

3.1 Characteristics of organic waste and water

Characteristics of organic waste and water used as substrates in methane formation are presented in Table 2. The parameters measured for organic waste characteristics were density, water content, volatile levels, C-organic and TKN while for water were pH, temperature, and COD.

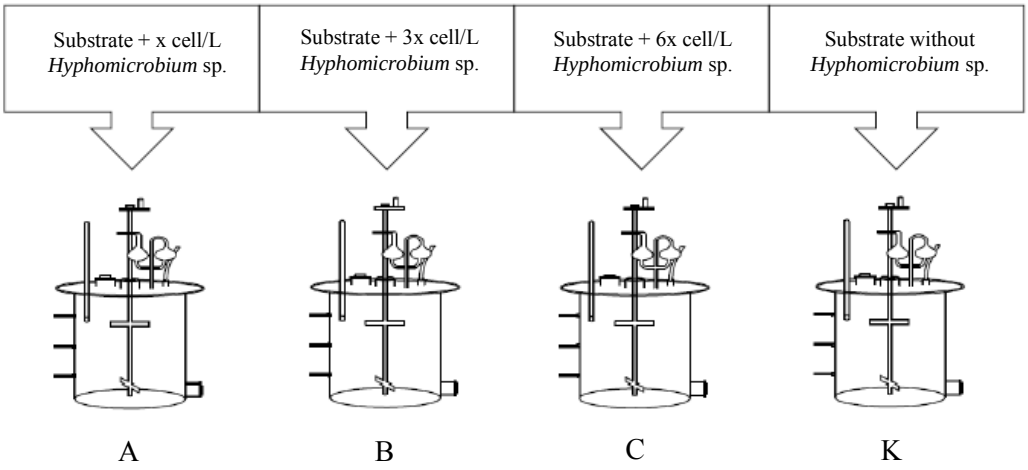

Figure 2. Variation of *Hyphomicrobium* sp. concentrations added to the reactors

Table 2. Characteristics of organic waste and water

No	Characteristics of organic waste			Water characteristics		
	Parameter	Results	Unit	Parameter	Results	Unit
1	Density	0.798	kg/L	pH	7.56	-
2	Water content	74.95	%	Temperature	29.25	°C
3	Volatile levels	86.92	%	COD	48	mg/L
4	C-Organic	50.04	%	-	-	-
5	TKN	2.24	%	-	-	-
6	C/N	22.33	-	-	-	-

Density is considered as one important factor that affects the rate of an organic waste decomposition process (Ramadanthi, 2008). The density on the organic waste used for this experiment was 0.798 kg/L while the water content was 74.9%. We considered analysis of the latter because water content would dissolve organic content and would act as a nutrient solvent for microorganisms (Rees, 1980). Note that the general value for water content in waste is about 50-80% (Tchobanoglous, 2004), hence the measured value was still within the range.

The volatile content of organic waste used was 86.92% which was lower than the values commonly reported for waste of about 95% (Tchobanoglous, 2004). Volatile level is important to determine the amount of organic matter contained in the waste. High volatile levels showed that organic waste was rich in organic materials which were easily degraded by bacteria (Ramadanthi, 2008). Carbon acts as a source of energy and nitrogen acts as a cell-builder for microorganisms. Therefore, the ratio of both is important for the anaerobic process (C/N ratio). The initial value of C/N ratio that is sufficient for starting the anaerobic processes should be within the range of 20-35 (Gotaas, 1956). In this research, organic waste used had a C/N ratio of 22.33% which showed sufficient amount of organic materials required to support the anaerobic process.

Temperature and pH are factors that greatly affect the growth of microorganisms in the process of anaerobic degradation of organic matter (Utami, 2010). According to Eckenfelder (1988), methanogenic bacteria worked well at pH 6.6-7.6. The pH of tap water used to dilute the organic waste was 7.56 so it was presumably suitable for growth and activity of the bacteria. The temperature of rinse water was 29.25 °C which was considered to be in an acceptable value to undergo the anaerobic process (Tchobanoglous, 2004). This indicated that the rinse water temperature may not potentially disturb the anaerobic process. The measured COD represents the organic content of the rinse water and the measured value was 48 mg/L which is an acceptable value for the process in the reactor.

3.2 Stages of acid formation

Acid formation was observed in this study as seen from the pH results from the 21 days run (Figure 3). It was clearly observed that pH values decreased up to the 6th day and continuously increased

afterwards. To prevent the formation of methane in the hydrolysis reactor, on the 21st day the substrate was transferred to the reactor for the methane generation stage. This is because we observed that the increasing concentration of TVA during the period of 10th - 21st day, the pH of the substrate slightly decreased from 5.1 to 5.0, thus methane formation may soon follow.

The pH value showed the activity of anaerobic bacteria in each process at its stage. As shown in Figure 3, during the period between the 2nd - 6th days, the pH on the substrate decreased from 6.1 to 4.4. The decrease in pH indicated that there has been an onset of the acidogenesis stage. During the period of the 6th - 10th days, the pH increased from 4.4 to 5.1 indicating the onset of the acetogenesis stage. While during the period of the 10th - 21st days, the concentration of TVA increased and the pH of the substrate slightly decreased from 5.1 to 5.0. The pH in the range of 5.5-6.5 is often reported as the optimal range for the acidogenesis stage (Mao et al., 2015), while acetogenesis has been reported to occur within a range of 6.0-6.2 (Ramos-Suárez et al., 2015).

Increased concentrations of TVA indicated the process of acid formation. It is shown in Figure 3 that the TVA concentration continued to increase. In the beginning, the TVA concentration was 1,463 mg/L and then increased to 11,552 mg/L after 21 days. The high TVA concentration could inhibit methane gas formation. According to Wilkie (2008), the concentration of the TVA of more than 10,000 mg/L could be toxic and act as an inhibitor in the anaerobic process. The increase of TVA over a given range did not cause a decrease in pH. This could be due to the buffering capacity in which the substrate could neutralize the formed acid. Therefore, it is necessary to measure the alkalinity to know the buffering capacity of the substrate.

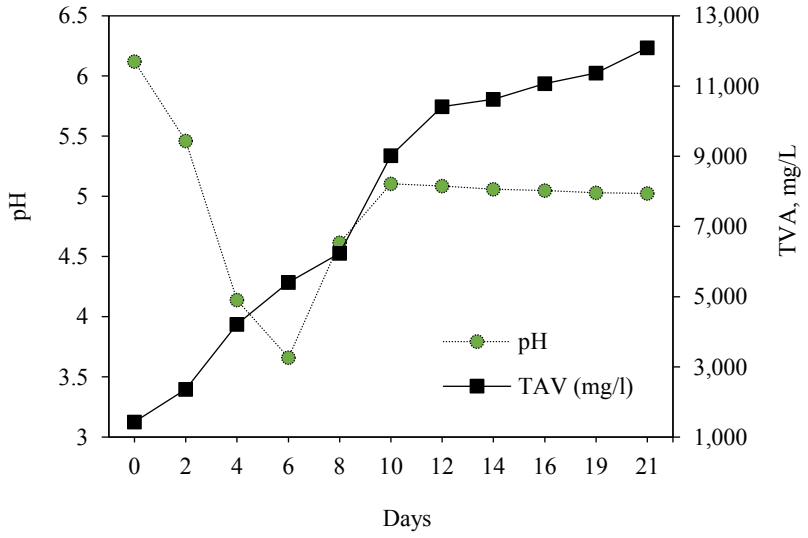

Figure 3. pH and TVA at hydrolysis reactor

Figure 4 shows that the temperature at the acid formation process ranged from 26.4 to 26.6 °C. According to Tchobanoglous (2004), the optimal temperature to support anaerobic degradation should be within the range of 25-35 °C. Temperature affects the growth of microorganisms and the rate of reaction in the formation of biogas. This is because microorganisms do not have temperature control systems in their cells (Widjajanti 2008). It can be said that the temperature range of the process carried out could support the anaerobic degradation.

Figure 5 showed that the decrease in COD concentration occurred during acid formation. Organic content from day 0 to day 21 decreased by 24.9% which was due to the degradation of organic compounds by the microbial activity (Ramadanthi, 2008).

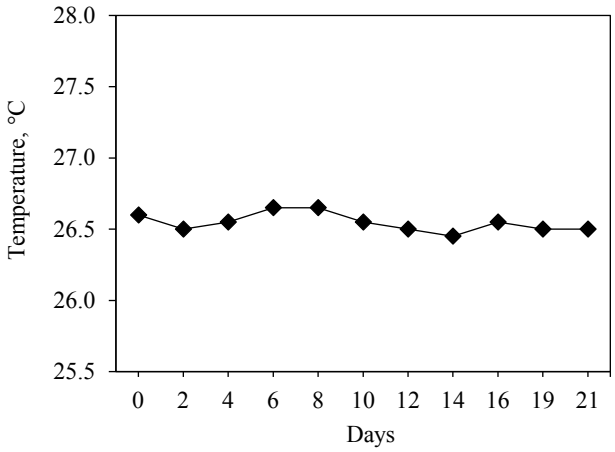

Figure 4. Temperature in the hydrolysis reactor

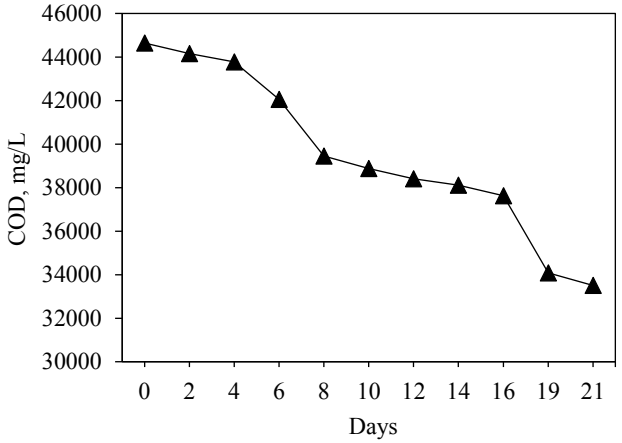

Figure 5. Concentration of COD in hydrolysis reactor

According to Malina et al. (1992), the suitable COD concentrations for anaerobic processing should range from 2,000 to 20,000 mg/L. The initial concentration of COD in the hydrolysis reactor was 44,640 mg/L. According to Syafila and Djajadiningrat (2003), this high concentration of organics would lead to more production of volatile acids from the acidogenesis process. Volatile acids formed in the acid formation process reached 11,552 mg/L. The amount of TVA content would interfere the process of methanogenesis, because acid is toxic to the methanogenesis bacteria.

On the 21st day, the liquid part of hydrolysis reactor, known as the substrate, was separated and transferred into the methanogen reactor. The initial characteristics of the starting substrate were measured and the results are presented in Table 3. The pH of substrate was 5.03 showing a typical acidic output from the acidogenesis stage. Substrate removal under these conditions was accomplished to prevent the occurrence of methane formation in the hydrolysis reactor. The temperature of the substrate was 25.6 °C, hence it can be said that the temperature of the substrate supports the anaerobic degradation. The concentration of the TVA in the substrate that was separated from the hydrolysis reactor was 13,253 mg/L. Under these conditions, the volatile acids contained in the substrate can be toxic to methanogenic bacteria, which would inhibit methane formation and growth of methanogenic bacteria (Grady and Lim, 1990).

Table 3. Substrate characteristics before entering methanogen reactor

No	Parameter	Concentration	Unit
1	pH	5.03	-
2	Temperature	25.60	°C
3	TVA	13,253	mg/L
4	COD	37,440	mg/L
5	BOD	31,049	mg/L
6	TKN	104	mg/L
7	Phosphate	2,319	mg/L
8	BOD/COD	0.83	-
9	COD/N	357	-
10	COD/P	16	-

The concentration of COD in the substrate that was separated from the hydrolysis reactor was 37,440 mg/L. High COD concentrations would result in high TVA concentrations, which are known to inhibit methane formation. The BOD/COD ratio was used as an early indication of the biodegradability of a material. The BOD/COD ratio of the substrate is 0.83 and this showed that the substrate could be used for methane formation through the biological process. Substrate characteristics in this study had COD/N 357 and COD/P ratio of 16. According to Veenstra and Lubberding (1993), the COD/N ratio should be 200 and COD/P of 1000. This indicated that the nutrient content was balanced with the existing organic content, so it is able to inhibit the growth of methanogen bacteria.

3.3 Stages of methane formation

Methanogenic process was identified clearly after 111 days of reactor run. We measured the parameters of temperature, pH, total volatile acid, alkalinity, COD and concentrations of methane gas. Figure 6(a) showed the time series of temperature and pH at four different methanogenesis reactors. There was little difference in temperature between reactor A, B, C and K. The temperature at the four

methanogenesis reactors ranged from 24 to 26.5 °C which was classified under mesophilic conditions (20-45 °C). Mesophilic bacteria had stability against the rapid changes in environmental temperature, so it was often used in anaerobic processing (Tchobanoglous, 2004). *Hyphomicrobium* sp. is a mesophilic bacteria (25-40 °C). The temperature in the methane formation process in this study supported the growth of *Hyphomicrobium* sp.

Figure 6(b) showed that the pH of the four methanogenesis reactors increased, but not significantly, after two days by only 0.01-0.02. On the second day, all four reactors had a similar pH of 5, while after 111 days the pH ranged from 5.4 to 5.5. The increase of pH at reactors A, B and C was relatively the same while the increase in pH of reactor K occurred slower. This can be because the concentrations of TVA at the reactor A, B, and C were lower than reactor K. However, after 47 days the pH at reactor K increased faster than reactors A, B and C. According Rahayu (2011), if the environmental conditions for the growth of methanogen bacteria are not suitable, then the bacteria will not consume acids anymore hence it further causes the increase in the TVA concentration.

Figure 7(a) showed that the concentration of the TVA in the four reactors tends to increase until the 44th day and the turn-over was seen after the 47th. The concentration of TVA at reactor K was higher than that measured in reactors A, B, and C that was due to the methanol content which was produced from the acid formation and was further utilized by *Hyphomicrobium* sp. bacteria for its growth (Wilkinson and Harrison, 1973).

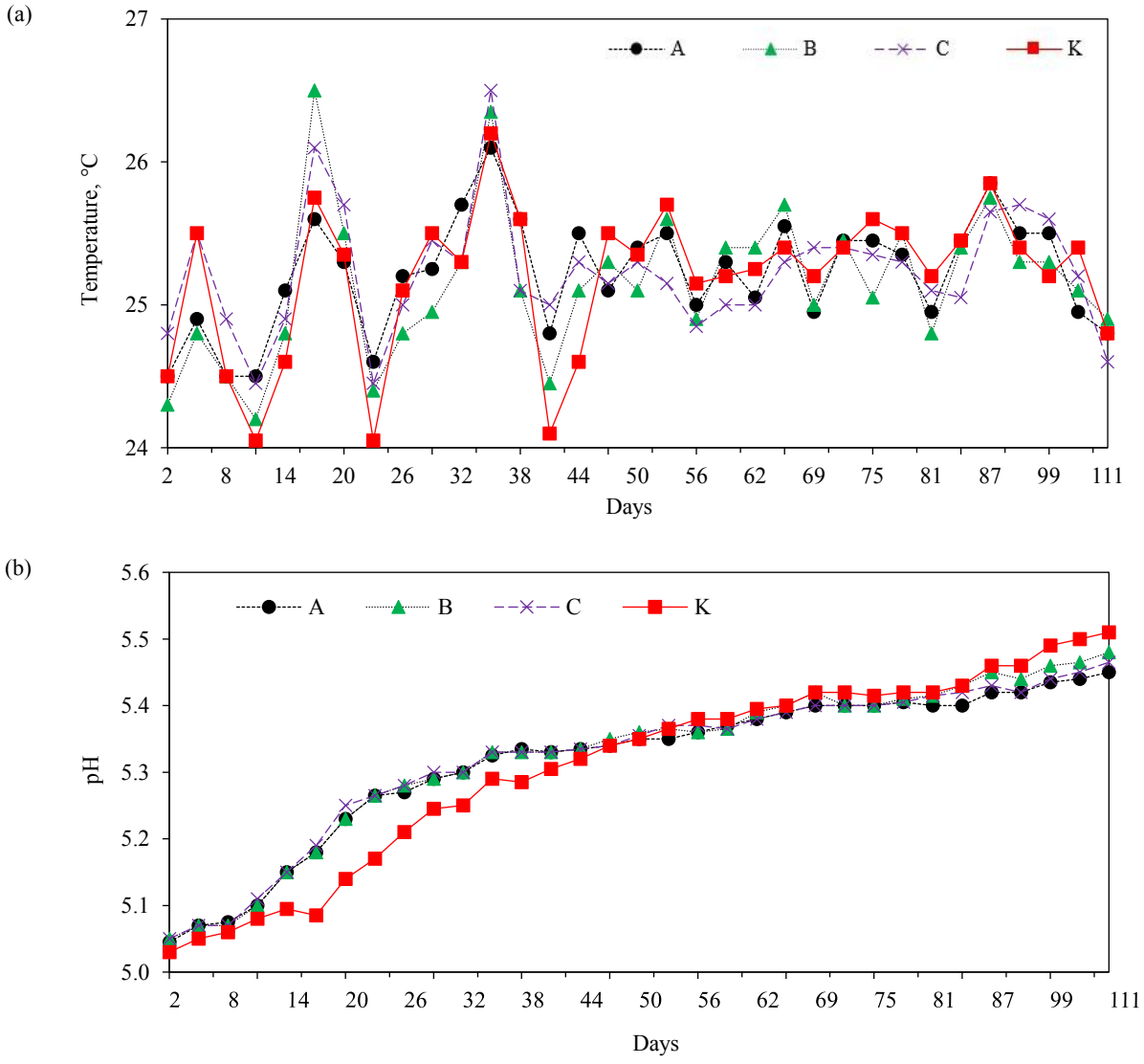

Figure 6. Measured parameters at the methanogen reactors: (a) temperature; and (b) pH

Alkalinity indicated buffer capacity in the anaerobic process, therefore high concentration of volatile acids would lead to the formation of acids, which in turn caused the pH to decrease. This showed that the buffer was not able to neutralize volatile acids. As a result, there was accumulation of volatile acids that could make the acidic environment of the microorganisms (Rahayu, 2011). The TVA/alkalinity ratio value of below 0.4 was a favorable condition for methanogen bacteria to grow so that methane gas production could be maintained (Grady and Lim, 1990).

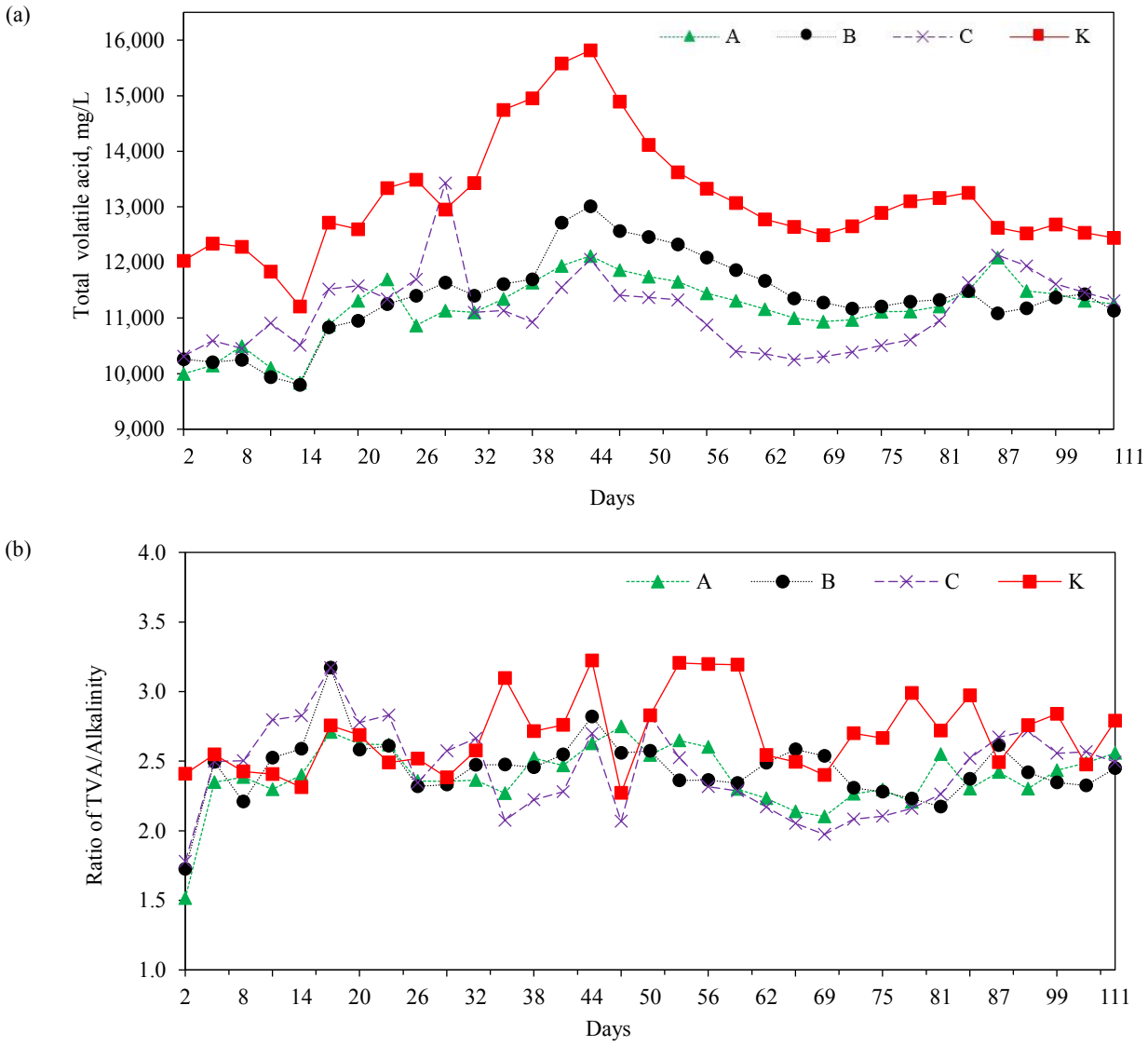

Figure 7. Measured parameters at the methanogen reactor: (a) TVA concentration; and (b) the ratio of TVA/alkalinity

Figure 7(b) presented the TVA/Alkalinity ratios of reactors A, B, C and K which ranged from 1.51 to 3.21. This indicated that the buffer could not neutralize excessive volatile acids. Figure 8(a) showed the concentration of bicarbonate alkalinity in reactor A was in the range of 3,064-5,530 mg/L, while in reactor B ranged from 2,167 to 5,066 mg/L. The associated concentrations in reactor C and K were 2,391-5,096 mg/L and 2,840-5,530 mg/L, respectively. According to Eckenfelder et al. (1988), the optimum bicarbonate alkalinity concentration for anaerobic treatment ranged from 2,500 to 5,000 mg/L. The average concentrations of bicarbonate alkalinity in all four reactors in the process are suitable for anaerobic treatment. The high ratio of TVA/Alkalinity was due to the high concentration of volatile acids formed in the process. Too high TVA or TVA/alkalinity ratio may suppress methane gas production because it will not provide suitable condition for the methanogen bacteria to grow in such environment.

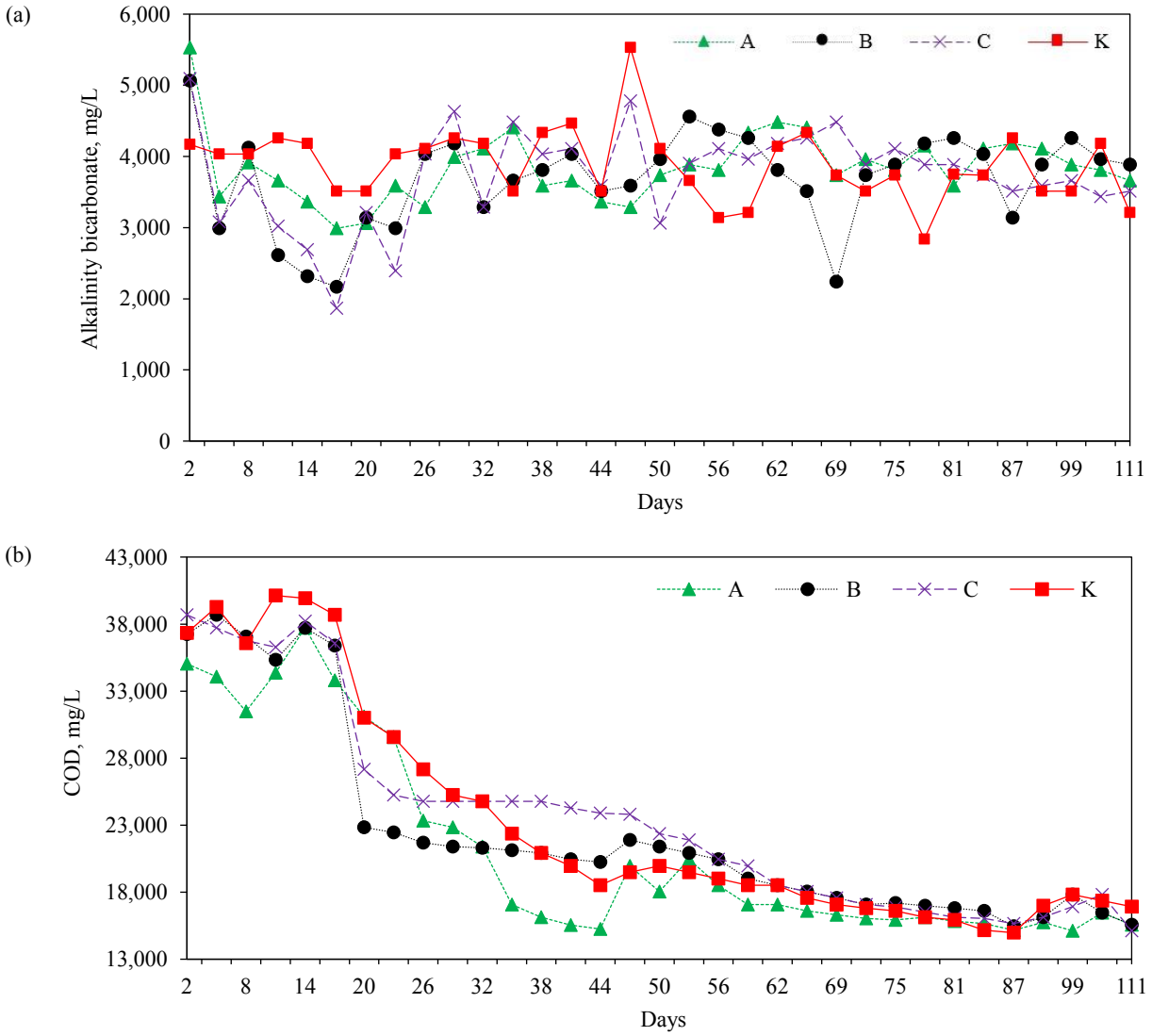

Figure 8. Concentrations of alkalinity bicarbonate: (a); and COD (b)

Figure 8(b) showed variations of COD concentration during the methane formation. The concentration of COD on the methanogenesis reactor tends to decrease. In reactor A, COD decreased by 49%, while in rectors B, C, and K were 52%, 53% and 54%, respectively. Decrease in COD concentration can be caused by the process of organic degradation by bacteria. Organic content is also used as a food source for bacteria present in the process (Ramadanthi, 2008).

3.4 Methane gas concentration

Methane gas is the final product of anaerobic processing. The methane gas formation is the result of the degradation of organic compounds, thus during the end of the operation (days 93rd -111th) we highlighted the changes of CH_4, TVA and COD concentrations in each reactor. The results are presented in Figure 9. There was a substantial decrease in methane gas concentration from day 93rd to day 111th. As shown in Figure 9(a), the concentration of methane gas at reactor K is higher than that of reactors A, B, and C. This is due to the absence of *Hyphomicrobium* sp. bacteria in reactor K as compared to others in which *Hyphomicrobium* sp. was added with different concentrations. *Hyphomicrobium* sp. is generally known as a methanol-beneficial bacterium where high methanol production can inhibit methane oxidation (Wilkinson and Harrison, 1973). As found in this study, the higher of the *Hyphomicrobium* sp. bacteria, the lower of methane gas would be produced.

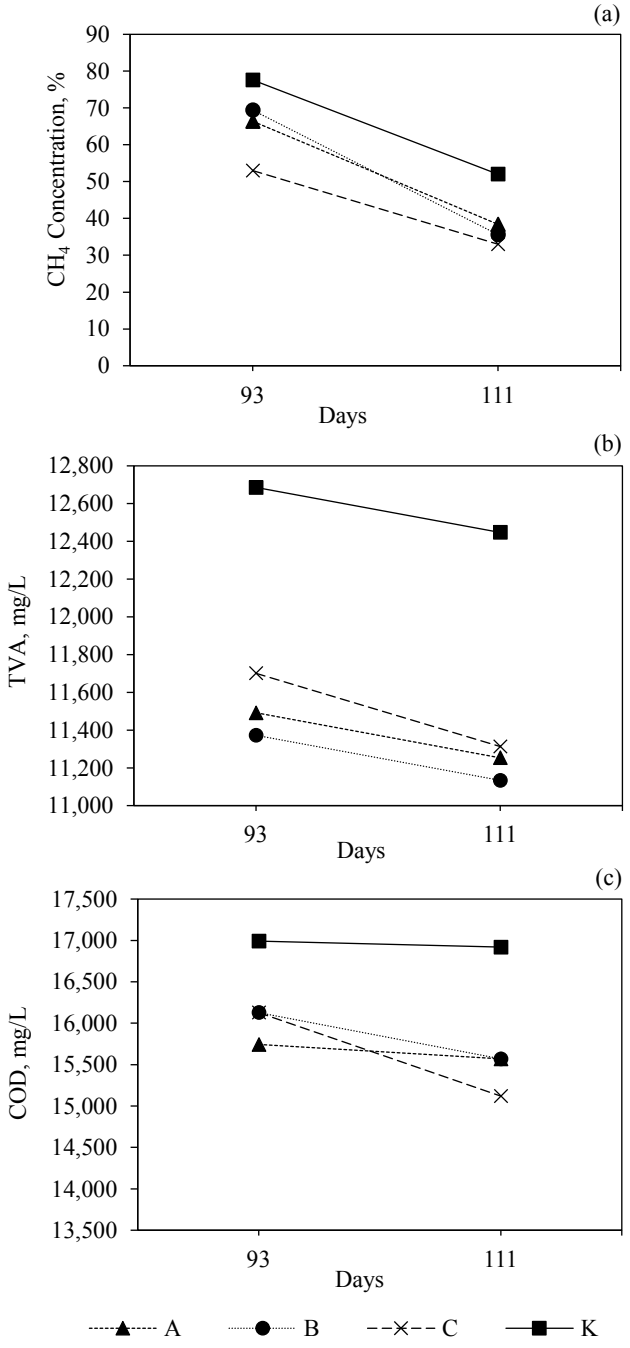

Figure 9. Concentrations of methane gas (a); TVA concentration (b); and COD (c)

Figure 9(b) showed a comparison between methane formation from the four variations of *Hyphomicrobium* sp. concentration in correlation with the TVA. The concentration of TVA in the K reactor was higher than that of reactors A, B, and C, and the methane gas concentration generated at the reactor K was greater than that of reactors A, B and C. According to Syafila and Djajadiningrat (2003) the amount of TVA would decrease as a result of the methanogenesis process which would convert the product from the process of acidogenesis into methane gas. In this study the decrease in TVA concentration was not accompanied by an increase in methane gas concentration which was caused by the addition of *Hyphomicrobium* sp. This would influence the formation of TVA because the *Hyphomicrobium* sp. bacteria utilized methanol for its growth (Wilkinson and Harrisons, 1973).

Methanol is one of the intermediate products of the acididogenesis stage in the formation of methane formation. The accumulation methanol production would inhibit the oxidation of methane, so that the methane concentration would be higher (Wilkinson and Harrisons, 1973). When methanol was utilized by *Hyphomicrobium* sp. bacteria, its concentration would decrease hence the methane formation would be limited. When a higher amount of the *Hyphomicrobium* sp. bacteria was added, the TVA concentration would be higher as well as the methane.

Concentration of COD in the four reactors (Figure 9(c)) tended to decrease along with the decrease of methane gas concentration in Figure 9(a). Reactors were operated in a batch system hence this affected the organic degradation to be slow which further bring in the lower production of methane. The concentration of methane gas would decrease along with the decrease in COD concentration. Under the condition of high concentration of *Hyphomicrobium* sp., the concentration of COD tended to be lower. Ramadanthi (2008) confirmed the situation that the decrease in COD concentration could be caused by the organic degradation process by bacteria.

In contrary to our previous hypothesis, the addition of *Hyphomicrobium* sp. seemed to be contra-productive to the methane production. The inoculum also consumed the organic hence reduced the TVA as compared to reactor K. Therefore, methane production in the reactor without inoculum was higher than those with the bacteria. *Hyphomicrobium* sp. was reported to use many carbon compounds (including methanol carbon as an intermediate compound for CH_4 production) as growth substrates (Wilkinson and Hamer, 1972). These explained our findings that higher concentration of *Hyphomicrobium* sp. did not yield higher concentration of CH_4, thus *Hyphomicrobium* sp. rich containing organic source should be avoided.

4. CONCLUSION

Maximum concentrations of methane gas produced from organic waste treated in the reactor were 66.4% (A), 69.4% (B), 53% (C) and 77.6 % (K).

It can be concluded that the concentration of *Hyphomicrobium* sp. has an effect on the formation of methane gas in the reactor however the production of methane was higher if it was not added. Inverse correlation was found between both parameters meaning that the higher concentration of *Hyphomicrobium* sp. would lead to lower concentration of the methane gas. This can be explained due to the formation of methanol as an intermediate product of the methane formation process which was further utilized by *Hyphomicrobium* sp. It also uses many carbon compounds such as methanol carbon as growth substrates. However, further research is required to investigate solely the role of *Hyphomicrobium* sp. on the methane gas formation in the reactor without contribution from the microbial activity from the substrate. Control experiment of tap water "+" inoculum and tap water only should be conducted in the future to isolate the impact of inoculum to the bio methane production.

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