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Effect of Substrate-to-Inoculum Ratio and Temperatures During the Start-up of Anaerobic Digestion of Fish Waste

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ABSTRACT: The high protein and lipid content of fish waste makes mono-digestion a difficult bioprocess for an anaerobic digestion (AD) system. On the other hand, the massive increase in fish and seafood consumption worldwide has led to an inevitable fish waste mono-AD. Therefore, this study was conducted to investigate the effects of food-to-microorganisms (F/M) ratios and temperatures during the start-up period of fish waste mono-digestion. F/M ratios of 0.5, 1, 2, and 3 on a g-COD/g-VSS basis were operated at 35°C and 45°C, representing mesophilic and hyper-mesophilic conditions, respectively. The increase in F/M ratio improved the maximum methane (CH_4) production rate at both temperatures. However, F/M ratio of 0.5 generated the highest CH_4 yield in mesophilic and hyper-mesophilic conditions (0.23±0.00 L-CH_4/g-COD_{input}). Further increase in F/M ratio decreased CH_4 yield up to 21.74% and 39.13% when the reactors were operated at 35°C and 45°C, respectively. When reactors were supplied with FM ratios of 0.5, 1, and 2, hyper-mesophilic temperature improved methanogenesis by up to 2.61% and shortened the lag phase by 22.88%. Meanwhile, F/M ratio 3 at 45°C decreased cumulative CH_4 production by up to 26.57% and prolonged the lag phase by 10.19%. The result of this study is beneficial to managing the input substrate of a batch-AD system that treats fish waste as a sole substrate.

KEYWORDS: Anaerobic digestion; F/M ratio; fish waste; mono-digestion; start-up

1. Introduction

Worldwide fish and other seafood consumption has shown a massive increase during the last several decades, from 6 kg/capita in 1950 to 20 kg/capita in 2018. The global harvest of captured fisheries and aquaculture production for human consumption reached 159 million tonnes in 2018 and is expected to reach 183 million tonnes in 2030 [1]. Despite the rising demand, a significant portion of captured fish and aquaculture harvests go to waste. Around 45% of the fish weight must be discarded during fish processing as it is not consumable for humans [2,3]. Fish farms also experience considerable economic loss as the harvested fish cannot pass the quality checks caused by the existence of pathogens and diseases.

Considering the bioeconomy of fish waste, some portions of it can be further processed for fish food, collagen, pectin, and chitin [4]. Meanwhile, another considerable amount of it remains as waste. This waste needs to be appropriately treated in order to avoid serious environmental issues. Anaerobic digestion (AD) is a well-known and consolidated bioengineering technology to treat fish waste as it produces methane (CH_4) as an energy source and is relatively more affordable compared to other waste treatment technologies [5]. Moreover, fish waste is a suitable substrate for AD as it has high organic content, mainly protein and lipid. Fish waste has been found to contain up to 60% protein and 20% lipid [6].

However, volatile fatty acids (VFAs) and ammonia (NH_3) generated from protein degradation are widely reported to cause process instability or even process failure in AD [2, 3, 7]. Process imbalances caused by VFA accumulation lead to acidification problems [7]. Meanwhile, in its high concentration, NH_3 leads the microbial cells to consume more energy to maintain extracellular pH and eventually disrupts the expression of key enzymes required for methanogenesis [8]. Moreover, long-chain fatty acids (LCFAs) produced by lipid degradation of fish waste also imposed an inhibitory effect on the methanogenic consortia by solubilizing the lipid bilayer or protein membranes. Eventually, it contributes to irreversible cell lysis [9].

Therefore, co-digestion of fish waste with a secondary substrate is preferable for an AD system rather than treating it as a sole substrate. In this regard, co-digesting fish waste with other substrates has been widely investigated, including vegetable wastes [10], cow manure [11], sisal pulp [12], waste activated sludge [3], agriculture wastes [13], and residual strawberry [14]. Previous studies reported that adding fish waste for co-digestion of AD improved CH_4 production [3,10–14]. However, a co-substrate suitable for fish waste AD may not always be available. Moreover, providing a secondary substrate may impose a challenging task for full-scale operation considering the transportation fee of the co-substrate. Therefore, mono-digestion is regarded as a favorable process operation.

Nevertheless, mono-digestion of fish waste AD may experience a challenging start-up caused by the production of intermediates from protein and lipid degradations [7, 11]. Start-up is a fundamental and crucial period for the success of an AD operation. Microbial communities require this period to acclimatize to the employed substrate and establish solid syntrophic cooperation. As the microorganisms adjust to a new substrate, process imbalances and undesired situations may occur, such as acidification, long lag periods, suboptimal removal of organic matter, and low CH_4 production [15,16].

A successful start-up is mainly affected by employing appropriate operational parameters; among those are adjusting organic loading and temperature [15]. In a batch system, the organic loading is adjusted by regulating the food-to-microorganisms (F/M) ratio [17]. The F/M ratio was reported to have the greatest influence during the start-up period of batch solid-state AD, from 75% to 38% at the beginning and end of the start-up period, respectively [18]. With an appropriate F/M ratio, well-established methanogenic consortia will degrade an employed substrate efficiently. The decent adaptability of microbial communities during the start-up period will then determine the reactor performance for successive digestion [16].

Meanwhile, temperature affects enzymatic and biological activities. Therefore, the substrate reaction rate will increase with the increase of temperature and vice versa [5]. In AD systems, mesophilic (35–40°C) and thermophilic (52–55°C) temperatures are commonly applied, considering their benefits [19]. Although the thermophilic condition is advantageous for increasing substrate degradation rate, it can cause a process imbalance by producing intermediates at rates that are difficult for methanogens to consume [20]. High energy requirements must also be invested in to maintain the thermophilic condition, leading to an increase in operational costs. Therefore, the mesophilic condition is widely applied in AD, especially in full-scale systems. However, the application of hyper-mesophilic temperatures (41–45°C) has attracted more attention in the last several years as it improves substrate degradation rates while maintaining the intermediate concentrations at non-inhibitory levels [21,22].

It is expected that different F/M ratios and temperatures applied to the system will affect the reactors' performance during the start-up in different ways. Therefore, this study was conducted to investigate the effect of substrate input by adjusting the F/M ratio on the performance of mono-digestion of fish waste AD, focusing on the start-up period in the batch mode. Mesophilic and hyper-mesophilic temperatures were also applied to investigate their effects on different F/M ratios. The result of this study is beneficial for managing mono-digestion of fish waste operating in a batch system.

2. Materials and Methods

2.1. Inoculum source and substrate

The inoculum source used in this study was collected from a full-scale anaerobic digester co-digesting primary sludge and food waste. The full-scale digester was located in Ulsan City, South Korea. Meanwhile, the substrate was fish waste powder (FWP) collected from a local fish waste processing industry that produced fish feed. The FWP consisted of approximately 80% dead fish and 10% squid gut collected from several local fish farms that cover around a fifth of the total area of South Korea. The remaining 10% contained soybeans and sesame to balance the carbon content required for fish feed. The characteristics of the inoculum source and the substrate used are provided in Table 1.

Table 1. Characteristic of inoculum source and fish waste powder.

Characteristics	Inoculum source in g/L	Fish waste powder in g/g
TS	33.08 ± 0.40	0.93 ± 0.00
VS	18.43 ± 0.24	0.85 ± 0.00
VSS	16.80 ± 0.07	0.50 ± 0.01
COD	13.63 ± 0.98	1.53 ± 0.11
Total carbohydrate	2.34 ± 0.25	0.22 ± 0.01
Kjeldahl Protein	8.94 ± 0.14	0.43 ± 0.00
Lipid	2.48 ± 0.18	0.18 ± 0.00

2.2. Experimental setup and operation conditions

The anaerobic batch tests were performed in serum glass bottles with a 100 mL working volume. Both the inoculum source and the FWP were sieved with a 0.45 mm pore size. The stock solution of FWP was prepared for the substrate. The concentration of the substrate stock solution was 362.70 g-COD/L, prepared to mimic the concentration of fish restaurant waste in the east coastal area of South Korea.

The F/M ratio and mesophilic temperatures were two independent variables applied in this study. The reactors were inoculated with F/M ratios of 0.5, 1, 2, and 3 g-COD/g-VSS. The reactors were inoculated with 100% seeding (16.80 g-VSS/L), following the seeding condition in the field that tends to avoid diluting the inoculum source with water as it will increase the operational cost. The volume of substrate and inoculum added into the reactors were calculated based on Eq. (1) and Eq. (2).

$$V_w = V_f + C_i \tag{1}$$

$$F/M\,ratio = \frac{(V_f)(C_f)}{(V_m)(C_m)} \tag{2}$$

where V_w is initial working volume, V_f is substrate volume (mL), V_m is inoculum volume (mL), C_f is substrate concentration (362.70 g-COD/L), and C_m is inoculum concentration (16.80 g-VSS/L).

Based on Eq. (1) and Eq. (2), the substrate concentrations added to the reactors were 8.16, 16.02, 30.74, and 44.25 g-COD/L for F/M ratios of 0.5, 1, 2, and 3, respectively. Each F/M ratio was operated at 35°C and 45°C, corresponding to mesophilic and hyper-mesophilic temperatures. Control treatments without the addition of substrate were conducted for both temperatures. The reactors were purged with 80% nitrogen (N_2) and 20% carbon dioxide (CO_2) for three minutes, and the pH was not adjusted until the end of the experiment. All treatments were conducted with quadruplicate reactors under 120 rpm continuous shaking. The experiment was conducted for 240 h (10 d), and the endpoint physicochemical analysis was conducted at 240 h.

2.3. Physicochemical analysis

Standard methods and protocols for measuring pH and total alkalinity (TA) were followed [23]. pH was analyzed using a pH electrode (Cole Parmer Instrument), and TA was measured using a SI Analytics TitroLine 5000 equipped with an automatic titrator. Biogas content was analyzed using gas chromatography (GC-HP; Agilent, Palo Alto, CA, USA) equipped with a thermal conductivity detector and HP-Plot Q packed column (Agilent). Helium (He) was used as a carrier gas with a 20 mL/min flow rate. The concentrations of volatile fatty acids ([VFAs], C_2–C_6) were determined using a GC-HP 6890 Plus (Agilent, Palo Alto, CA, USA) equipped with a flame ionization detector and an Innowax capillary column (Agilent). He was also used as a carrier gas with a 10:1 split ratio and a 2.5 mL/min flow rate. The results of the analysis items mentioned in this section were presented as the mean standard deviation of quadruplicate reactors.

Standard methods and protocols were used to measure solids (total solid TS, volatile solid VS, and volatile suspended solid VSS), chemical oxygen demand concentration ([COD]), and Kjeldahl protein concentration ([Kjeldahl protein]) of the inoculum source and FWP [23]. A spectrophotometer (Hach DR 6000) was used to analyze [COD], and a Kjeltech 2300 Analyzer unit was used to measure [Kjeldahl protein]. Meanwhile, total carbohydrate concentration was measured using the phenol-sulfuric acid method. Lipid concentration ([lipid]) was determined based on the gravimetric method, using chloroform-methanol (1:2 v/v) as solvents for lipid extraction.

2.4. Statistical analysis

The modified Gompertz model (Eq. (3)) was employed in this study to estimate cumulative CH_4 production (CMP), maximum CH_4 production rate (R_m), and lag phase of methanogenesis from fish waste (λ).

$$M_{CH_4}(t) = P_{CH_4} x \; exp\left\{-exp\left[\frac{R_m \, x \, e}{P_{CH_4}}\right] x \; (\lambda - t) + 1\right\} \tag{3}$$

where $M_{CH_4}(t)$ is CMP at the digestion time t (d) in L/L, P_{CH_4} is the ultimate CMP in L/L, and $e = 2.71828$.

A principal component analysis (PCA) with Euclidean distance was performed to investigate the environmental factors that shaped the performance of fish waste mono-digestion. PCA input variables included R_m, CMP, λ, CH_4 yield, final pH, final TA, temperatures, residual [acetate] (Res_[Ac]), and residual [propionate] (Res_[Pro]). Before conducting the PCA analysis, standardization (Eq. (4)) was performed independently on all input variables to avoid the dependency of PCA on certain variables that had larger values. Standardization was also performed to minimize the effect of outliers on the model.

$$z_i = \frac{x_i - u}{s} \tag{4}$$

where z_i is the standardized value of data entry i, x_i is the unstandardized value of data entry i, u is the mean of input variables, and s is the standard deviation. The standardized value had zero mean, and the resulting distribution had unit variance.

To obtain insight into how the data clustered on PCA, cluster analysis (CA) was performed with Euclidean distance using the paired group algorithm and group constraints. Analysis of similarity (ANOSIM) with Euclidean distance and 9999 permutations was also conducted to analyze the significant difference between the clusters obtained from CA. A two-sample t-test was also performed to evaluate the significant difference in R_m between the actual and predicted values and the significant difference in lag phase on the tested temperatures. Fitting of the modified Gompertz model was performed using Sigma Plot v. 12 software (Systat Software Inc., USA). PCA, CA, ANOSIM, and two-sample t-tests were performed using PAST 4.05 software. Standardization was performed using Python 3.8 with the sklearn module.

3. Results and Discussion

3.1. Effect of F/M ratio and applied temperatures on CH_4 production

The F/M ratio is a critical parameter representing the organic loading of digesters operated in batch mode. A high F/M ratio may provoke an imbalance in the AD process. In this regard, several studies reported that the F/M ratio above 3 on VS/VS basis and COD/VS basis did not improve CH_4 production rate as methanogens require a longer time to consume residual VFAs. Meanwhile, a certain low F/M ratio may prevent the generation of enzymes required for biodegradation. CH_4 yield was also substantially reduced at F/M ratio below 0.25. Moreover, the range of F/M ratios between 0.5 – 0.7 is recommended for many substrates, including dairy manure, waste activated sludge, food waste, synthetic wastewater, straw, and maize [17,24]. The finding of those previous studies oriented the implementation of F/M ratios 0.5, 1, 2, and 3 in this study.

The profiles of cumulative and daily CH_4 production for mesophilic and hyper-mesophilic conditions with different F/M ratios are shown in Figure 1 and Figure 2, respectively. Meanwhile, the parameters obtained from fitting the modified Gompertz model are provided in Table 2. During the observation, the CMP increased with the increase of the

F/M ratio from 2.03 ± 0.02 and 2.00 ± 0.01 L/L at F/M ratio 0.5 to 9.07 ± 0.01 and 6.66 ± 0.01 L/L at F/M ratio 3 for mesophilic (35°C) and hyper-mesophilic (45°C) temperatures, respectively (Figure 1 (a) and Figure 2 (a)). In hyper-mesophilic conditions, the CMP of F/M ratios 1 and 2 was 2.61% and 1.58% higher than those in mesophilic conditions. Meanwhile, the CMP of F/M ratio 3 was 26.57% lower than that of mesophilic conditions (Table 2).

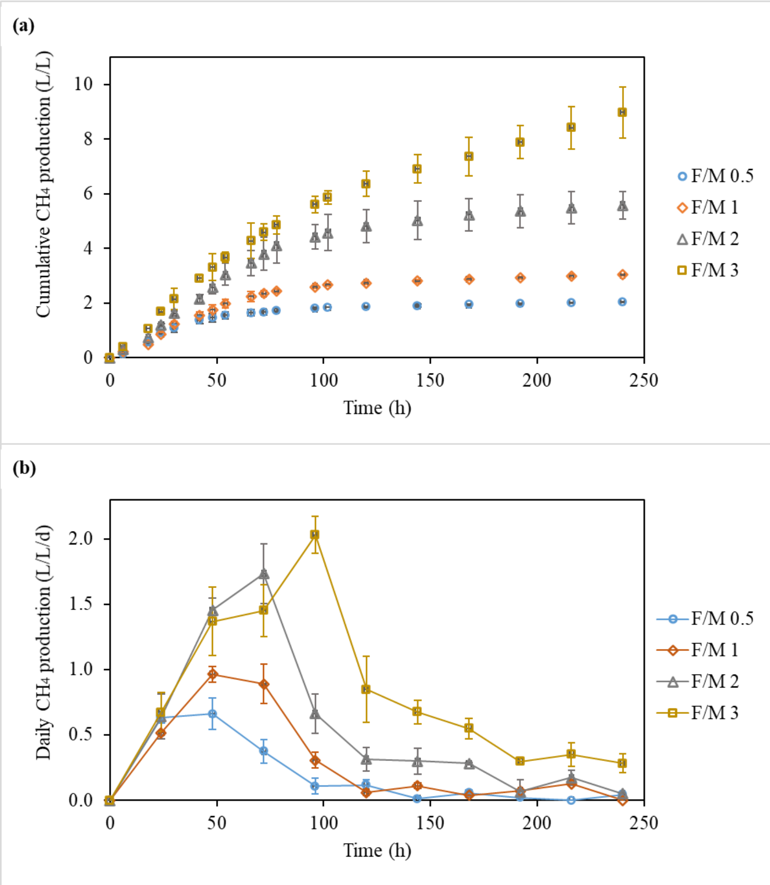

Figure 1. Methane production of the anaerobic batch-test treated at 35°C: (a) cumulative CH_4 production and (b) daily CH_4 production. The values were subtracted from control reactors.

Lu et al. (2020) defined the start-up period of a batch system until it reaches the first peak of daily CH_4 production [25]. As shown in Figure 1 (b) and Figure 2 (b), the peak of daily CH_4 production occurred at 48–96 h for mesophilic conditions and 48–72 h for hyper-mesophilic conditions. After that, the daily CH_4 production gradually decreased toward zero until the remaining observation time, indicating the end of the start-up period. The result indicates that higher temperature shorten the time required by the system to reach the peak of daily CH_4 production. Therefore, the hyper-mesophilic temperature shortened the start-up period. However, the two-sample t-test showed that there was no significant difference in R_m between the two different temperatures, both for predicted and actual values ($p > 0.05$).

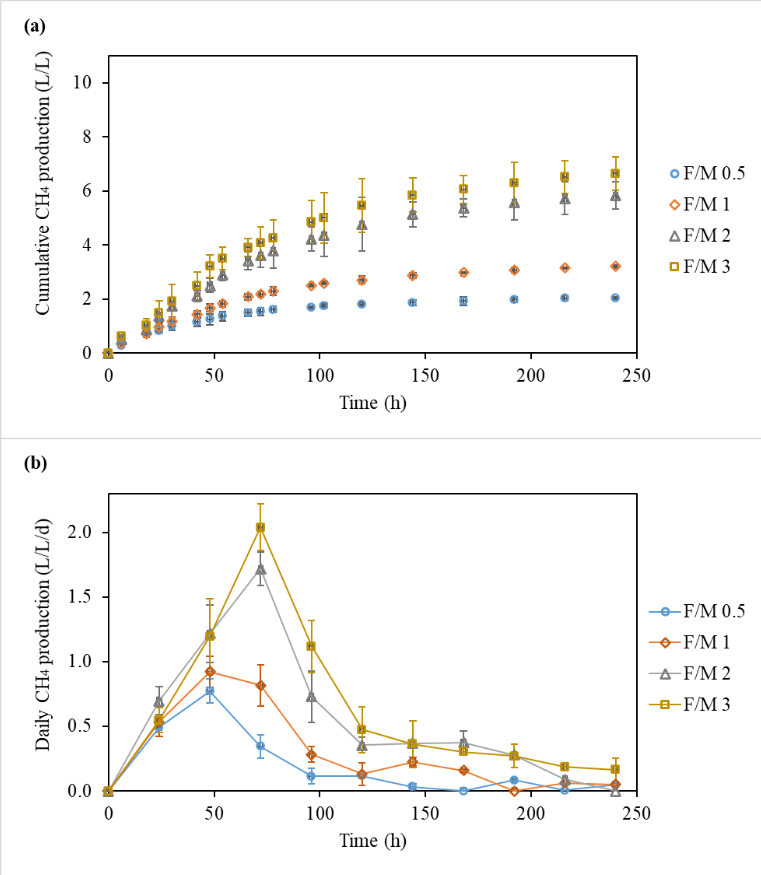

Figure 2. Methane production of the anaerobic batch-test treated at 45°C: (a) cumulative CH_4 production and (b) daily CH_4 production. The values were subtracted from control reactors.

Figure 1 (b) and Figure 2 (b) also showed the actual maximum CH_4 production rate (R_m) when daily CH_4 production showed the highest peak for each F/M ratio. The actual values were then compared with the predicted values obtained from fitting the modified Gompertz model. In general, the predicted parameters obtained from fitting the modified Gompertz model are close to the actual values, suggesting that the obtained parameters are reliable.

Table 2. Comparison of parameters obtained from modified Gompertz model and its actual values

Treatment		Cumulative CH_4 production (L/L)		R_m (L/L/d)		λ (h)	R^2
		Predicted	Actual	Predicted	Actual		
Mesophilic	F/M 0.5	1.96 ± 0.02	2.03 ± 0.02	0.72 ± 0.05	0.66 ± 0.03	3.89 ± 0.07	0.99 ± 0.00
	F/M 1	2.97 ± 0.01	3.06 ± 0.00	1.04 ± 0.02	0.97 ± 0.05	12.90 ± 0.36	0.99 ± 0.00
	F/M 2	5.51 ± 0.01	5.68 ± 0.01	1.58 ± 0.03	1.73 ± 0.02	15.46 ± 1.32	0.99 ± 0.00
	F/M 3	9.01 ± 0.04	9.07 ± 0.01	1.60 ± 0.02	2.03 ± 0.01	18.16 ± 1.20	0.99 ± 0.00
Hyper-mesophilic	F/M 0.5	1.91 ± 0.01	2.00 ± 0.01	0.75 ± 0.13	0.77 ± 0.09	3.00 ± 0.72	0.99 ± 0.00
	F/M 1	3.06 ± 0.01	3.14 ± 0.02	0.89 ± 0.10	0.92 ± 0.05	9.48 ± 1.93	0.99 ± 0.00
	F/M 2	5.69 ± 0.00	5.77 ± 0.01	1.40 ± 0.00	1.72 ± 0.02	13.58 ± 0.07	0.99 ± 0.00
	F/M 3	6.44 ± 0.02	6.66 ± 0.01	1.66 ± 0.02	2.04 ± 0.01	20.22 ± 0.08	0.99 ± 0.00

Although the temperature showed no significant effect on R_m in general, the hyper-mesophilic temperature shortened the lag phase of CH_4 production by 22.88%, 26.51%, and 12.1% when the reactors were supplied with F/M ratios of 0.5, 1, and 2, respectively. When the reactors were supplied with F/M ratio 3 (Table 2), the lag phase was 10.19% longer than at mesophilic temperature (Table 2).This finding suggests that hyper-mesophilic temperatures activate methanogenesis more quickly until a certain organic loading is reached. However, under higher organic loading, methanogens require more time to adapt to the process imbalance caused by higher production of residual [VFAs] (Table 3), resulting in a longer lag phase.

The temperatures also had no significant effect on the lag phase of CH_4 production, as indicated by $p > 0.05$ in the two-sample t-test. However, the increase in the F/M ratio prolonged the lag phase at either mesophilic or hyper-mesophilic temperatures. This result is in line with the study conducted by Koksoy and Sanin (2010) and Ma et al. (2019), who stated that a longer lag phase was observed to occur in the reactors with a high F/M ratio that caused an imbalance between acidification and methanogenesis as an effect of the lower dose of inoculum seed [17,26].

The lowest F/M ratio employed in this study (F/M ratio 0.5) produced the highest CH_4 yield in mesophilic and hyper-mesophilic conditions with a 0.23 ± 0.00 L-CH_4/g-COD input. When the reactors were operated at 35°C and 45°C, an increase in the F/M ratio reduced CH_4 yield by up to 21.74% and 39.13%, respectively (Table 3). This result was in accordance with a previous study, which reported that higher CH_4 yields were also obtained at lower F/M ratios. It was caused by the efficient conversion of VFAs to CH_4 as a higher inoculum dose provides more methanogen population [26].

Theoretical CH_4 yields at 35 °C and 45 °C calculated based on [27] were 0.39 and 0.41 L-CH_4/g-COD_{input}, respectively. At the end of the start-up period, mesophilic temperature achieved 58.97%, 46.15%, 43.59%, and 48.72% of theoretical CH_4 yield at F/M ratios of 0.5, 1, 2, and 3, respectively. Meanwhile, in hyper-mesophilic conditions, 56.09%, 43.90%, and 34.15% of theoretical CH_4 yield were achieved by F/M ratios of 0.5, 1, 2, and 3, respectively. The lower CH_4 yield obtained at 45°C with F/M ratio 3 (Table 3) was caused by an imbalance between acidification and methanogenesis as the effect of a higher temperature on substrate degradation rate [21,22]. Therefore, the hyper-mesophilic condition improves substrate degradation rate while maintaining the intermediate concentrations at non-inhibitory levels when the reactors were inoculated with F/M ratios of 0.5, 1, and 2.

3.2. Effect of F/M ratio and applied temperatures on pH recovery

Besides temperatures, pH also influences enzymatic activities, which eventually affects the digester performance [5]. The pH profiles over a period of time are shown in Figure 3. For comparison, the pH of control reactors at mesophilic and hyper-mesophilic temperatures throughout the observation time was 7.45 ± 0.04 and 7.45 ± 0.06, respectively. At mesophilic conditions, the increasing organic loading from F/M ratio 0.5 to F/M ratio 3 decreased the initial pH from 7.45 (control reactors) to 7.28, 7.27, 7.19, and 7.17 for FM ratios of 0.5, 1, 2, and 3, respectively. At hyper-mesophilic temperatures, the increasing organic loading also decreased the initial pH from 7.45 (control reactors) to 7.31, 7.26, 7.17, and 7.17 for FM ratios of 0.5, 1, 2, and 3, respectively. From those initial values, pH showed a decreasing trend during the first 30 h in all F/M ratios and gradually recovered until the end of the start-up period, reaching approximately 7.42–7.51 at mesophilic temperature and 7.12–7.59 at hyper-mesophilic temperature (Figure 3 (a) and (b)). It is worth noting that at a hyper-mesophilic temperature, only F/M ratio 3 showed poor pH recovery until the end of the start-up period, with the final pH reaching 7.12 ± 0.01 (Figure 3 (b)).

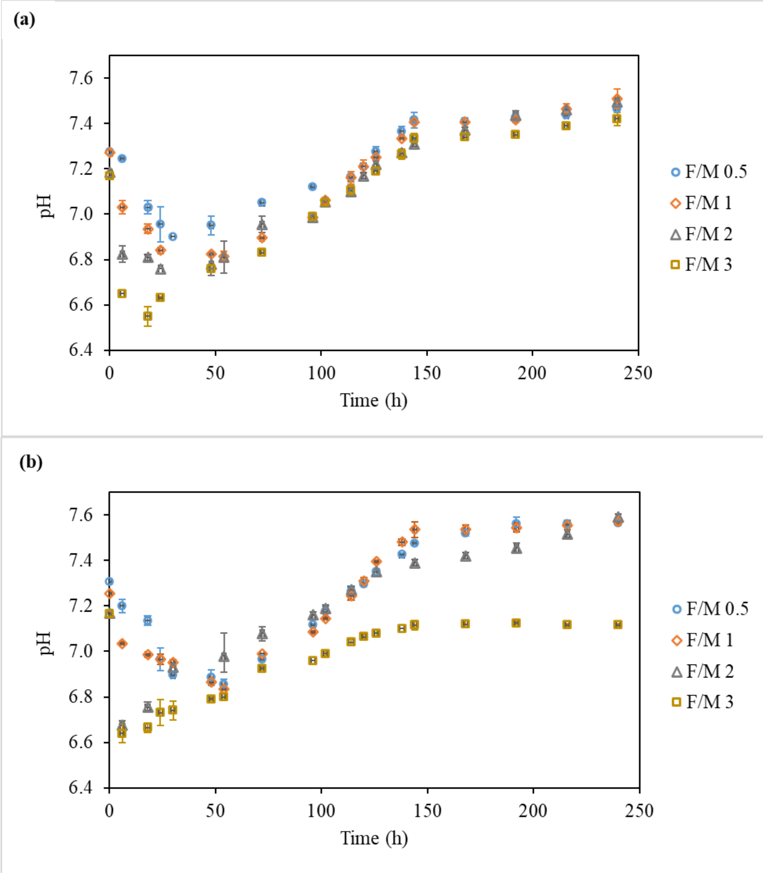

Figure 3. pH profiles of the anaerobic batch-test over 10 d of observation treated at: (a) 35°C, and (b) 45°C.

The decrease in pH during the initial operation was caused by the activity of acidogens and acetogens in producing VFAs. However, as the methanogens consumed the VFAs, followed by the production of alkalinity, the pH was gradually increased and stabilized [17]. This explains the profiles of pH recovery that were in accordance with the daily CH_4 production profiles (Figure 1(c) and (d)). Gerardi (2003) stated that an anaerobic digester that operates properly would have a pH in the range of 6.8–7.8 as the VFAs are converted to CH_4 and CO_2. Amongst methanogenic consortia, methanogens are the most sensitive microbial group to pH change, as pH lower than 6.8 inhibits their activities, ultimately reducing CH_4 production [5]. This was observed to occur at F/M ratio 3 treated at a hyper-mesophilic temperature that showed a lower CMP by 26.57% than reactors treated at 35°C with the same F/M ratio (Figure 1 (a) and Figure 2 (a); Table 2).

Table 3. Performance of batch reactors at the end of start-up period.

Treatment		**Initial TA (g-CaCO$_3$/L)**	**Final TA (g-CaCO$_3$/L)**	**Residual [Acetate] (g/L)**	**Residual [Propionate] (g/L)**	**Residual [TVFAs] (g/L)**	**CH_4 yield (L-CH_4/ g-COD input)**	**[Propionate] / [Acetate] ratio**
Meso-philic	F/M 0.5	4.93 ± 0.03	6.80 ± 0.05	0.05 ± 0.00	0.00 ± 0.00	0.05 ± 0.00	0.23 ± 0.00	0.00 ± 0.00
	F/M 1	5.00 ± 0.08	8.18 ± 0.15	0.04 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.18 ± 0.01	0.00 ± 0.00
	F/M 2	5.04 ± 0.02	10.27 ± 0.25	0.04 ± 0.00	0.00 ± 0.00	0.08 ± 0.00	0.17 ± 0.01	0.00 ± 0.00
	F/M 3	5.07 ± 0.05	12.93 ± 0.18	0.05 ± 0.00	0.00 ± 0.00	0.10 ± 0.00	0.19 ± 0.00	0.00 ± 0.00
Hyper-mesop hilic	F/M 0.5	5.05 ± 0.06	7.17 ± 0.01	0.04 ± 0.00	0.00 ± 0.00	0.07 ± 0.00	0.23 ± 0.00	0.00 ± 0.00
	F/M 1	5.07 ± 0.13	8.40 ± 0.02	0.04 ± 0.00	0.00 ± 0.00	0.08 ± 0.00	0.18 ± 0.01	0.00 ± 0.00
	F/M 2	5.03 ± 0.10	10.79 ± 0.06	0.06 ± 0.02	0.00 ± 0.00	0.11 ± 0.03	0.18 ± 0.02	0.00 ± 0.00
	F/M 3	5.16 ± 0.01	13.52 ± 0.12	0.46 ± 0.01	1.59 ± 0.01	3.13 ± 0.05	0.14 ± 0.00	3.46 ± 0.01

The increase in pH at the end of the start-up period can be associated with the increase in final TA. At the beginning of the experiment, the average TA of all treatments ranged from 4.93 to 5.16. Meanwhile, at the end of the start-up period, the final TA increased with the increase in the F/M ratio (Table 3). As VFAs generated by protein and lipid degradations are converted to CH_4 and CO_2, pH gradually reaches a stable operational range as CO_2 may be present in soluble forms. Therefore, the release of CO_2 contributes to the generation of carbonic acid, bicarbonate, and carbonate alkalinity. In addition, NH_3 generated by protein degradation also contributes to the increase in alkalinity [5].

3.3. Environmental factors that oriented the reactors' performance

The PCA was performed in order to investigate the environmental factors that shaped the performance of fish waste mono-digestion during the start-up period of batch operation. Next, the clustering of three treatment groups, as shown in Figure 4, was obtained from CA. Two experimental groups within the dash lines represent ≥ 50% similarity based on Euclidean distance. To validate the significant difference between cluster_1 and cluster_2, ANOSIM was also performed. The ANOSIM R-value of the test was 0.67 ($p \leq 0.05$), indicating that the two clusters varied significantly in response to input variables. Meanwhile, the ANOSIM test on cluster_3 could not perform as it only consists of one constituent.

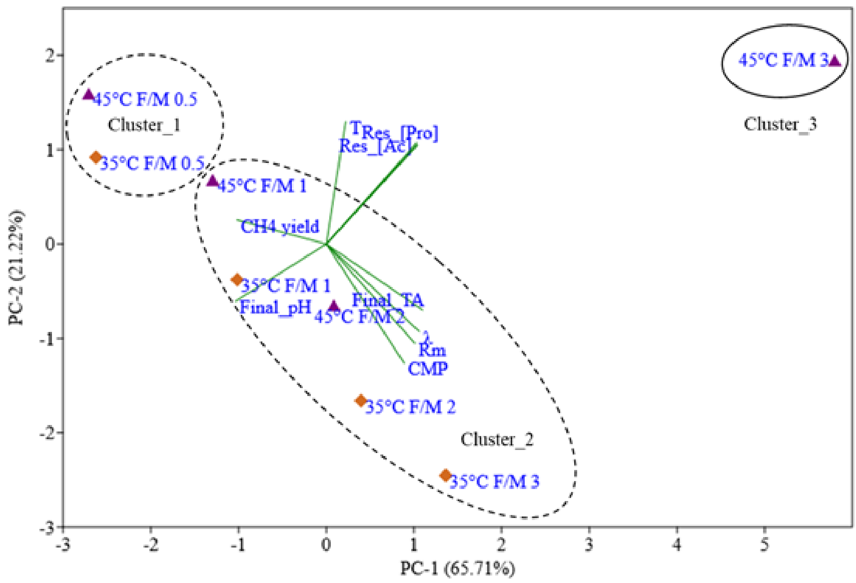

Figure 4. Principal component analysis of the performance of each batch test. ♦ treated at 35°C, ▲ treated at 45°C, Res represents residual, [Ac] and [Pro] are [acetate] and [propionate], respectively.

Cluster_1 consists of F/M ratio 0.5 treated at two different temperatures, and the grouping was mainly affected by CH_4 yield as these two treatments produced the highest CH_4 yield compared to other treatments (Table 3). On the other hand, the clustering of cluster_2 was affected by the increase of final TA, CMP, R_m, and prolonged lag phase. Meanwhile, the clustering of cluster_3 was affected by the highest residual [VFAs], in which its production is regulated by hyper-mesophilic temperature.

A study reported that the ratio between propionic and acetic acid is one of the important indicators of the stability of anaerobic digesters. In a co-AD system treating fish waste and cow manure that employed a gradual increase in organic loading, the propionic-to-acetic acid ratio increased from 0.1 during the stable condition to 0.4 when the reactors reached system failure [11]. Based on the reported study, it can be concluded that F/M ratio 3 treated at 45°C experienced system imbalance as the propionic-to-acetic acid ratio reached 3.46 ± 0.01 at the end of start-up period (Table 3). Based on thermodynamic point of view, methanogenesis of propionate (C_3) is more challenging compared to other VFAs caused by positive Gibbs free energy (+76 KJ per mole). Its anaerobic degradation also occurs when hydrogen partial pressure is less than 10^{-4} bar. These explain the cause of process imbalance when propionate is accumulated far above the acetate level [28]. The accumulation of propionates was also observed in the co-digestion of fish waste with manure and waste activated sludge as co-substrates [2].

Another considerable amount of residual VFAs that was accumulated in reactors with a F/M ratio of 3 treated at 45°C was i-valeric acid, which reached 1.06 ± 0.02 g/L at the end of start-up period. This result is in accordance with a previous study that showed co-accumulation of propionic and i-valeric acid in co-AD treating fish waste and cow manure. I-valeric acid also contributes to process imbalance because it is slowly degradable and eventually hinders CH_4 production [11].

4. Conclusions

A study of mono-digestion of fish waste operated in batch mode was conducted to investigate the effects of F/M ratios and two different mesophilic temperatures during the start-up period of batch-AD. The results showed that the increase in F/M ratio improved the ultimate CH_4 production at mesophilic (35°C) and hyper-mesophilic (45°C) temperatures but did not improve the CH_4 yield. Furthermore, 45°C improved cumulative CH_4 production by up to 2.61% and shortened the lag phase of methanogenesis by 22.88% when the reactors were supplied with F/M ratios of 0.5, 1, and 2. The results indicate that hyper-mesophilic temperature are recommended for mono-digestion of fish waste AD within those ranges of F/M ratios. Meanwhile, at a high F/M ratio, hyper-mesophilic caused a process imbalance by decreasing cumulative CH_4 production by up to 26.57% and prolonging the lag phase by 10.19%. This study is beneficial to managing fish waste for mono-digestion of AD.

Author contributions

Arma Yulisa: Conceptualization, Investigation, Formal analysis, Data curation, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Chayanee Chairattanawat:** Data curation, conceptualization, Writing – review & editing. **Sang Hyeok Park:** Conceptualization, Methodology, Data curation. **Md Abu Hanifa Jannat:** Data curation, conceptualization. **Seokhwan Hwang:** Funding acquisition, project administration, Resources, Supervision.

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Competing Interest

The authors have no conflict of interest to declare related to this study.

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