Study on Highly Efficient Methane Fermentation by Adoption of Ethanol Fermentation Pretreatment of Food Waste

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Chapter 1. Introduction

1.1 The current situation and utilization technology of food waste

Food waste (FW) was defined by the UN Food and Agriculture Organization (FAO) and includes any healthy or edible substance that is wasted, lost, degraded at every stage of the food supply chain [1]. Approximately 1.3 to 1.6 billion tons of FW are generated globally each year and the amount of FW is expected to increase in the next 25 years [2, 3]. FW is currently a serious issue in megacities worldwide. FW, which is the dominant fraction of organic fraction of municipal solid waste, is putrescible; when buried in a landfill, it decomposes to form methane, a greenhouse gas with a global warming potential 25 times greater than CO_2 on a 100 year time scale [4]. In Japan, ~19 million tons of FW was generated annually, including ~ 11.3 million tons from wholesale, retail, catering, and restaurant activities for food manufacturing and ~ 7.7 million tons from household preparation and cooking [5]. Presently, the most common FW treatment and utilization methods are incineration, landfilling, use as animal feed, methane fermentation, and composting. In particular, incineration or landfillin has been the main process for discarding and reducing the volume of FW from restaurants and households [6]. However, Japan is short of available land for landfilling, and a large amount of harmful substances (dioxins) and greenhouse gases (CO_2) are produced during incineration [5]. Therefore, more sustainable and environmentally friendly management strategies for food waste should be addressed and developed.

Compared with other waste types, FW is rich in organic matter, oil, salt, and nutrients [7, 8]. FW is a high organic waste which is rich in starch, fat, protein and cellulose, and has an average water content of 80% [9]. Compared to landfilling, composting and

incineration, AD is regarded as an economic and eco-friendly method has been widely applied in the disposal of FW. Due to its ability of converting organic substrates to methane and organic fertilizers from FW simultaneously, largely reducing the risk of FW to human health and the environment [5, 10]. This process in which organic substrates are degraded in the absence of oxygen is based on the metabolism and interspecies interactions of diverse microorganisms [11, 12]. AD has various advantages as a method for waste treatment and energy generation. AD of food waste is a highly ranked alternative method to recycle food waste when it is not practical to utilize food waste as feed or fertilizer, e.g., while separating inadequate matter is difficult or when the utilization site is located far from urban areas. In particular, biogas power generation that is attractive because of the comparatively higher prices of other renewable energy sources that are subjected to tariffs [13]. In Japan, acquisition price of solar power (between 10 and 250 kW) in 2020 was 12~13 yen (plus tax) per 1 kW, and methane fermentation power was 39 yen (plus tax) [14].

The driving force of development of anaerobic digestion of FW also derived from serious shortage of fossil fuels and the urgent demand for renewable and sustainable alternative fuels [15]. The mobilization and extensive use of organic FW as a renewable bioenergy production source have high potential to help secure a safe energy supply [16]. Therefore, methane fermentation with low greenhouse gas emission and higher profit which meets growing energy demand in promoting renewable alternatives [17].

1.2 Research progress and development trend on improving the efficiency of biogas fermentation of food waste

1.2.1 Existing pretreatment technology for FW

As mentioned in the previous section, methane fermentation technology has many economic and sustainability benefits. Consequently, food recycling methods have gained more attention and many recycling projects are based on AD. However, methane fermentation processes have certain disadvantages, methanogens grow more slowly than bacteria; this may readily lead to an imbalance between acidification and methanation [18]. In addition, long hydraulic retention times (HRTs), dependence on sluggish decomposition, and decreases in pH due to the accumulation of VFAs (Cesaro & Belgiorno et al., 2014; Ma et al., 2018).

AD comprises a series of reactions which are performed by different groups of microbial populations. AD mainly involves four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis [19]. During AD, VFAs are decomposed to acetate, CO_2 and H_2 which are subsequently utilized as substrates for methanogenesis [20]. Many FW pretreatment methods have been explored in order to make AD go smoothly. FW pretreatment methods can be divided into four categories, namely heat treatment; mechanical treatment, including microwave, ultrasonic, high-pressure and pulse discharge; biological treatment, including enzymatic hydrolysis and aerobic composting; and chemical treatment, including addition of acid, alkali and oxide [21, 22]. As well as combinations of these have been applied for improving methane production and increase processing load of AD.

In addition, adding a membrane into a fermenter effectively prevents the runoff of anaerobic microorganisms with low growth rates, providing the long sludge retention

time (SRT) needed to maintain substrate degradation while enabling operation with short HRT [23–25]. In theory, the AnMBR is more suitable to treat highstrength wastewater due to the prolonged SRT [26]. Therefore, more and more researchers are attempting to use AnMBRs for the treatment of organic solidwastes. Cheng et al. (2018) and Amha et al. (2019) reported achieving high degradation efficiency up to an organic loading rate (OLR) of 10 g-COD/L/d in mesophilic methane fermentation of FW using anaerobic membrane bioreactors (AnMBR). AnMBR effectively prevent the washout of these slow growing methanogens, enabling operation at longer solid retention times than HRT.

1.2.2 Pretreatment of food waste with ethanol to improve anaerobic biodegradability

Batch biomethanation experiments using an ethanol fermented artificial food waste substrate showed an increased methane ratio in biogas, indicating that carbon dioxide generation decreased without affecting methane generation [29]. Ethanol fermentation using organic waste is generally performed to produce liquid fuel. To obtain further energy, biomethanation is available for ethanol fermented residues following evaporating [30–32]. However, the methods for gaseous and liquid fuels necessitate collection and refinement, which are complex processes; thus, integrating biomethanation will be simpler than described previously. Therefore, ethanol fermentation was adopted to improve the biodegradability process for methanation but not for biofuel collection process. The secondary advantage of ethanol fermentation as pretreatment for biomethanation is that higher methane content is obtained in biogas than that obtained via conventional biomethanation. Under direct methanation, 1 mol of glucose would be stoichiometrically converted to 3 mol of methane and 3 mol of carbon dioxide, as depicted in **Eq. (1-1)**. Thus, approximately 50% of the biogas would be methane:

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad \textbf{(1-1)}$$

Furthermore, the ethanol fermentation of glucose in an open vessel, as shown in **Eq. (1-2)**, would release 2 mol of carbon dioxide. Moreover, 2 mol of ethanol produces 3 mol of methane and 1 mol of carbon dioxide through acetic acid, as shown in **Eq. (1-3)**, resulting in a biogas with a methane content of 75%:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \textbf{(1-2)}$$

$$2C_2H_5OH \rightarrow 3CH_4 + CO_2 \quad \textbf{(1-3)}$$

Kalyuzhnyi and Davlyatshina demonstrated that the biogas obtained through anaerobic decomposition of ethanol depicted a methane content of 79% and that the carbon dioxide generation was decreased to one-third [33].

Food waste from retail outlets and restaurants is rich in starch [31, 32, 34], indicating that pretreatment would be effective for methane fermentation using such waste. Other reports have indicated that improved methane yields could be obtained using ethanol fermentation pretreatment [35, 36]. Ethanol fermentation pretreatment (EP) in biological pretreatment is an effective treatment with bacteriostatic action on FW, increasing the methane fermentation system's buffering capacity while maintaining hydrolytic acidification [36–38].

1.3 Objective and structure of the dissertation

Methane fermentation is a technology that can be expected to reduce the volume of waste and produce energy. Methane fermentation using food waste has been introduced in large-scale factories, but it is expected that it will be introduced in shopping centers and small-scale factories in the future due to the recent attention to biomass resources. Therefore, research and development for miniaturize the methane fermentation facility will become more important in the future. In this study, to investigated the effectiveness of ethanol pretreatment fermentation for improving the biodegradation rate in methane fermentation of FW. The feature of this study is that ethanol is not recovered and is fermented with methane as it is. Since food waste contains a relatively large amount of carbohydrates, it can be expected that ethanolization will reduce the molecular weight of the carbohydrates, improve the decomposition rate and methane concentration, and reduce the amount of sludge generated.

This chapter as the first chapter, in chapter 2, a sequential batch experiment was demonstrated by supplying ethanol fermented artificial food waste. The feasibility of the operation in a stable state was discussed, and the biomethanation characteristics were compared with those of a control group whose substrate was not ethanol fermented. However, it should be considered that solid–liquid separation has to be performed to keep the biomass in the reactor.

In chapter 3, examined whether anaerobic membrane bioreactor (AnMBR) can contribute to prevent the runoff of anaerobic microorganisms, and investigate whether stable continuous operation and pretreatment effects can be obtained of this method, and by testing different load with the aim to investigate the degradation characteristics of an ethanol fermented substrate on an AnMBR system. The results are expected to contribute

to the comprehensive understanding of the reaction rate of FW and the reduction rate of generated sludge, thus providing a reference for further studies and engineering applications.

In Chapter 4, based on the knowledge obtained in Chapters 2 and 3, aimed to systematically compare control series (substrate: FW) and EP series (substrate: ethanol fermented FW) performance of both substrates using AnMBRs. Moreover, the substrate with a moisture content of 80 ~ 85% is used, which is close to the moisture content of actual food waste. In the present study, a laboratory-scale mesophilic semi-continuous AD reactor, bacterial communities present in the stable AD reactor were analysed by Illumina MiSeq high-throughput sequencing. Both series were operated step-by-step increasing the load to compare performance and operating limits. This study examined whether EP of FW can allow for more effective high load operation in an AnMBR, examining whether pretreatment effects can be obtained in a stable state and determining the effect of EP on the maximal organic loading rate (OLR), providing a reference for further potential engineering applications.

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Chapter 2. Production of methane-rich biogas and minimization of sludge by adopting ethanol fermentation for the pretreatment of biomethanation

2.1 Introduction

In this chapter, researchers have focused on ethanol fermentation as a pretreatment for biomethanation of FW. Although we can know from the other reports in the chapter 1 have indicated that improved methane yields could be obtained using ethanol fermentation pretreatment [1, 2] ; however, previous research were only reported in short-term studies such as batch experiments. Thus, it is necessary to understand the characteristics of the process under continuous operation in a stable state to discuss the feasibility of its use in real-time facilities, which are usually operated through continuous feeding.

In this chapter, a sequential batch experiment was demonstrated by supplying ethanol fermented artificial food waste. The feasibility of the operation in a stable state was discussed, and the biomethanation characteristics were compared with those of a control group whose substrate was not ethanol fermented.

2.2. Materials and Methods

2.2.1 Substrate (Artificial food waste)

An artificial food waste (AFW) was created comprising boiled rice (300 g), cabbage (90 g), carrot (90 g), chicken (60 g), and small dried sardines (48 g), measured on a wet basis. The chicken and dried sardines were weighed after boiling. The material weight ratios in the AFW were estimated based on a survey that was conducted in a university cafeteria [3]; the materials were combined; and their mixture was further homogenized into a paste using a grinder (Grindomix GM200, Retsch, Haan, Germany) without sterilization process. The total solids (TS) of the AFW were adjusted to 100 g/L by adding distilled water purged by nitrogen gas. The average composition of the AFW was 98 g/L of volatile solids (VS), 46 g/L of total organic carbon (TOC), and 83 g/L of total sugar.

2.2.2 Pretreatment (ethanol fermentation)

The AFW adjusted to 100 g TS/L was saccharized by adding 7.5 mL glucoamylase as an enzyme (Novozymes, Spirizyme Fuel) per 1 L of food waste at 50 °C for 2 h with constant stirring. The saccharized AFW's glucose concentration was 65 g/L. Subsequently, the saccharized AFW was fermented by adding 10 g of commercial yeast (Alcotec, Alcotec 48 Turbo Yeast) per 1 L of saccharized AFW at 26 °C for 65 h while stirring. The fermentation procedure yielded an ethanol concentration of 33 g/L and TOC of 43 g/L for the fermented AFW. Substrate of control was not conducted the pretreatment procedure.

2.2.3 Biomethanation (sequential batch experiment)

An anaerobic digester (AF10-2, Miyamoto Corp., Osaka, Japan), as depicted in **Fig. 2-**

1, was used to perform the sequential batch experiment. Two 10 L vinyl chloride cylindrical reactors were set at 37 °C, and the contents were stirred using a paddle at 90 rpm. One reactor was supplied with ethanol fermented in pretreated series AFW, whereas the other was supplied with AFW without any pretreatment in the control series. Next, 250 mL of the substrate was fed once each day, except on Sundays, and a solution of trace minerals dissolved in water was added—100 mg of Fe as $FeCl_3 \cdot 6H_2O$, 10 mg of Co as $CoCl_2$, and 10 mg of Ni as $NiCl_2$ (per 1 L of AFW)— simultaneously. The digested liquid was drawn from the reactor just before feeding. To maintain the reactor's biomass concentration, half of the drawn liquid was spun in a centrifuge (H-201F, Kokusan, Tokyo, Japan) at 10,000 rpm for 5 min after which the solid components were returned to the reactor. Seed sludge from livestock was acclimated to the experiment by adding AFW over a period of 6 months. pH, biomass concentration, and biogas generation volume were checked to determine whether a stable state had been reached. Experimental conditions were hydraulic retention time for 47 days with a VS volumetric load of 2.5 g /L/day in the control experiment and 2.7 g L/day in the pretreatment experiment along with the presence of enzyme (0.306 gVS/mL) and yeast (0.580 gVS/g-yeast).

2.2.4 Analysis method

Biomethanated sludge was sampled just before feeding the substrates and analyzed the samples immediately afterward. TS, VS, suspended solids (SS), and volatile suspended solids (VSS) were analyzed following standard methods [4]. Total sugar was analyzed using the phenol sulfuric acid method [5], whereas glucose, ethanol, and volatile fatty acids (VFAs) were measured after filtration using a PTFE filter (DISMIC-25HP, ADVANTEC, Tokyo, Japan). Glucose concentration was analyzed using the glucose

oxidase method (Glucose kit, Glucose CIItest Wako), and total carbon in the AFW was analyzed by combustion catalytic oxidation and non-dispersive infrared (NDIR) methods (SSM-5000A, Shimadzu). The total organic carbon (TOC) and inorganic carbon (IC) from the digestion liquid was also analyzed using the combustion catalytic oxidation and NDIR methods (TOC-V, Shimadzu, Kyoto, Japan). Ethanol was quantified using a flame ionization detector gas chromatography (GC14B, Shimadzu) using a Gasukuropack-55 column (GL Sciences, Tokyo, Japan) with helium as the carrier gas, and VFAs were quantified using ion chromatography (Organic Acid Analysis System, Shimadzu) with a Shimpack column SCR-102H (Shimadzu) and a mobile phase of 5 mM p-toluenesulfonic acid, 20 mM Bis–Tris buffer, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). Biogas from the methane fermentation tank was collected by gas bag and quantified by performing thermal conductivity detector (TCD) gas chromatography (GC14B, Shimadzu) using a ShinCarbon ST 50/80 column (Shinwa Chemical Industries, Kyoto, Japan) with argon as the carrier gas. The biogas volume was measured at 23–26 °C, which was the room temperature in the laboratory.

2.3 Results and discussion

2.3.1 Feasibility of operating in a stable state

Feasibility of operating in a stable state **Fig. 2-2a** shows the VSS concentration variations in the biomethanation reactor. The pretreatment and control series depicted the VSS concentrations of 17.4 g/L at the beginning of the experiment. In the control series, the VSS concentrations were observed to stabilize at approximately 16 g/L after the 14th day, and the pH, VFA, and other characteristics were also stabilized. The control series could operate for 70 days, from the 14th to the 84th day, at a stable state. Thus, the operation was terminated on the 84th day, because it was deemed that sufficient data had been obtained. In contrast, the pretreatment series depicted a decrease in its VSS concentration to 12.6 g/L by the 42nd day. It was feared that decreasing biomass in the reactor would lead to the failure of biomethanation. Therefore, to maintain the biomass concentration equal to that of the control, all effluents that were observed after the 42nd day were centrifuged, and the sediments were returned to the reactor except for sampling for analyzing. Consequently, operation term was longer than that required for the control, the VSS concentration recovered to become 13 g/L after the 77th day and further went on to stabilize. The pH and VFA values also stabilized; thus, the pretreatment series could operate for 56 days, from the 77th to the 133rd day, in a stable state. **Table 2-1** shows the average result of substrate, reactor, biogas and effluent during the stable state. In the pretreatment series, lactic acid and acetic acid existed in addition to ethanol in the ethanol fermented AFW; therefore, the pH was low at 4.2. In contrast, although VFA was observed to mainly comprise acetic acid in the biomethanation reactor, it depicted a low concentration of approximately 0.2 g/L. In addition, the pH in the reactor was 7.9, and ethanol could not be detected. As stated previously, methane fermentation supplying the

ethanol fermented substrate would not cause VFA inhibition by maintaining operation of the sludge in the reactor, thereby allowing the sequential batch operation to be maintained in a stable state.

2.3.2 Characteristics of the biomethanation of pretreated substrate

Fig. 2-2b shows the variations in the methane content of the biogas. The methane contents stabilized in both series; it reached a value that was between 50 and 55% in the control series, which was within the range of results that were observed in studies related to food waste (50–65%) [6–11]. In the pretreatment series, the methane contents were between 65 and 70% after the third day, which was higher than that in the control series. The average biogas methane values were 53.1% for the control series and 67.5% for the pretreatment series (**Table 2-1**), approximately 15% greater than in the control series. The biogas yield in the pretreatment series was 680 mL/gVSadded, which was smaller than that in the control series (800 mL/gVSadded). In contrast, the methane yields were 420 mL/gVSadded for the control series and 460 mL/gVSadded for the pretreatment series, which were almost identical. The methane yields that were observed in previous studies ranged between 400 and 500 mL/g VS [6, 8, 10, 12] for food waste, which indicated that this study fell within the same range illustrated by those studies.

Fig. 2c shows the variations in the accumulated drawn sludge. The drawn volume in the control series stabilized; therefore, it was increased linearly. In the pretreatment series, all the sludge drawn after the 47th day was centrifuged. Thus, the sludge generation in the pretreatment series was lower than that observed in the control series. The sludge generation that was calculated using the results in a stable state was 1.7 gVSS/day for the control series and 0.3 gVSS/day for the pretreatment series. Thus, sludge generation in

the pretreatment series was observed to be one-fifth of that in the control series. As their values were expressed as the sludge yield per added substrate, the control series value was 0.082 gVSS/gVSadded, and the pretreatment series value was 0.014 gVSS/gVSadded. Qiang indicated that the biomass yield of food waste was between 1.5 and 15% on the COD base [8]. Although it was a different indicator, the yield obtained from the control series in this study was similar to that obtained in Qiang's study.

The VS decomposing ratios calculated using the substrates (excluding enzyme and yeast) and drawn residue (**Table 2-1**) are 94% for the pretreatment series and 87% for the control series. Cho reported a VS base decomposing ratio of 90% for food waste that contained 73% dry weight of boiled rice [13], which was similar to that observed in this study.

As stated previously, biomethanation installed ethanol fermentation pretreatment caused a 15% improvement in methane content of the produced biogas without any loss in the volume of generated methane and with a decrease in sludge generation to one-fifth of the VSS base.

2.3.3 Improving biogas methane content by pretreatment

Fig. 2-3 shows the material balances of carbon in the stable state conditions. Values from biomethanation were calculated from the concentration and volume of all effluents and biogas during the stable state, whereas carbon dioxide from biomethanation was analyzed for biogas and drawn effluent. Carbon dioxide generated from ethanol fermentation was calculated by produced ethanol concentration using **Eq. (1-2)**. In the control series, the generated weights of methane and carbon dioxide from the substrate were 5.2 and 5.0 gC, respectively. Therefore, the methane to carbon dioxide ratio was

1:0.96. The total sugar content of the AFW was 72% on carbon base. Therefore, it was considered that similar amounts of methane and carbon dioxide were generated following **Eq. (1-1)**. In the pretreatment series, the weight of the monosaccharide generated by saccharification was 6.8 gC, and almost all of the monosaccharide was consumed during ethanol fermentation. The generated weight of ethanol in this series was 4.5 gC, which nearly agreed with the theoretical value that was calculated using **Eq. (1-2)**. In addition, the calculated carbon dioxide was 2.3 gC. In case of biomethanation after ethanol fermentation, the generated weights of methane and carbon dioxide were 6.0 and 3.6 gC, respectively. The higher methane generation in the pretreatment series (6.0 gC) than in the control series (5.0 gC) was because of the added carbon from the enzyme, yeast, and an improved decomposing ratio, as discussed later. The generated weights of methane and carbon dioxide were 6.0 and 5.9 gC, respectively, for the whole reaction, which indicated a ratio that was identical to that of the control series (1:0.98). These results indicate that the final products correspond with that of the control series although they use different pathways and that the methane content of the biogas is improved by decreasing the volume of carbon dioxide without any loss in the volume of methane during biomethanation.

2.3.4 Decreasing sludge generation and improving degradation ratio through pretreatment

As stated previously, sludge generation in the pretreatment series decreased on a VSS base. With the carbon material balance shown in **Fig. 2-3**, the particulate organic carbon (POC) of residue in pretreatment was 0.5 gC/day, which was smaller than that of the control (1.0 gC/day). Both indicator VSS and POC showed smaller sludge generation

from biomethanation in the pretreatment. There are two reasons for decreasing sludge generation. First, the biomass yield from the ethanol substrate was less than that obtained from starch. Heijnen et al. indicated that the molecular base biomass yield of ethanol was 0.028 C-mol/(C)-mol of substrate, which was one-sixth of that of glucose (0.176 C-mol/(C)-mol substrate). They stated that this was caused, because the substrate carbon number near a biomass cell requires less energy for chemotrophic growth [14]. Second, the substrate energy available for cell growth is decreased by ethanol fermentation. Variations in free energy resulting from the presence of methane and carbon dioxide as final glucose metabolites are calculated in **Eqs. (2-1)** and **(2-2)** using Gibbs free energy of formation [15]:

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad \textbf{(2-1)}$$

$$\Delta Gf^0 = 3 \times (-50.75) + 3 \times (-386.02) - (-917.2) \quad \textbf{(2-2)}$$
$$= -393.11 \text{ kJ mol}^{-1}.$$

In contrast, in case of the generation of ethanol from glucose, the free energy changes are calculated using **Eqs. (2-3)** and **(2-4)**:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \textbf{(2-3)}$$

$$\Delta Gf^0 = 2 \times (-181.75) + 2 \times (-386.02) - (-917.2)$$
$$= -218.34 \text{ kJ mol}^{-1}. \quad \textbf{(2-4)}$$

When biomethanation occurs after ethanol fermentation, it is calculated using **Eqs. (2-**

5) and **(2-6)**:

$$2C_2H_5OH \rightarrow 3CH_4 + CO_2 \qquad \textbf{(2-5)}$$

$$\Delta Gf^0 = 3 \times (-50.75) + 1 \times (-386.02) - 2 \times (-181.75)$$
$$= -174.77 \text{ kJ mol}^{-1}. \qquad \textbf{(2-6)}$$

Using **Eqs. (2-4)** and **(2-6)**, the variation of free energy that was obtained through ethanol fermentation is observed to case of ethanol as a substrate, it is − 174.77 kJ mol^{-1}, which is half of that of a glucose substrate. Thus, the free energy that the bacterial cells can obtain through biomethanation by conducting ethanol fermentation as a pretreatment mechanism decreases; therefore, growth becomes difficult.

In addition, from **Fig. 2-3**, the decomposing ratio on the carbon base in the pretreatment series was calculated to be 93%, which was higher than 85% that was observed in the control series. Therefore, it was considered that the lower biomass yields also contribute to the improved degradation ratio of the substrate.

2.4 Summary

Ethanol fermentation as a pretreatment process for biomethanation of food waste was conducted in this study. A sequential batch biomethanation experiment was performed for 130 days using AFW that was saccharized and ethanol fermented. A stable state was feasible at least for 56 days of the experiment. Furthermore, the results described an improvement in the methane content of the biogas, a reduction in sludge generation. The results of the sequential batch experiment followed stoichiometry, and thermodynamics indicated that a biomethanation system that includes pretreatment is a theoretically controllable system. However, it should be considered that solid–liquid separation has to be performed to keep the biomass in the reactor.

Table 2-1 Average substrate, reactor, biogas, and effluent in a stable state.

Description	Control	Pretreatment
Substrate		
Substrate	Artificial food waste	Ethanol fermented artificial food waste
pH	6.5	4.2
Total solid (g/L)	100	–
Volatile solid (g/L)	98	–
Total organic carbon (g/L)	46	43
Total Kjeldahl nitrogen (g/L)	3.2	3.4
Ethanol (g/L)	0	33
Lactic acid (g/L)	0	10
Acetic acid (g/L)	0	2
In the reactors		
pH	7.5 (0.02)	7.9 (0.01)
Total solid (g/L)	25.0 (0.85)	25.5 (1.2)
Volatile solid (g/L)	21.3 (0.93)	18.0 (0.71)
VS/TS	0.86 (0.01)	0.71 (0.03)
Total suspended solid (g/L)	18.1 (0.37)	16.4 (0.61)
Volatile suspended solid (g/L)	16.0 (0.36)	13.1 (0.34)
VSS/TSS	0.88 (0.004)	0.80 (0.02)
Total organic carbon (g/L)	12.3 (0.31)	10.7 (0.33)
Dissolved organic carbon (g/L)	1.8 (0.15)	1.6 (0.23)
Dissolved total nitrogen (g/L)	2.1 (0.14)	3.7 (0.28)
Propionic acid (mg/L)	ND (0)	33 (65.5)
Acetic acid (mg/L)	12 (16.4)	220 (28.9)
Ethanol	ND (0)	ND (0)
Biogas		
Methane ratio (%)	53.1 (0.59)	67.5 (1.2)
Biogas yield (mL/g VSadded)[a]	800	680
Methane yield (mL/g VSadded)[a]	420	460
Effluent		
Drawn residue (gVS/day)[a]	3.3	1.5
(gVSS/day)[a]	1.7	0.3
Sludge yield (gVSS/gVSadded)[a]	0.082	0.014

The values in the brackets are standard deviations

[a] The values calculated from mass balance during entire stable state period

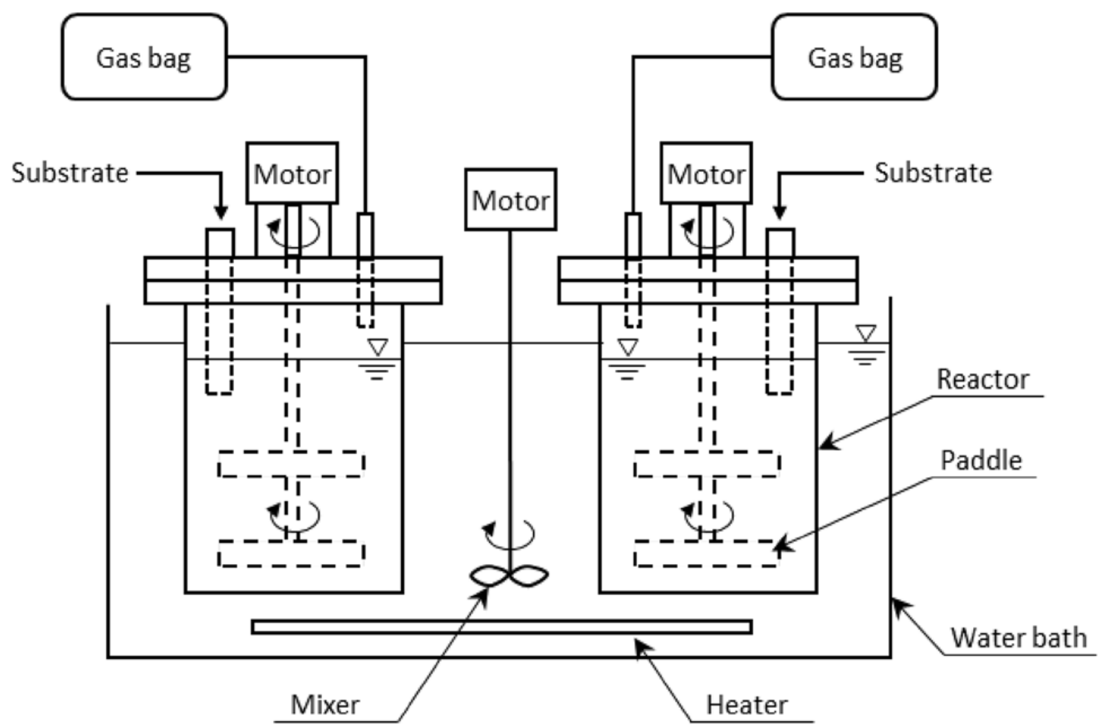

Fig. 2-1 Experimental apparatus.

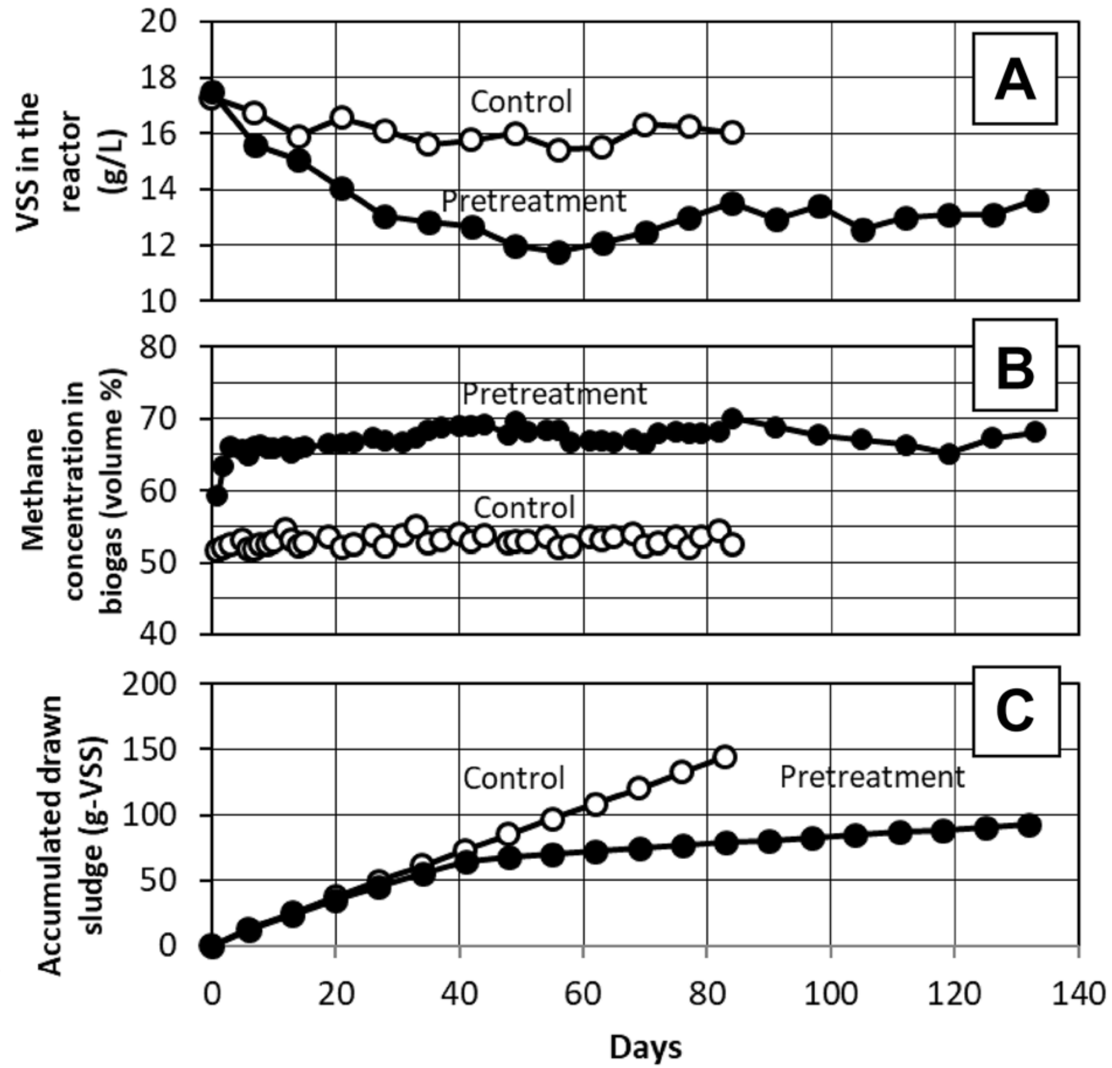

Fig. 2-2 Variation of biomethanation during the sequential batch experiment.

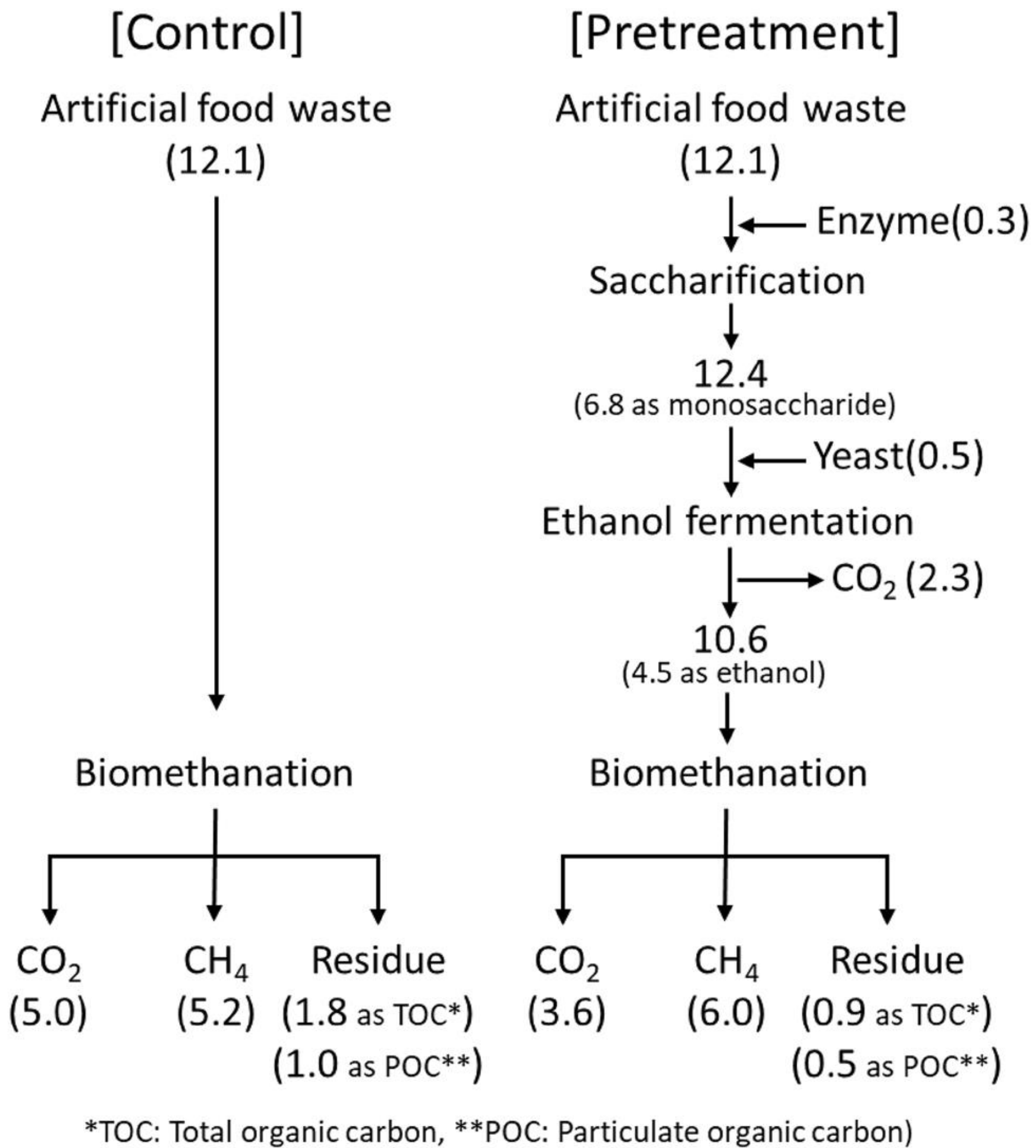

Fig. 2-3 Material balance of carbon in steady-state conditions (unit: g carbon per day).

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Chapter 3. Research on the possibility by adoption of ethanol fermentation pretreatment for methane fermentation of food waste using an anaerobic membrane bioreactor

3.1. Introduction

In chapter 2, confirmed reduced sludge generation, improved biogas methane concentration, and long-term operation possibilities in a sequential batch EP experiment using starch-rich food waste [1]. Because EP improves reaction rates and reduces sludge generation as compared with conventional processes.

However, anaerobic bacteria grow very slowly. A loss of bacteria also occurs when the processing load is increased by raising the feed volume, thus limiting the treatment of higher loads. Treatments utilizing the traditional methodology can only operate on a long HRT. It is problematic to extend the SRT and simultaneously shorten the HRT. Previous studies [2, 3] have used the supernatant of centrifuged sludge as treated water and have returned the sediment to the tank after centrifuging, thus separating and controlling the HRT and SRT. However, the use of centrifuges is not economical in practical terms. AnMBRs have recently evinced promise as viable alternatives to conventional anaerobic digesters for the treatment of organic waste. The membrane separation in AnMBRs decouples the SRT and HRT, enabling operations at longer SRTs [4–7]. Problems involving the loss of bacteria can hence be effectively resolved. The membrane can treat food waste in the anaerobic reactor and can also be used for the treatment of organic sludge. Cheng et al. (2018) were able to operate the AnMBR system at a higher load by adding a membrane unit reactor after the continuous stirred tank reactor. However, their design required two reactors, bioreactor which needs mixing and membrane separation

tank which needs membrane washing by biogas circulation. Further, Amha et al. (2019) employed a flat-sheet membrane and were able to actualize the former project using a single reactor. Despite improvements, this process required the circulation of biogas to flush the membrane and must be mandated the use of an impeller to mix the reactor. The flushing of the hollow fiber membrane only required one biogas spout underneath, while the flushing of the flat-sheet membranes needed multiple spouts to be positioned beneath the membrane. Thus, hollow fiber membranes were also found to be suitable for small reactors. In this chapter, the authors of this paper were able to actualize the abovementioned project using a single reactor. The proposed method allows the circulation of biogas to simultaneously flush the hollow fiber membrane and enable the mixing of the reactor; hence, this design makes system simplify and reduces operational difficulties.

In addition, very few studies have investigated AnMBR treatment of only FW, and there is no studies have investigated AnMBR treatment of ethanol fermented FW. Therefore, in this chapter, examined whether AnMBR can contribute to prevent the runoff of anaerobic microorganisms, and investigate whether stable continuous operation and pretreatment effects can be obtained of this method, and by testing different load with the aim to investigate the degradation characteristics of an ethanol fermented substrate on an AnMBR system. The results are expected to contribute to the comprehensive understanding of the reaction rate of FW and the reduction rate of generated sludge, thus providing a reference for further studies and engineering applications.

3.2. Materials and Methods

3.2.1 Substrate (Artificial food waste)

An artificial FW (AFW) was created consisting of boiled rice (300 g), cabbage (90 g), carrot (90 g), chicken (20 g), and small dried sardines (48 g). The AFW components were measured on a wet basis and the chicken and dried sardines were weighed after boiling. Material weight ratios in the AFW were estimated based on a survey conducted in the Osaka Institute of Technology's cafeteria [8]. The mixtures were then further homogenized into a paste using a Grindmix GM 200 grinder (Grindmix, Retsch, Haan, Germany) without a sterilization process. The total solids (TS) content of the AFW were adjusted to 100 g/L by adding distilled water purged with nitrogen gas. The average AFW composition was 98 g / L of volatile solids (VS) and 46 g/L of total organic carbon (TOC). Additionally, a lack of trace minerals in substrates has been reported to severely limit the growth and metabolism of hydrogenotrophic and acetoclastic methanogens [9, 10]. Therefore, a solution of trace minerals dissolved in water was added—100 mg of Fe as $FeCl_3 \cdot 6H_2O$, 10 mg of Co as $CoCl_2$, and 10 mg of Ni as $NiCl_2$ (per 1 L of AFW) — simultaneously [1].

3.2.2 Pretreatment (ethanol fermentation pretreatment)

The TS-adjusted AFW was then saccharized by reacting 7.5 mL of glucoamylase (Spirizyme Fuel, Novozymes, Denmark) per 1 L of AFW for 2 hours at 50 °C with constant stirring. The glucose concentration was 80 g/L. Subsequently, the saccharized AFW was fermented by adding 10 g of commercial yeast (48 Turbo Yeast, Alcotec, UK) per 1 L of saccharized AFW for 65 hours at 27 °C with stirring. The substrate characteristics before and after pretreatment are shown in **Table 3-1**. The fermentation

procedure yielded an ethanol concentration of approximately 40 g/L, and the control substrate was not pretreated. In addition, trace minerals were added to the substrate after the pretreatment.

3.2.3 Methane fermentation using AnMBR

The AnMBR system used in this study is shown in **Fig. 3-1**. Because this system was divided into a control series and an EP series, two identical systems were prepared as shown in **Fig. 3-1**. A hollow fiber membrane (LSPMW-02, Sumitomo Electric Industries, Japan) used in both systems has an average pore diameter of 0.2 μm and an effective filtration area of 0.1 m^2 of polytetrafluoroethylene (PTFE). The membrane module was immersed in the reaction vessel with an effective volume of 4.5 L and maintained at 37 °C using a water jacket, and the substrate tank was maintained at 4 °C using a water jacket. The AFW without pretreatment and the pretreated AFW were stored in the substrate tank for the control and EP series, respectively. The substrate was supplied from the substrate tank to the reaction tank using roller pumps, the roller pumps were operated under cycle of 1 minutes on, 239 minutes off, and treated water was permeated from the membrane module. The membrane was washed by circulating biogas from the head space to the bottom of the reactor using a diaphragm gas pump.

The fermentation reactor was purged with O_2-free N_2 for 15 min before seed sludge was added. Seed sludge was collected from the mesophilic anaerobic digester at the sewerage treatment plant and FW digester, and was acclimated with AFW (without pretreatment) in both series with a 20 d HRT for 3 months before use.

In the AnMBR system, HRT reduced stepwise from 20 to 5 days to increase the load by raising feeding volume. The corresponding OLR was 6.6 to 26.5 g-COD/L/d.

3.2.4. Analysis method

TS, VS, suspended solids (SS), volatile suspended solids, and alkalinity were analyzed by standard methods [11]. Glucose, ethanol, and volatile fatty acids (VFA) were measured after filtration with a PTFE filter (DISMIC-25HP, ADVANTEC, Japan). Glucose concentration was analyzed using the glucose oxidase method (Glucose kit, Glucose CIItest, Wako, Japan), and total carbon (TC) in the AFW was analyzed using combustion catalytic oxidation and a non-dispersive infrared (NDIR) method (SSM-5000A, Shimadzu, Japan). Dissolved organic carbon (DOC) and inorganic carbon from the permeate were also analyzed using combustion catalytic oxidation and a NDIR method (TOC-V, Shimadzu, Japan). Ethanol and VFA were quantified using a flame ionization detector gas chromatography (GC14B, Shimadzu, Japan) using a Gasukuropack-54 60/80 column (GL Sciences, Tokyo, Japan) with helium as the carrier gas (44 mL/min). 1 μm of sample was injected using airtight syringes and with injector, column, and FID temperatures of 250 °C, 200 °C, and 250 °C, respectively. Biogas from the AnMBR was collected with a gas bag and quantified by performing thermal conductivity detector gas chromatography (GC14B, Shimadzu, Japan) using a ShinCarbon ST 50/80 column (Shinwa Chemical Industries, Kyoto, Japan) with argon as the carrier gas (50 mL/min). 0.5 mL of biogas was injected into gas chromatograph and with injector, column, and TCD temperatures of 200 °C, 40°C (12 min. hold)–200°C, and 200 °C, respectively. Analysis of chemical oxygen demand (COD) used a spectrophotometer (DR900, HACH, USA) and CODcr reagent (HACH 4236, HACH, USA).

3.2.5. COD balances in the AnMBR

COD mass balance was investigated to understand material balance behavior during

methane fermentation. Influent AFW was a fractionate component of COD and outflow COD was assumed to be distributed as four components: (1) soluble COD derived from the drawn sludge, (2) permeate COD, (3) solid content COD, and (4) COD transformed into collected methane gas. Therefore, solid content COD was defined as the value obtained by subtracting soluble COD from total sludge COD. Additionally, permeate was defined as soluble COD. COD resulting from biomass growth was calculated from drawn sludge and COD of methane gas was estimated using conversion of methane to carbon dioxide by oxidation. The COD equivalent of measured methane gas volumes was determined as 1mol CH_4 = 64 g COD [12].

3.3. Results and Discussion

3.3.1 Higher loading operation by pretreatment

The daily change of main measurement items is shown in **Fig. 3-2**. **Table 3-2** shows the operating index average under each operating condition. In the AnMBR system, HRT reduced stepwise from 20 to 5 days to increase the load by raising feeding volume. The corresponding OLR was 6.6 to 26.5 g-COD/L/d. The experimental equipment divided the control and EP series, and HRT values that can be operated were 20 and 15 days for the control series and 20, 15, 12.5, 10, 7.5, and 5 days for the EP series. The corresponding OLR was 6.6, 8.8 g-COD/L/d, and 6.6, 8.8, 10.5, 13.2, 17.7, 26.5 g-COD/L/d.

Table 3-2 indicates that the control series had methane yields corresponding to these HRT values of 380 and 370 mL-CH_4/g-VSadded, respectively. The EP series had corresponding values of 440, 420, 410, 420, 410, and 390 mL-CH_4/g-VSadded, respectively. However, as shown in Fig. 3-2, when HRT was further reduced to 12.5 days in the control series, methane gas concentration decreased to 41.7%, the amount of generated biogas sharply decreased to 0.43 L/L/d, and pH decreased to 5.1. This indicate that control series failed by irreparable inhibition. The failure of control series was not only suddenly pH drop but caused by unstable in the reactor during the 12.5-day control series HRT. Since the alkalinity has decreased from the 60th day, it has earlier than the pH drop. The system state was seemed to be unstable by weaken the buffering capacity. On the other hand, the alkalinity of the EP series was maintained at a high concentration. Although the accumulation of VFA at 5-day EP series HRT, it was considered that the buffering capacity maintained the stable of the operation.

In the control series, inhibition occurred between 8.8 and 10.5 g-COD/L/d. Cheng et al. (2018) noted that the OLR was inhibited from 9.72 to 14.58 g-COD/L/d, and the

operational load in this study's control series was similar to that in Cheng et al. Therefore, the OLR capability in this study was like that of previous research. In the EP series, even if the HRT was further raised to 5 days, OLR = 26.5-COD/L/d, methane gas concentration was the same as a lower load, and methane yield was only slightly lower. Additionally, pH stabilized at around 7.5, enabling operation at three times the load than the control series. This clarified that methane production in an AnMBR system using ethanol fermentation for pretreatment was superior to other AnMBR system operation methods.

3.3.2 Upgrading methane production by pretreatment

Fig. 3-2 shows that the biogas production rate under each operating condition tended to increase in proportion to HRT shortening. HRT values that could be operated simultaneously on both series were 20 days and 15 days. The control series had a methane gas concentration of 53–54% and that of the EP series was 69–71%. Previous studies explained stoichiometry as the reasons for increased methane concentration generating from ethanol fermentation due to lower carbon dioxide generation from subsequent methane fermentation [13, 14]. As shown in **Table 3-2**, comparing the methane gas yield per 1 g of added VS in the measured values of 20 day and 15 day HRT, the control series yields were 380 and 370 mL and the EP series yields were 440 and 420 mL, respectively. Thus, the EP series enables a 15–18% higher methane concentration while keeping the same methane yield.

3.3.3 Analysis of COD mass balance

COD mass balance was determined to understand material reaction behavior during methane fermentation. As shown in Table 3-3, COD output was based on soluble COD

derived from the drawn sludge, permeate COD, solid content COD, methane COD in biogas. In pretreatment, COD input is AFW before adding enzyme and yeast in the EP series. COD mass balances were calculated based on methods from Jeong et al. (2017). Calculation results show that the errors of inflowing and outflowing material are within 5%, which is clear evidence of the AnMBR system's stability and the reliability of the experimental results [15]. After anaerobic digestion, methane gas accounted for the most recoverable component, with the EP series being slightly higher than the control series under OLR conditions of 6.6 and 8.8 g-COD/L/d. Even under a 10.5 ~ 26.5 g-COD/L/d OLR condition, the EP series had a methanation ratio maintained at 80–90%, and an operation loading three times higher than the control series was possible. Through the calculation of solid content of drawn sludge rate, at three times the load of the control series, EP series can still reduce the sludge yield by 27-46%. The above results indicate that pretreatment maintains satisfactory substrate biodegradability.

3.3.4 Investigation of the cause of high load operation of the EP series

As the results show, methane fermentation in the EP series could be performed series by avoiding acidification caused by VFA accumulation. In addition, it was confirmed that the EP series could operate at three times the substrate level of the control series. To clarify the cause of the higher load operation possible in the EP series, the authors examined variations of parameters in each feeding period.

In this experiment, the substrate was fed every 240 minutes. **Fig. 3-3** and **Table 3-4** shows material fluctuation over 240 minutes from one substrate feeding to the next feeding on experiment day 73 (HRT 12.5 d), with the substrate fed at 0 minutes. The VFA generation amount is shown in a stacked area chart, with the height of the stacked area

representing the sum of VFA concentrations (TVFA). VFA was shown as an acetic acid conversion value (mg/L) as follows [16].

$$\mathrm{TVFA} = \mathrm{Acetic\ \ acid} + \left(\frac{\mathrm{Propionic\ \ acid}}{74.08} + \frac{(\mathrm{Isobutyric\ \ acid}) + (\mathrm{n-butyric\ \ acid})}{88.11} + \frac{\mathrm{Isovaleric\ \ acid}}{102.13} \right) \times 60.05 \quad \textbf{(3-1)}$$

As shown in **Fig. 3-3**, the maximum amount of TVFA in the control series was 230 mg/L 5 minutes after substrate feeding. After that, it decreased to 37 mg/L at 180 minutes and then stabilized. In the EP series, the decomposition most part of ethanol was completed around 50 min after the substrate feeding, this trend was the same as previously reported [14]. TVFA increased in the meantime, reaching a maximum of 540 mg/L 50 minutes after feeding. Then the TVFA dropped sharply to 11.5 mg/L by 180 minutes. Although the maximum VFA was higher in the EP series, the time to stabilize was the same. Therefore, the reaction on easily degradable matter was completed before the next substrate feeding. In contrast, the biogas production rate in the control series reached a maximum of 0.72 L/L/hr after 5 minutes, dropped sharply to 0.3 L/L/hr after 20 minutes, and then decreased slowly. In the EP series, the biogas production rate did not reach the same level as in the control series, with a 0.4 L/L/hr gas production rate continuing until 60 minutes and then suddenly dropping to 0.25 L/L/hr. After that it decreased slowly until 180 minutes, after which it dropped again to 0.04 L/L/hr. Considering VFA concentration, the EP series completed degradation of easily degradable substrate after 180 minutes, which was before the next feeding. In contrast, the gas generation rate in the control series was 0.18 L/L/hr, which was about four times greater than that of the EP series. This

indicates that the easily degradable substrate did not completely degrade. In the control series, the produced VFA amount was increased over the degradation of VFA due to the next substrate feeding occurring despite continuing degradation of the substrate. Therefore, it was thought that VFA accumulation would decrease pH, lead to the failure of the anaerobic fermentation process.

The ratio of propionic acid to acetic acid is an important indicator used to assess anaerobic fermentation process stability [17]. In a previous study, this ratio was generally used to predict digestion system stability and generally ratios ≤ 0.5 resulted in faster methane production and VFA decomposition than ratios ≥ 1 [13, 18]. In addition, as shown in **Table 3-4**, the propionic acid to acetic acid ratio in the control series increased sharply the feeding, with a peak ratio of 2. However, the ratio of propionic acid to acetic acid was significantly lower in the EP series. This indicates that ethanol fermentation of the substrate contributes to stable methane fermentation.

Table 3-5 shows the main reactions of glucose, which is a constitutional unit of starch in the substrate in both series [19, 20]. In the control series, hydrolyzed saccharides in the substrate are degraded to acetic acid and propionic acid following **eqs. 3-2** and **3-3** under anaerobic conditions. In the anaerobic reaction, propionic acid is produced in an endergonic reaction and is thus difficult to progress spontaneously. When hydrogen produced from the decomposition of glucose as seen in **eq 3-2**, the reaction of propionic acid of **eq 3-4** is maintained at a very low rate [21]. By comparing the Gibbs free energy changes for the methane conversion are $\Delta G^o = -31.0$KJ/mol for acetic acid and $\Delta G^o = +68.7$KJ/mol for propionic acid, as show in **eq 3-4** and **3-6**. In the control series, the major VFA produced by acidogenesis in the reactor was propionic acid. Thus, due to propionic acid tends to be accumulated in the reactor. Causing the control series failed due to

irreparable inhibition. In contrast, the VFA produced from the anaerobic digestion of ethanol in the EP series was acetic acid, as shown in **eq 3-5**. When acetic acid and H_2 are formed, the reactions described in **eqs 3-6** and **3-7** easier proceed than propionic acid in control series. This indicates that acetic acid was easily converted to methane than propionic acid under high load conditions.

3.4. Summary

The aim of this chapter was operation in the AnMBR system was attempted by using EP for AFW rich in starch. Ethanol fermented substrate was fed to the AnMBR several times per day. The HRT reduced stepwise from 20 to 5 days to increase the load by raising feeding volume. The OLR was 6.6 to 26.5 g-COD/L/d. The control series (without pretreatment) was operable to an OLR of up to 8.8 g-COD/L/d, whereas EP series was 26.5 g-COD/L/d. At three times the load of the control series, EP series can still reduce the sludge yield by 27-46%. By comparing the Gibbs free energy changes for the methane conversion, EP was demonstrated that effective in avoiding accumulation of propionic acid. The methane fermentation process generated a large proportion of acetic acid in VFA generation whereas in the control series it was propionic acid. Therefore, experiments have proved that using AnMBR treatment of EP is feasible, and EP significantly improves AnMBR performance. The use of pretreatment in AnMBR shows that it avoids the serious accumulation of propionic acid and the subsequent decreased pH, and membrane contribute to prevent the runoff of anaerobic microorganisms. The author considered that this method has the potential to operate under higher loads.

Table 3-1

Characteristics of artificial food waste (AFW) added in the control and ethanol fermentation pretreatment (EP) series.

Parameters	Control	Pretreatment
Substrate	AFW	Ethanol-fermented AFW
pH	6.5	4.2
TS (g/L)	100	-
VS (g/L)	98	-
T-organic carbon (g/L)	46	43
T-kjeldahl nitrogen (g/L)	3.2	3.4
T-phosphorus (g/L)	0.2	0.2
T-CODcr (g/L)	131	135
Ethanol (g/L)	0	40

Table 3-2

Main operating conditions and performance of the control and ethanol fermentation pretreatment series.

Series	Control			Pretreatment					
Operation feasibility	Enable	Enable	Failure	Enable	Enable	Enable	Enable	Enable	Enable
Operating conditions									
OLR (g-COD/L/d)	6.6	8.8	10.5	6.6	8.8	10.5	13.2	17.7	26.5
HRT (d)	20	15	12.5	20	15	12.5	10	7.5	5
Operating performance									
Biogas									
Methane ratio (%)	53.1 ± 0.55	53.7 ± 0.15	51.4→41.7	69 ± 0.55	70.2 ± 0.35	69.9 ± 0.3	70.9 ± 1.75	69.3 ± 0	71.4 ± 0.4
Biogas yield (mL/g-VSadded)	720	690	690→60	640	600	580	600	600	544
Methane yield (mL/g-VSadded)	380	370	360→20	440	420	410	420	410	390
In the reactors									
pH	7.3 ± 0.02	7.3 ± 0.03	7.4→5.1	7.7 ± 0.01	7.7 ± 0.03	7.7 ± 0.05	7.7 ± 0.04	7.7 ± 0.02	7.5 ± 0.04
TS (g/L)	35.1 ± 0	39.8 ± 1.5	43.1→48	37.8 ± 2.92	38.7 ± 0.53	41.7 ± 1.84	47.7 ± 3.94	55.9 ± 4.03	59.2 ± 2.01
VS (g/L)	30.6 ± 0.01	34.8 ± 1.3	38.3→43.5	32.11 ± 2.2	32.2 ± 0.34	34.4 ± 1.49	39.2 ± 3.37	46.9 ± 3.42	50.8 ± 1.9
TC (g/L)	14.6 ± 0.54	17.4 ± 0.45	19.6→32.4	16.5 ± 0.98	18.3 ± 0.08	17 ± 1.13	27.5 ± 1.63	24.5 ± 1.14	27.3 ± 0.94
Dissolved organic carbon (g/L)	0.3 ± 0.08	0.27 ± 0.01	0.23→0.43	0.36 ± 0.11	0.58 ± 0.04	0.68 ± 0.02	0.79 ± 0.01	0.89 ± 0.03	2.57 ± 0.48
Dissolved inorganic carbon (g/L)	1.33 ± 0.16	1.34 ± 0.04	1.23→0.23	1.82 ± 0.13	1.95 ± 0.15	2.12 ± 0.01	2.15 ± 0.04	1.98 ± 0.1	1.38 ± 0.19
Dissolved total nitrogen (g/L)	1.82 ± 0.32	1.78 ± 0.06	1.5→1.15	2.38 ± 0.1	2.63 ± 0.16	2.67 ± 0.01	2.73 ± 0.06	2.63 ± 0.08	2.73 ± 0.16
T-CODcr (g/L)	48.9 ± 0.57	55.7 ± 1.28	64.9→86.1	50.8 ± 0.25	55.5 ± 0.45	58.2 ± 2.43	61.8 ± 0.9	79.3 ± 4.98	90.6 ± 2.78
D-CODcr (g/L)	1.08 ± 0.09	0.97 ± 0.11	0.72→10.3	1.65 ± 0.19	1.74 ± 0.06	2.18 ± 0.11	2.52 ± 0.08	3.03 ± 0.09	7.99 ± 1.69
Alkalinity (g/L)	6.73 ± 0.07	6.58 ± 0.18	5.55→1.25	9.58 ± 0.08	10.1 ± 0.13	10.3 ± 0.1	10.2 ± 0.08	9.78 ± 0.18	8.14 ± 0.04

Table 3-3

Chemical oxygen demand (COD) mass balance of the anaerobic membrane bioreactor system.

Parameters	Control		Pretreatment					
OLR (g-COD/L/d)	6.6	8.8	6.6	8.8	10.5	13.2	17.7	26.5
COD input (%)	100	100	100	100	100	100	100	100
Biogas production (%)	82.9	81.1	93.2	88.4	86.0	89.6	87.3	82.1
Solid content of drawn sludge (%)	14.2	15.0	6.61	11.7	13.3	9.93	7.65	11.0
Dissolved content of drawn sludge (%)	0.34	0.21	0.14	0.19	0.25	0.22	0.21	1.07
Permeate (%)	0.35	0.56	1.08	1.07	1.28	1.43	1.99	2.88

Table 3-4

Variations in one cycle of feeding on the 73rd day of the continuous experiment in both series.

Retention time	0 min [a]	0 min [b]	5 min	20 min	50 min	70 min	120 min	180 min	240 min [a]
Control									
Acetic acid (mg/L)	29	56	77	68	79	78	78	32	33
Propionic acid (mg/L)	0	0	152	112	71	44	7.4	5.1	0
Isovaleric acid (mg/L)	0	0	0	2.6	3.8	3.8	2.9	0	0
Issoquissoic acid (mg/L)	0	0	3.2	4.1	4.6	4.9	2.9	0	0
Propionic acid/Acetic acid	0	0	2	1.6	0.9	0.6	0.1	0.2	0
pH	7.6	7.5	7.2	7.1	7.2	7.2	7.3	7.4	7.6
Pretreatment									
Acetic acid (mg/L)	19	23	130	261	468	425	202	11	17
Propionic acid (mg/L)	0	0	29	37	65	36	0	0	0
Isovaleric acid (mg/L)	0	0	0	2.6	4	2.3	0	0	0
n-butyric acid (mg/L)	0	0	6.1	4.4	4.9	2.6	0	0	0
Issoquissoic acid (mg/L)	0	0	0	2.4	2.3	2.9	1.9	0	0
Propionic acid/Acetate acid	0	0	0.2	0.1	0.1	0.1	0	0	0
Ethanol (mg/L)	0	518	514	304	31	0	0	0	0
pH	7.8	7.8	7.9	7.9	8.1	8.1	8.1	8.1	7.8

In this table, 0 min is the time of substrate feeding, and 240 min is immediately before the next feeding.

[a] Immediately before feeding

[b] Immediately after feeding

Table 3-5

Different CH_4 fermentation pathways and free energy change yielded from different substrates.

Control		
Acidogenesis & Acetogenesis		
$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^-$		
$+ 4H^+ + 4H_2$	$\Delta G^o = -206.1 KJ/mol$	(3-2)
$3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COO^- + 2CH_3COO^-$		
$+ 2HCO_3^- + 8H^+$	$\Delta G^o = -940.4 KJ/mol$	(3-3)
$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^-$		
$+ H^+ + 3H_2$	$\Delta G^o = +68.7 KJ/mol$	(3-4)
Pretreatment		
Acidogenesis & Acetogenesis		
$C_2H_5OH + 2H_2O \rightarrow 2CH_3COO^- + 2HCO_3^-$		
$+ 4H^+ + 4H_2$	$\Delta G^o = +9.6 KJ/mol$	(3-5)
Control & Pretreatment		
Methanogenesis		
$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$		
	$\Delta G^o = -31.0 KJ/mol$	(3-6)
$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$		
	$\Delta G^o = -135.6 KJ/mol$	(3-7)

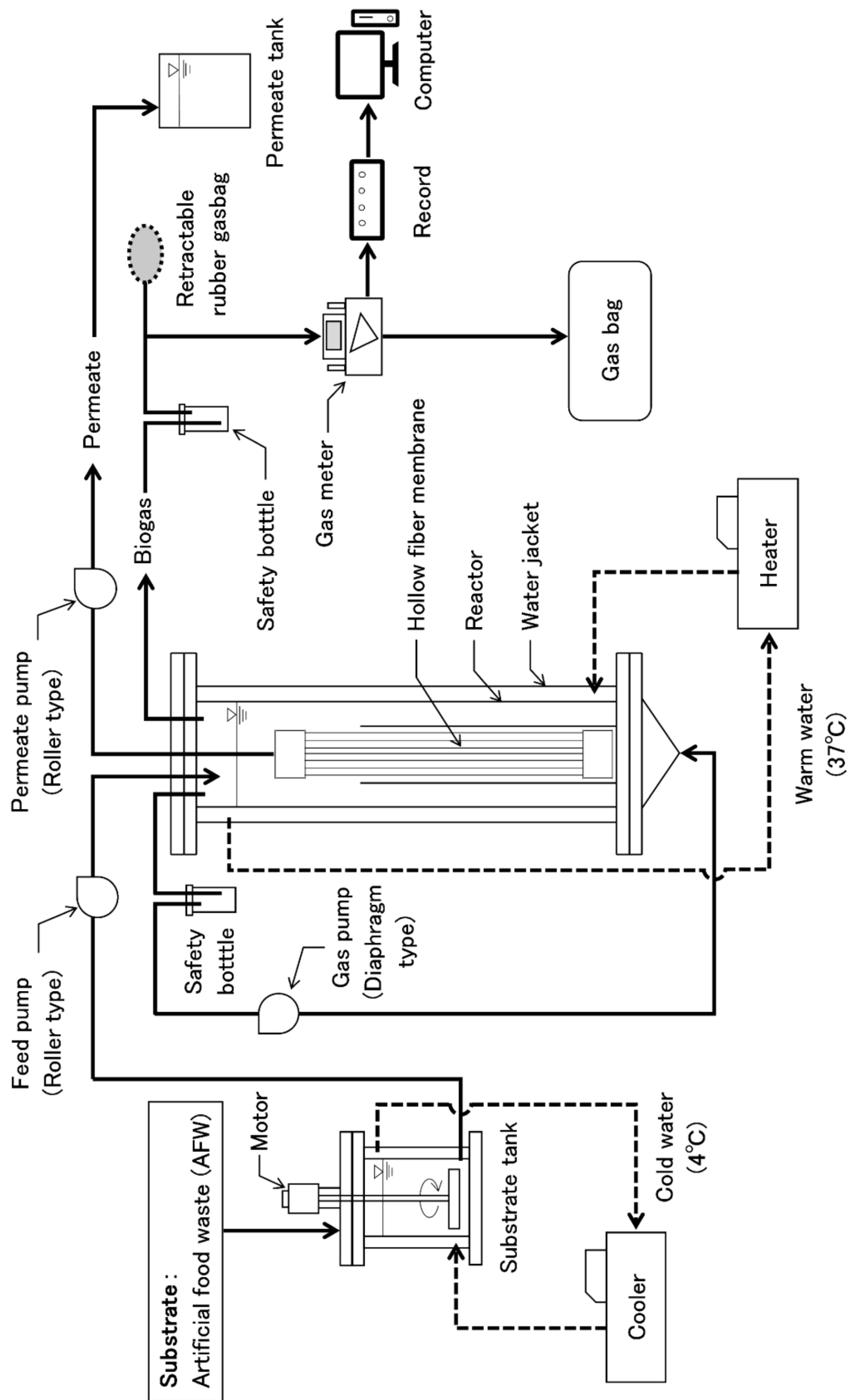

Fig. 3-1. Experimental apparatus.

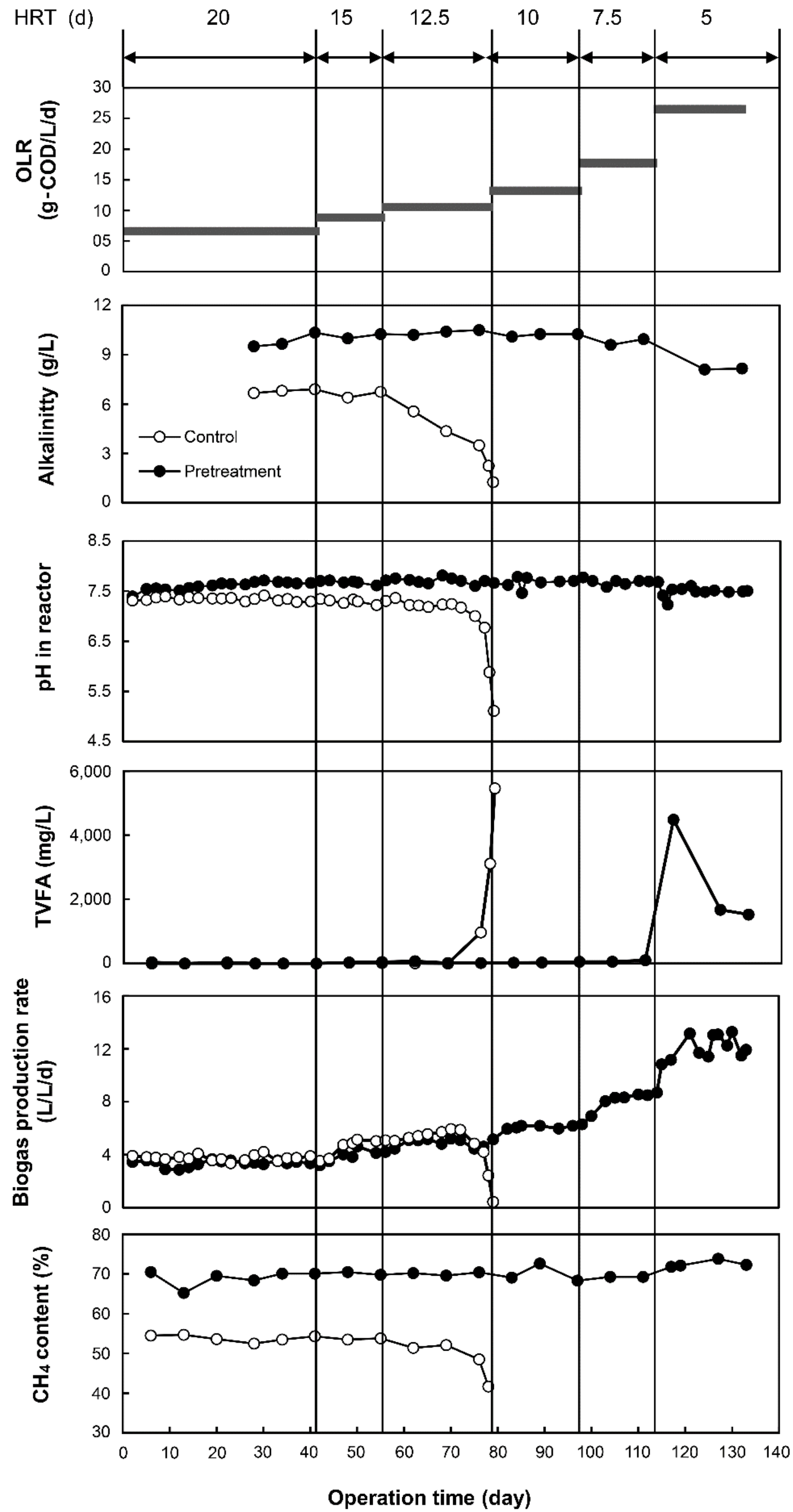

Fig. 3-2. Time course of changes in alkalinity, pH, total volatile fatty acid (TVFA) levels, biogas production rates, and CH4 content in the anaerobic membrane bioreactor.

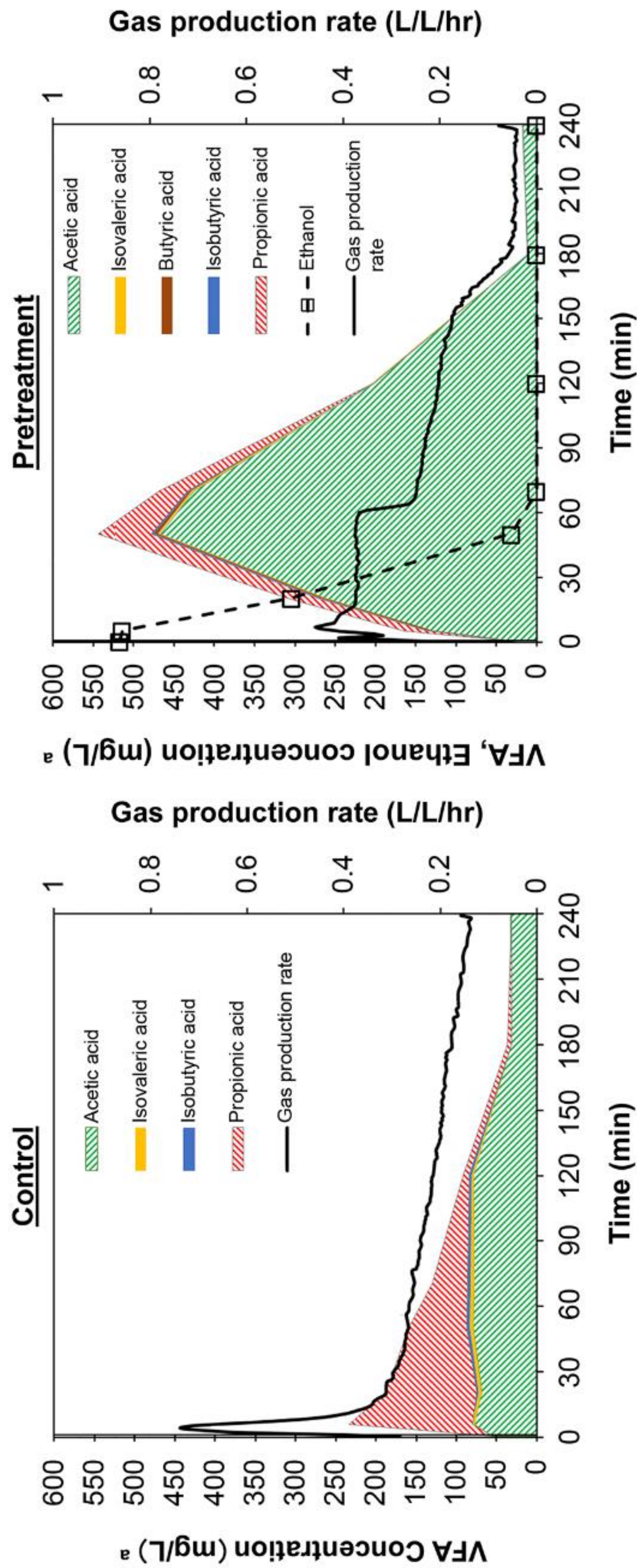

Fig. 3-3. Variation of the concentrations of ethanol and volatile fatty acids (VFAs) in the one feeding period at the methane fermentation stage.

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Chapter 4. Higher load operation by adoption of ethanol fermentation pretreatment for methane fermentation of food waste using an anaerobic membrane bioreactor: Performance and microbial community

4.1. Introduction

The disposal of food waste (FW) has become a social problem in recent years. However, owing to its high moisture content, and rich organic matter content, FW is considered as a potentially valuable material [1]. Consequently, food recycling methods have gained more attention and many recycling projects are based on anaerobic digestion (AD). AD mainly involves four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis [2]. Many FW pretreatment methods have been explored in order to improve digestion performance, and to make these four stages go smoothly. Many pretreatment methods including chemical [2, 3], thermal [4, 5], and biological disintegration [6, 7], as well as combinations of these have been applied for improving methane production and increase processing load of AD.

However, some characteristics of FW are the most problematic features in the process of AD, resulting volatile fatty acid (VFA) inhibition, affecting the stability and sustainability of AD [8]. Among of VFA, the propionate is not easily converted to acetate, which leads to the accumulation in the methanogenesis stage [9]. At high VFA concentrations, causes a decrease in pH, and causing the acid–base imbalance of intracellular environment, inactivating the cells, reducing the degradation rate of organic acids and H_2/CO_2 and eventually leading to the failure of anaerobic fermentation [1, 10]. Of these pretreatment methods, ethanol fermentation pretreatment (EP) has been demonstrated to be effective in avoiding VFA accumulation which can cause a subsequent

decrease of pH, affecting reactor performance [11]. In addition, EP could improve the methanogenic carbon conversion rate and speed of substrates [12, 13].

However, the excessively slow growth rate of anaerobic microorganisms utilized in this process when compared with that of conventional fermenters is a drawback in keeping sufficient biomass in the rector to maintain efficient operation [14]. Anaerobic membrane bioreactors (AnMBR) effectively prevent the washout of these slow growing methanogens, enabling operation at longer solid retention times (SRT) than hydraulic retention times (HRT).

Up to date, very few studies have investigated AnMBR treatment of ethanol fermented FW [11], and no research has evaluated microbial community dynamics within AnMBR treatment of ethanol fermented FW. In particular, there is no report indicating how high the load in AnMBR can be operated by using ethanol fermented FW. Therefore, in this work we aimed to systematically compare control series (substrate: FW) and an EP series (substrate: ethanol fermented FW) performance of both substrate using AnMBRs. In the present study, a laboratory-scale mesophilic semi-continuous AD reactor, bacterial communities present in the stable AD reactor were analysed by Illumina MiSeq high-throughput sequencing. Both series were operated at incremental OLRs up to 43.5 g COD L/day to compare performance and operating limits. This study examined whether EP of FW can allow for more effective high load operation in an AnMBR, examining whether pretreatment effects can be obtained in a stable state and determining the effect of EP on the maximal organic loading rate (OLR), providing a reference for further studies and potential engineering applications.

4.2. Materials and methods

4.2.1. Substrate (Artificial food waste)

Artificial FW (AFW) was created using boiled rice (300 g), cabbage (90 g), carrots (90 g), chicken (20 g), and small dried sardines (20 g). The specific production process of AFW was according to the methods described by Sun et al. (2020a). As shown in **Table 4-1**, the total solids (TS) content of AFW was 200 g/L. The average AFW composition was 190 ± 4.9 g/L of volatile solids (VS) and 91.3 ± 2.6 g/L of total organic carbon (TOC). Trace minerals dissolved in water was added—100 mg of Fe as $FeCl_3 \cdot 6H_2O$, 10 mg of Co as $CoCl_2$, and 10 mg of Ni as $NiCl_2$ (per 100 g-TS of AFW)—simultaneously [14]. In addition, when the total solids (TS) content of the AFW were needs adjusted to 150 g/L, which adding distilled water purged with nitrogen gas.

4.2.2. Pretreatment (ethanol fermentation pretreatment)

AFW with a total solids (TS) content of 200 g/L was used for pretreatment. The TS-adjusted AFW was then saccharized by reacting 5 mL of glucoamylase (Spirizyme Fuel, Novozymes, Denmark) per 100g-TS of AFW for 2 h at 50 °C with constant stirring. The saccharized AFW was fermented by adding 1.9 g of commercial yeast (Maurivin POP Yeast, AB MAURI, UK) per 100g-TS of saccharized AFW for 44 h at 27 °C with stirring. The substrate characteristics before and after pretreatment are shown in **Table 4-1**. The fermentation procedure yielded an ethanol concentration of approximately 85.4 ± 2.26 g/L, and the control substrate was not pretreated. In addition, trace minerals were added to the substrate after the pretreatment same as **4.2.1**.

4.2.3. Methane fermentation using AnMBR

The AnMBR system was divided into a control series and an EP series, two identical systems were prepared as shown in **Fig. 4-1**. A hollow fiber membrane (LSPMW-02, Sumitomo Electric Industries, Japan) used in both systems has an average pore diameter. The membrane module was immersed in the reaction vessel with an effective volume of 4.5 L, and the temperature was maintained at 37 °C using a water jacket. In addition, the substrate tank was maintained at 4 °C using a water jacket. The AFW without and with pretreatment were stored in the substrate tanks for the control and EP series, respectively. The substrate was supplied from the substrate tank to the reaction tank using roller pumps, which were operated under a cycle of 1 min on and 359 min off on OLR load from 6.5 to 9.6 g-COD/L/day; 1 min on and 239 min off from 13.0 to 43.5 g-COD/L/day. The treated water was permeated from the membrane module to the treated permeate tank using a roller pump. Before substrate was supplied, most of the biodegradation had been completed [15]. Thus, the roller pump was intermittent relaxation operated for 60 min before the substrate was supplied, intermittent relaxation operation can reduce the fouling rate of the module [16]. The membrane was washed by circulating biogas from the head space to the bottom of the reactor using a diaphragm gas pump.

The seed sludge was collected from the mesophilic anaerobic digester at the sewage treatment plant and FW digester and was acclimated with AFW (without pretreatment) in other reactor (volume of 10 L) using an OLR of 1.1 g-COD/L/day at 6 months before added in AnMBR. The AnMBR was purged with O_2-free N_2 for 15 min before seed sludge was added. In the AnMBR system, the OLR load was increased stepwise to 6.5 g-COD/L/day for 1 months; it was confirmed that the gas generation amount and pH were in a steady state, and they were used for the formal experiment.

In the formal experiment, the AnMBR system was operated from 6.5 to 43.5 g-COD/L/day by increasing the feeding load.

4.2.4. Analysis biogas production rate

The use of the diaphragm gas pump in this study caused the pressure of biogas to fluctuate, raising the potential of error in the gas meter. Therefore, a retractable rubber gasbag (113401, DEBIKA, Japan) was installed before the biogas entered the gas meter so that generated biogas passed through the gas meter at a constant speed. Biogas production was measured using a wet tipping gas meter (μFlow, Bioprocess control, Sweden). The operating of these gas meters is based on the "tipping bucket" principle in which liquid is displaced by gas in a specially-designed chamber [17]. This gas meter allowed the measurement of each tipping of the container. The measurement of signal from the gas meter was processed by the data logger and sent to the computer for calculation. Biogas production rate was calculated by dividing the volume of biogas by the time elapsed since the previous tipping.

4.2.5. Analysis method

TS, VS, suspended solids (SSs), volatile SSs, and alkalinity were analyzed using standard methods (American Public Health Association, 2005). Glucose, ethanol, and volatile fatty acids (VFAs) were measured after filtration with a PTFE filter (DISMIC-25HP, ADVANTEC, Japan). The glucose concentration was analyzed using the glucose oxidase method (Glucose kit, Glucose CIItest, Wako, Japan), and total carbon (TC) in the AFW was analyzed using combustion catalytic oxidation and a non-dispersive infrared (NDIR) method (SSM-5000A, Shimadzu, Japan). Dissolved organic carbon (DOC) and

inorganic carbon from the permeate were also analyzed using combustion catalytic oxidation and a NDIR method (TOC-V, Shimadzu, Japan). Ethanol and VFA levels were quantified by flame ionization detection-gas chromatography (GC14B, Shimadzu, Japan) using a Gasukuropack-54 60/80 column (GL Sciences, Tokyo, Japan) with helium as the carrier gas (44 mL/min). In total, 1 μL of sample was injected using airtight syringes, and the injector, column, and FID temperatures were 250 °C, 200 °C, and 250 °C, respectively. Biogas from the AnMBR was collected using a gas bag and quantified by thermal conductivity detection-gas chromatography (GC14B, Shimadzu, Japan) using a ShinCarbon ST 50/80 column (Shinwa Chemical Industries, Kyoto, Japan) with argon as the carrier gas (50 mL/min). In total, 500 μL of biogas was injected into the gas chromatograph, with injector, column, and TCD temperatures were 200 °C, 40 °C–200 °C, and 200 °C, respectively. Analysis of chemical oxygen demand (COD) used a spectrophotometer (DR900, HACH, USA) and CODcr reagent (HACH 4236, HACH, USA).

4.2.6. Metagenomic analysis

DNA extraction from samples, stored at 4℃, was conducted by DNA extraction kit (Nippon Gene, ISOIL for Beads Beating). DNA concentration was measured with DNA analyzing kit (life technologies Qubit ds DNA BR Assasy kit) and analyzer (life technologies, Qubit2.0 fluorometer). 16S rRNA gene sequencing analysis was conducted by MiSeq system (Illumina). The primer sets were selected 16S Amplicon PCR forward primer/reverse primer : 5’- CCTACGGGNGGCWGCAG -3’ / 5’- GACTACHVGGGTATCTAATCC -3’ to amplify V3-V4 region. Library denaturing was carried out using MiSeq Reagent kit v2500 cycle (Illumina).

Quality of reads were confirmed by FastQC to judge their use, or not. Sequence analysis was conducted using the Quantitative Insight Into Microbial Ecology (Qiime2 ver. 2020.2) [18]. Reads were joined and primers were trimmed. Low quality reads which were under 80% base pairs of $Q < 20$ on quality value were detected and removed by FastX tool kit. Chimeric reads were detected and removed using usearch61. They were clustered at 97% read identity by de novo otu picking method. Representative reads in OTUs were selected by PyNAST and assigned to taxonomy using Qiime2's uclust-based taxonomy assigner referring GreenGenes (ver. 13_8) database. Read numbers were counted in each OTU and totalized on each taxonomy.

4.3. Results and discussion

4.3.1 Higher loading operation by pretreatment

The daily changes in the main measurement items are shown in **Fig. 4-2**. **Table 4-2** presents the average operating index under each operating condition. In the AnMBRs systems, the OLR values that could be operated were 6.5 to 13.0 g- COD/L/d for the control series and 6.5 to 43.5 g- COD/L/d for the EP series. At OLR 6.5 g- COD/L/d, the VFA of the EP system increased. This may be due to the EP series has not adapted to the high TS of the FW temporarily, which caused the decomposition of VFA to weaken. As a countermeasure, on the 55th day, the TS of the FW before ethanol fermentation has been reduced from 20% to 15% and maintained OLR remains unchanged. In addition, at the OLR43.5 of the EP series, the TS of the substrate before ethanol fermentation has been increased to 20%. Although the TS the substrate has increased, AnMBR can still maintain a high decomposition rate and operate stably. Previous studies explained stoichiometry as the reason for the increased methane concentration generated from ethanol fermentation, because of the lower carbon dioxide generation from subsequent methane fermentation [14].The control series had a methane gas concentration of 52–57%, whereas that of the EP series was 71–74% under operable load.

Methane production rate (primary y-axis) and methane yield (secondary y-axis) are shown in Fig. 3. When the OLR was 6.5, 7.8, 9.8, and 13.0 g-COD/L/d, the control and EP series both had methane yields that stabilized at approximately 0.3 L/g-COD (**Fig. 4-3**). However, when the OLR was further increased to 19.6 g- COD/L/d in the control series, the amount of generated biogas sharply decreased to 0 COD/L/d, VFA correspondingly increased to 7.7 g L^{-1} and was accompanied by a significant pH and alkalinity drop (to pH 4.2, alkalinity 0 mg/L). The methane gas concentration also

decreased to 47.5% accompanied by a sharp decrease in the amount of generated methane, indicating severe inhibition of the anaerobic fermentation process. Low pH (< 6.2) and high VFA concentrations (> 5.8 g/L) have been shown to completely inhibit methanogens (Cheng et al. 2018; Amha et al., 2019). In contrast, for the EP series, when the OLR was further raised to as high as 43.5 g- COD/L/d, methane yield remained the same as that seen at lower loads.

In the control series, inhibition occurred between 13.0 and 19.6 g- COD/L/d. Many researchers indicated that the OLR was inhibiting from 10 to 15 g- COD/L/d in an AnMBR system [5, 7]; the operational load of the control series in this study was similar to that used by Amha et al. (2019), and therefore the results observed correlate well with previous research on maximum OLR. In the EP series, the pH stabilized at around 7.7, enabling operation at 3 times the load than that seen for the control series. These results demonstrate that methane production in an AnMBR system using ethanol fermentation as a pretreatment strategy produces superior performance to that seen with a conventional AnMBR system.

At the highest load of the EP system, the author discovered that the sludge would expand by about 10% due to the substantial increase in biogas production after the substrate was feeds. If the load is increased more, the sludge will be sucked into the biogas pump and clogged due to the limited head space. Therefore, it can be considered that the maximum operable load in the EP series was 43.5 g-COD/L/d. If it is operated at a higher load, the author recommends increasing the head space or shortening the interval between feedings to stabilize biogas production.

4.3.2 Effect of EP on biogas production rate kinetics

Since this experiment was semi-continuous experiment, the substrate was fed every 240 min at the beginning of OLR 13.0. **Fig. 4-4** shows biogas generation rate over 240 min from first substrate feeding to the next feeding on different loads, with the substrate fed at 0 min. **Fig. 4-4A** shows the biogas production rate of the control series under the highest stable operating load. In a previous study knows that, three steps can be distinguished in these curves: *step 1* a rapid increasing and then decreasing phase of biogas production (CH_4 formation by hydrogenotrophic methanogens); *step 2* constant high biogas production (CH_4 formation by acetotrophic methanogens) and *step 3* constant low biogas production and there are transition stage between the last two steps [17]. Transition moved backward as the load increased. But under operable loads, the transition will remain stable. **Fig. 4-4B, C** and **D** shows the AD of acidosis at OLR19.6. The second step was gradually longer, until the transition disappears, which is consistent with the results of previous studies. [15, 17]. From day 155, daily biogas production decreased due to severe acetotrophic methanogens inhibition of *step 2* leading to a drop in pH. Finally, all these conditions resulted in a total inhibition of all methanogens.

Previous research on EP series show that methane produced from hydrogen generated by the decomposition of ethanol in the first step; the second step was mainly acetic acid to methane production [11]. In EP series, it was interesting that this transition was relatively late compared to the control system. If refer to the experience of the control series would suggest that a relatively late transition of the EP series might increase the inhibition risk of methanogenesis. However, although the transition in the EP series was after 180 minutes, the position of the transition does not change much even when the load increases to OLR 43.5. It can be considered that whenever the load increases, the EP

series can adapt and decompose the increased substrate, thereby maintaining a constant biodegradability. In addition, when the two series were at the same load of OLR 13.0, the gas production rate of the EP series in the third step was lower than that of the control series. This shows that before the next feeding, the EP system has less undecomposed substrates than the control system, thus avoiding excessive substrate accumulation.

In the EP series, as the load increases, it can be clearly observed that the advantages of the first step gradually appear. In **Fig. 4-4H**, it can be observed that the duration of the first step was significantly longer than **Fig. 4-4A**. This may be due to the gradual increase of hydrogen bacteria to become the dominant species, which improves the utilization efficiency of hydrogen and enables high-load operation. However, the analysis of the kinetics of the biogas production rate alone is not enough to explain what changes have taken place in the microbial community for the decomposition and pretreatment of FW. Therefore, it is necessary to analyze the microbial community.

4.3.3 Microbial community

4.3.3.1 Bacterial taxonomic identification

The results of the bacterial community structure (**Fig. 4-5**) showed that the *Clostridium* genus was the most abundant bacterium in both AnMBRs. The genus *Clostridium* belongs to the *Firmicutes* phylum and includes a number of species that are known to degrade complex biopolymers such as cellulose [8]. In this work, the EP series substrate contains ethanol. Ethanol is a common intermediate produced by hydrolytic and fermentative bacteria during methanogenesis [19], and so it can be easily used by bacteria. There are some species of the *Clostridium* are uses the ethanol which converted into VFAs and H_2 [20, 21]. *Clostridium* are also the main bacteria for the hydrolysis of lipids, similar

function of these bacteria were also reported in previous studies [22, 23]. In the control and the EP series, its proportion in the genus classification did not change much, but significant changes occurred in the unclassified species classification. Although they belong to the same genus, the decomposition of starch and ethanol may not be the same species. *Paludibacter* belongs to *Bacteroidales* utilize various sugars and produce acetate and propionate as major fermentation end-products with succinate as a minor product [24]. In the previous anaerobic fermentation research using food waste as the substrate, the abundant genus *Paludibacter*, showed a significantly negative correlation with HRT, suggesting *Paludibacter* to be robust at high OLR and low HRT. [25]. This was consistent with the obvious increase when *Paludibacter* was at the highest load in the EP series. *Candidatus Cloacamonas* was a genus of bacteria in the family *Cloacamonaceae,* which the bacterial genus can produce acetate, hydrogen, and carbon dioxide from various organic materials such as amino acids, lactate, succinate, and propionate previously found to be hydrogen-producing, and the reaction is thermodynamically favorable under low hydrogen pressure [26].

In a previous study, the major bacterial genera were also similar among the AnMBRs with changes in OLR (i.e., 1.5–6 kg VS/m^3/d), because the anaerobic hydrolytic/acidogenic bacteria have strong resistance to process disturbance [27]. In this study, the control series was operated with a higher load 9.8 g-COD/L/d (7.5 kg VS/m^3 /d) beyond the range, so it can be considered that the reason for the significant change in the bacterial community compared with the low-load (OLR<1 kg VS/m^3/d) seed sludge. Another reason might be that the control series into AnMBR, which was caused by relatively low HRT and high SRT.

In addition, it is interesting that this study found that the bacterial communities of the

control series (OLR 9.8) and the EP series (OLR 19.6) are also very similar in the two AnMBR systems with different substrates. It has been reported that ethanol had little effect on bacterial microflora, but changed the microflora structure of archaea significantly, changing the gas-producing pathway from acetoclastic to hydrogenotrophic [28]. The author found that the proportion of some bacteria in the EP series operating under the highest load (OLR 43.5) has changed. This may be due to the adaptation to the high-load substrate feeding and the formation of a new syntrophy with bacteria and archaea.

4.3.3.2 Archaeal taxonomic identification

Fig. 4-6 shows the methanogenic microbial composition at the genus level. There were two main of archaea in seed sludge: obligate aceticlastic methanogens of *Methanosaeta* accounted for 62.2% and hydrogenotrophic methanogenesis of *Methanolinea* accounted for 29.3%. With the increase of the load, the microflora structure of archaea of the EP series has undergone a fundamental change, and the hydrogenotrophic (*Methanomassiliicoccus*, *Methanobacterium*, and *Methanoculleus*) increased from 36.4% (OLR 19.6) to 84.7% (OLR 43.5). However, we found that the proportion of hydrogenotrophic was only 38% in the control series under the OLR 9.8. EP caused an increase in hydrogenotrophic methanogenesis, which increased the rate of conversion of hydrogen to methane. Further caused a reduction in the hydrogen partial pressure, thereby promoting the conversion of ethanol, propionate, and butyrate to acetate, consistent with the results of previous studies [13, 29]. This showed that the AnMBR was adapted to the matrix containing ethanol as the substrate by changing the methane production pathway, which well explained the reason why the high biodegradation rate can be maintained

under high load.

In the EP series (OLR 43.5), the total proportion of obligate hydrogenotrophic methanogens (Methanomassiliicoccus, Methanobacterium, and Methanoculleus) was identified as the dominant methanogen. Many researchers indicated obligate aceticlastic methanogens were more sensitive to ammonium inhibition compared with hydrogenotrophic methanogens [28, 30]. It is interesting that as the one and the only important facultative aceticlastic methanogen, Methanosarcina is the most metabolically and physiologically versatile methanogen that can convert different substrates, such as acetate, H_2, and methyl containing groups to CH_4 [31]. In other words, *Methanosarcina* are both acetoclastic and hydrogenotrophic methanogens. Recent studies have confirmed the diversification of the metabolic pathways of *Methanosarcina* through the analysis of key enzymes in the anaerobic fermentation facilities of municipal sewage treatment plants [32]. Many researchers indicated that Methanosarcina also robust to ammonia inhibition [31, 33]. This may be because *Methanosarcina* are not obligate aceticlastic methanogens. Therefore, the inhibition of ammonium may weaken the VFAs decomposition ability of others aceticlastic methanogen and cause the accumulation. In the EP system, the decrease of obligate aceticlastic methanogens (*Methanosaeta*) and the increase of hydrogenotrophic methanogens will avoid the above risks. On the other hand, ammonium is the main component of alkalinity in AD, and the produced ammonium further improved the AD system buffer capacity [34]. **Table 4-3** shows that the control series had a NH_4-N concentration of below 2000mg/L and that of the EP series was 2900–3900 mg/L. Therefore, EP could enable the AnMBR reactor to biologically decompose FW with a higher relative nitrogen concentration and ensure the stable production of methane.

On the other hand, when the load of the EP system was from OLR 19.6 to 43.5, the

proportion of *Methanosarcina* decreased from 40.8% to 15.2%, and the proportion of *Methanomassiliicoccus* increased from 16.8% to 65.4%. Therefore, it can be inferred that although *Methanosarcina* can convert part of the hydrogen and methyl compounds (i.e., methanol, dimethylamine, and monomethylamine) to produce methane, as the load increases, the proportion of *Methanomassiliicoccus* increases significantly and replaces the *Methanosarcina*. It is interesting that when methanogenic bacteria use hydrogen and methyl compounds to produce methane, the appropriate reduction of *Methanosarcina* in AnMBR may be beneficial in mode of energy conservation in methanogenic archaea. A common method to compare methanogenic archaea with respect to the efficiency of their energy conserving systems is the determination of growth yields. The growth yield is defined as the amount of dry cell mass (g) which is obtained per mole of methane that is formed and is referred to as Y_{CH4} [35]. **Table 4-3** shows the different species display remarkable differences in specific growth yields (Y_{CH4}), i.e., the amount of biomass formed per mole of methane produced at a given growth condition [36]. In previous studies, the growth yield was determined for *Methanomassiliicoccus* on methanol + H_2 and trimethylamine + H_2 which resembled the growth yield of hydrogenotrophic methanogens with 2.4 g of cell dry mass being formed per mole methane, respectively [35]. When *Methanosarcina* depredated hydrogen and methanol to produce methane, the amount of cells produced is two to three times that of acetic acid. However, when *Methanomassiliicoccus* replaces *Methanosarcina* to decompose hydrogen and methanol, the efficiency of methane production of AnMBR is significantly improved and the increase of sludge can be reduced. However, in this study the ratio of acetic acid, hydrogen, and methanol to methane produced by *Methanosarcina* is still an unknown question. In future research, new methods (such as key enzyme analysis) are needed to

determine the specific methane production pathway of *Methanosarcina*.

4.3.3.3 The main metabolic pathways of the microbial communities

In anaerobic processes, syntrophic bacteria play an important role for reducing metabolic intermediates which can inhibit methanogenic activity or supplying substrate on methanogens [29]. In particular, for anaerobic processes treating high-strength organic such as food wastes, syntrophic relationship among the functional microbial communities is closely related to stable process performances [27]. In order to summarize the main metabolic pathways of ethanol pretreated food waste degradation and methanogenesis for high load in the AnMBR, which combined the results of this experiment and previous research to draw **Fig. 4-7.** The lipids were degraded by bacteria (the genus *Clostridium*). The starch in carbohydrates has been converted into ethanol by ethanol fermented process, and the remaining cellulose is converted into monosaccharides by bacteria (the genus *Clostridium* and *Caldicobacter*), and then into VFAs. On the other hand, most of ethanol was converted to acetic acid, which reduced the accumulation of VFAs such as propionic acid [13]. The major bacteria for protein decomposition were genus *Candidatus Cloacamonas*, etc. The increase of the genus *Candidatus Cloacamonas* produced a large amount of methyl compounds and ammonium, and the produced ammonium further improved the AnMBR buffer capacity [34]. The methyl compounds, hydrogen and carbon dioxide produced in it could supply hydrogenotrophic methanogens to generate biogas. On the highest load of EP, hydrogenotrophic methanogens and obligate aceticlastic methanogens (*Methanosaricina*) were identified as the dominant methanogens, and all of them which with a high tolerance of ammonium inhibition.

Another the decrease of *Methanosarcina* and the increase of *Methanomassiliicoccus*

may be caused by changes in their respective co-nutritive bacteria. While *Methanomassiliicoccus* increased greatly, it was found that the family *Cloacamonaceae* multiply at the same time. It can be speculated that it may form a co-nutritive relationship with *Methanomassiliicoccus*. *Cloacamonaceae* produces a large amount of methyl intermediates and hydrogen in the process of protein conversion, which provides energy for *Methanomassiliicoccus*. This reaction is thermodynamically favourable only when the large amount of *Methanomassiliicoccus* reduces the partial pressure of hydrogen, which in turn makes *Cloacamonaceae* work more smoothly. A virtuous cycle was formed between bacteria and archaea in the stable and high-rate AnMBR. Therefore, using EP for FW is a promising alternative to improve AD stability, gas production, methane concentration and operating load.

4.4. Summary

A higher operating load was at tempted using EP for AFW in an AnMBR system by using a sequential batch experiment, where ethanol fermented substrate was fed to the AnMBR. The control series (without ethanol fermentation) was operable to an OLR of 13.0 g COD/L/d, while the EP series was operable to an OLR of 43.5 g COD/L/d, 3 times higher than the capability of the control series. In high load operation, EP also proved effective in maintaining a stable methane yield and stable long term operation of the reactor; together, this demonstrates that EP of AFW significantly improves anaerobic digestion performance. The abundance of varieties of hydrogenotrophic methanogens rate in the AnMBR were determined to be enhanced using EP for FW through high-throughput sequencing analysis. The large amount of hydrogenotrophic methanogens reduces the partial pressure of hydrogen, thereby promoting the biodegradation of the substrate by bacteria. The hydrogenotrophic methanogens with a high tolerance of ammonium inhibition, and higher ammonium then improves the AD buffer capacity. Therefore, a virtuous cycle was formed in the EP series as an additive that was also responsible for the stable and high operational loading by adoption of ethanol fermentation pretreatment.

Table 4-1

Characteristics of artificial food waste (AFW) added in the control and ethanol fermentation pretreatment (EP) series. When the total solids (TS) content of AFW was 200 g/L.

Parameters	Control	Pretreatment
Substrate	AFW	Ethanol fermented AFW
TS (g/L)	200	-
VS (g/L)	190 ± 4.9	-
pH	4.9 ± 0.2	4.2 ± 0.2
T-organic carbon (g/L)	91.3 ± 2.58	69.7 ± 2.25
T-kjeldahl nitrogen (g/L)	4.3 ± 0.09	5.0 ± 0.01
T-phosphorus (g/L)	0.40 ± 0.002	0.46 ± 0.008
T-CODcr (g/L)	266 ± 14.8	261 ± 5.3
Ethanol (g/L)	0	85.4 ± 2.26

Table 4-2

Main operation conditions and performance of control and EP series.

Series	Control					EP (Ethanol fermentation pretreatment)						
Operation feasibility	Enable	Enable	Enable	Enable	Failure	Enable	Enable	Enable	Enable	Enable	Enable	Enable
Operating condition												
Operating days (d)	0→70	71→98	99→126	127→146	147→162	0→70	71→98	99→116	117→126	126→148	148→175	176→210
OLR (g-COD/L/d)	6.5	7.8	9.8	13.0	19.6	6.5	7.8	9.8	13.0	13.0→32.6	32.6	43.5
HRT (d)	30.0	25.0	20.0	15.0	10.0	30.0	25.0	20.0	15.0	15→6	6.0	6.0
SRT (d)	95	105	57	40	35	315	315	210	180		39	25
Biogas												
Methane ratio (%)	57.3	54.9	54.6	51.8	49.4→47.5	73.9	73.9	72.7	71.3		70.9	70.3
Methane yield (L/g COD)	0.31	0.31	0.29	0.30	0.18	0.33	0.33	0.32	0.31		0.31	0.31
In the reactors												
pH	7.4	7.4	7.3	7.3	7.2→4.2	7.8	7.8	7.8	7.8		7.7	7.8
TS (g/L)	58.4	61.4	69.2	64.7	71.1→83.6	43.9	51	59	68.2		75.7	76.3
VTS (g/L)	51.7	55.5	56.5	59.6	65.8→78.9	35.4	45.6	51.8	56.4		64.5	67.5
TC (g/L)	33.9	30.2	34.5	31.6	35.7→44.6	25.3	26.2	30.3	35.8		38.8	39.8
DOC (g/L)	0.68	0.94	0.56	0.39	0.33→5.65	2.08	1.32	1.34	1.33		1.11	2.07
DIC (g/L)	1.81	1.57	1.95	1.51	0.91→0.05	2.94	3.26	3.95	2.61		2.39	2.93
T-CODcr (g/L)	80.7	86	91.1	90.7	90.0→135	66.8	58.9	77.9	93.2		105	111
D-CODcr (g/L)	2.1	2.6	2.1	1.3	1.0→26	7.4	3.9	4.7	4.6		4.1	6.7
TVFA (mg/L)	48	2200	42	13	13→7700	4400	480	190	150		230	433
Alkalinity (g/L)	7.5	7.8	6.8	5.6	5.5→1.6	10.1	11.2	12.6	12.2		9.7	12
NH_4-N (mg/L)	2150	1750	1980	1716	950→638	3550	3900	3550	3250		2900	3750

Table 4-3

Growth yields of methanogenic archaea growing.

Organism	Substrate	Y_{CH4} (g cells/mol CH_4)	Reference
Methanosarcina barkeri	$H_2 + CO_2$	6.4	[37]
	Methanol	7.2	
	Acetate	2.1	[38]
Methanosarcina sp. strain 227	$H_2 + CO_2$	8.7	[39]
	Methanol	6.0	
	Acetate	2.7	
Methanomassiliicoccus luminyensis	Methanol + H_2	2.4	[35]
	Trimethylamine + H_2	2.4	
Methanobacterium bryantii	$H_2 + CO_2$	2.5	[40]
Methanobacterium marburgensis	$H_2 + CO_2$	3.0	[41]

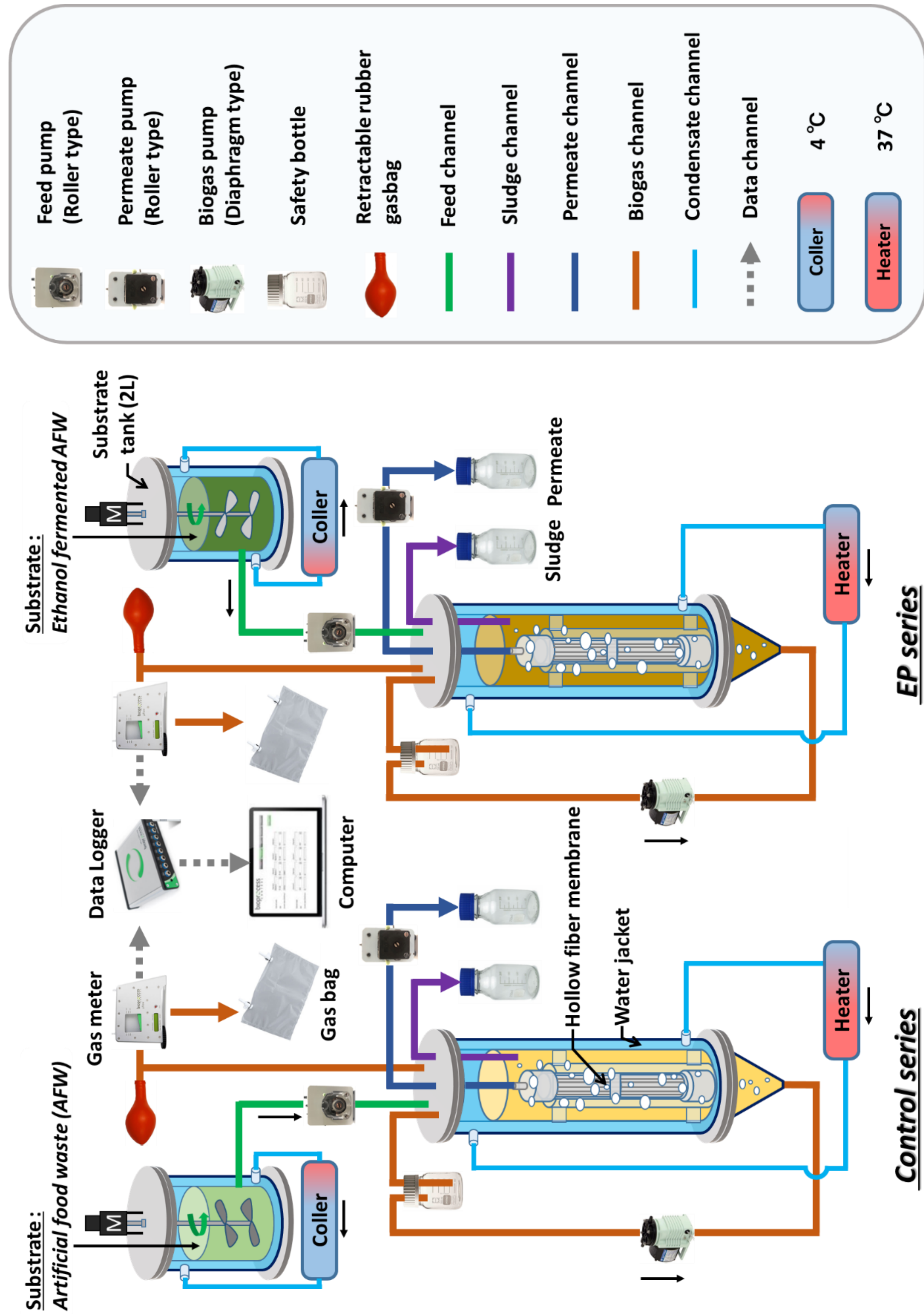

Fig. 4-1. Experimental apparatus, a submerged hollow fiber type AnMBRs. The left side is the control series, the right side is the EP series.

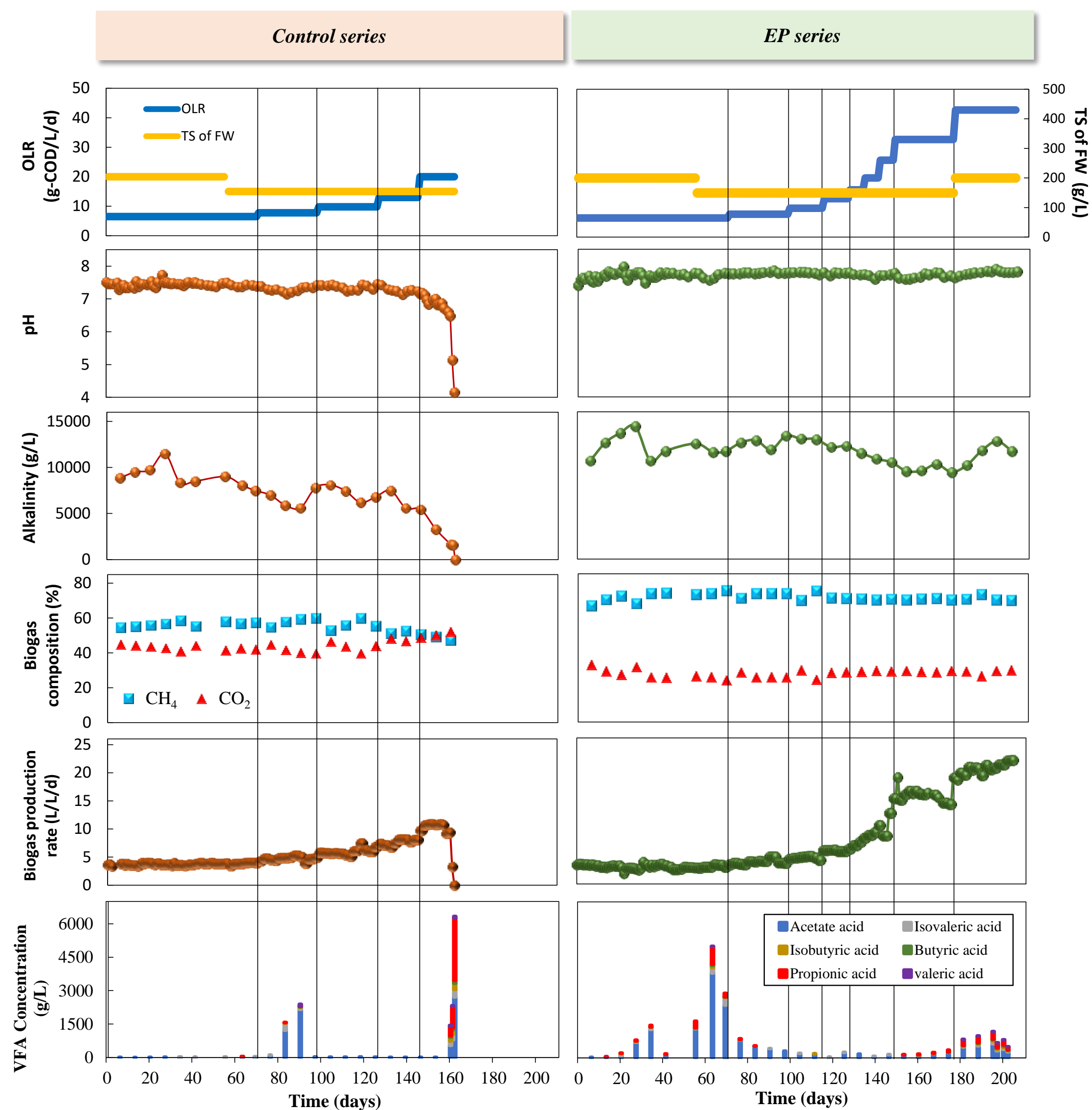

Fig. 4-2. Time course of changes in alkalinity, pH, total volatile fatty acid (TVFA) levels, biogas production rates, and CH_4 content in the anaerobic membrane bioreactor.

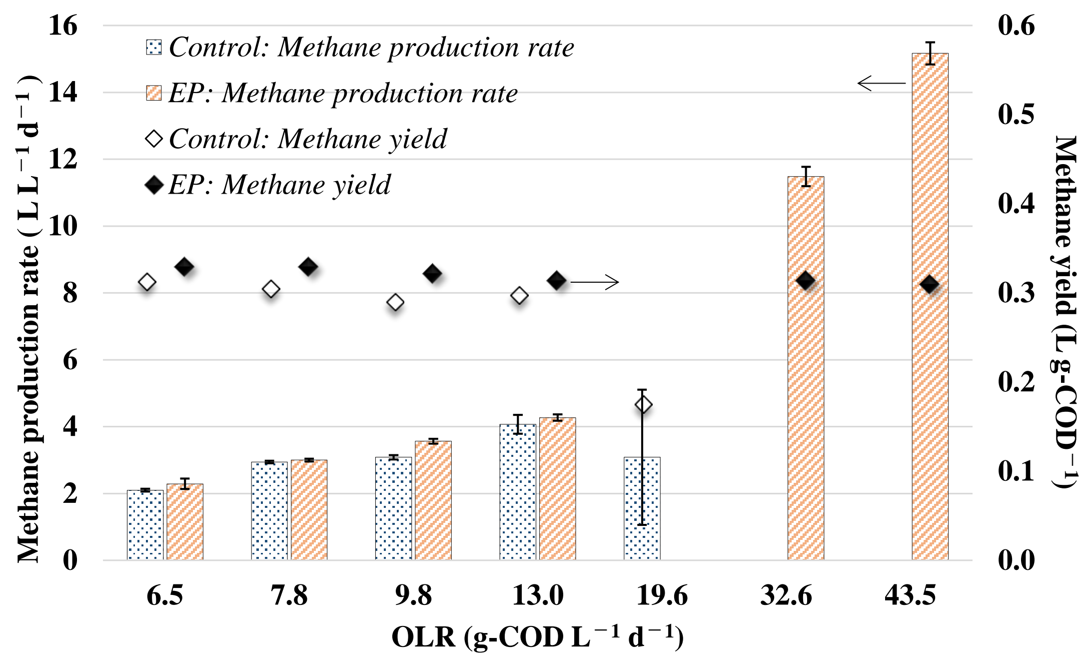

Fig. 4-3. Methane production rate (primary y-axis) and methane yield (secondary y-axis). Pattern fill shows control series and solid fill signifies EP series. The error bars indicate in methane production rate for each OLR are the standard deviations.

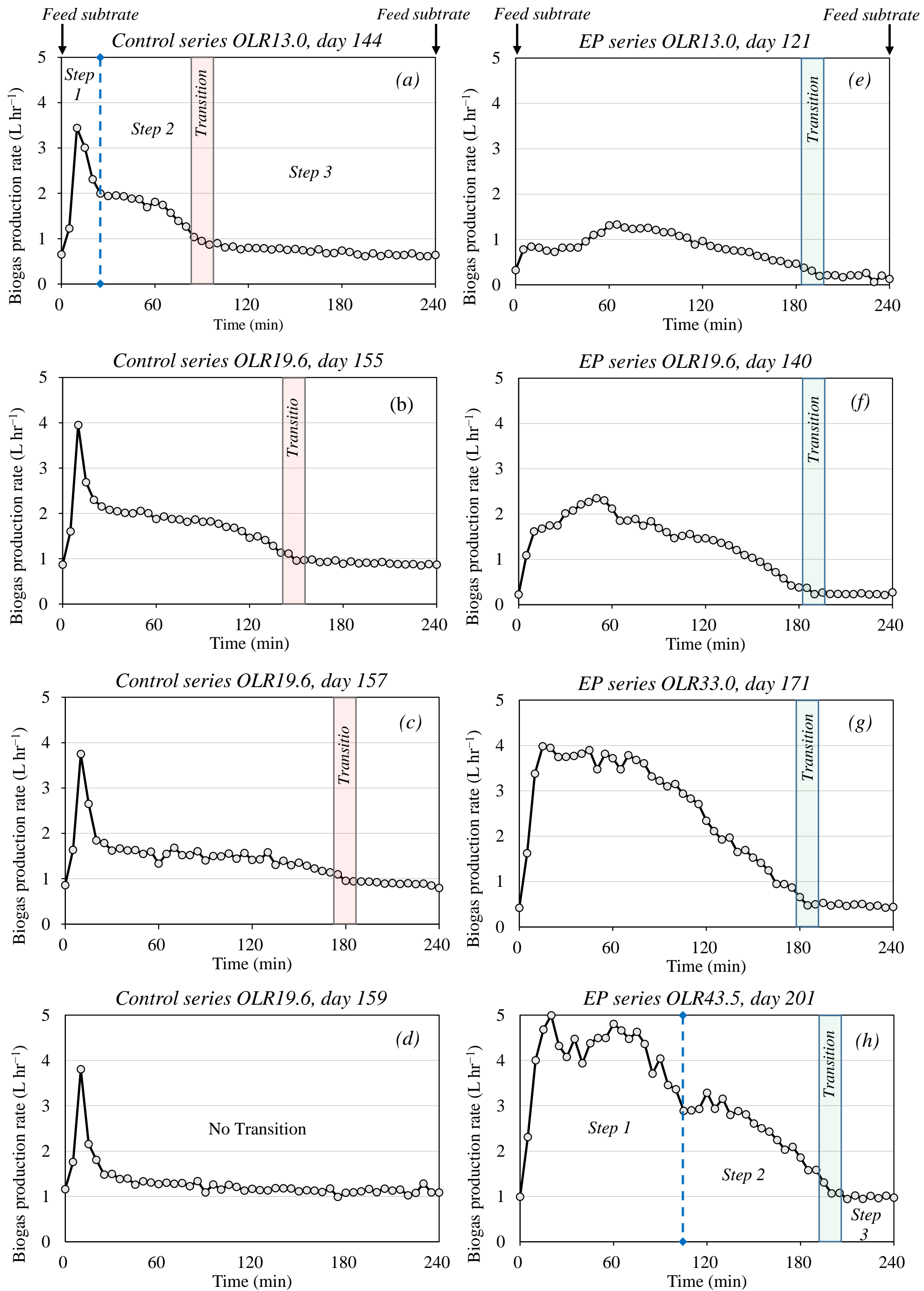

Fig. 4-4. Biogas production rate kinetics acquired at different OLR of the digester operation in experiments.

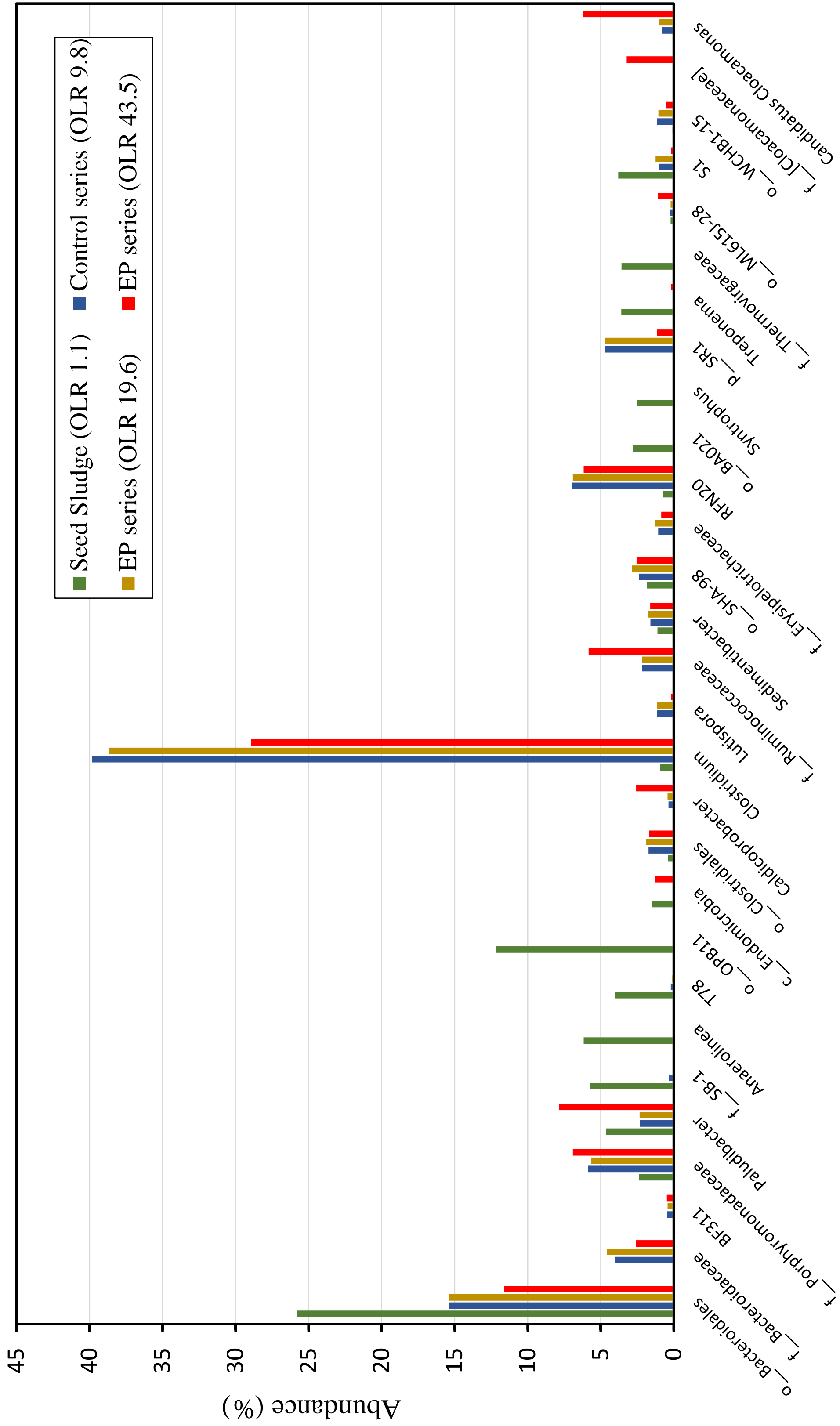

Fig. 4-5. Microbial community composition of methanogenic sludge fed at different series and OLR under the genus level.

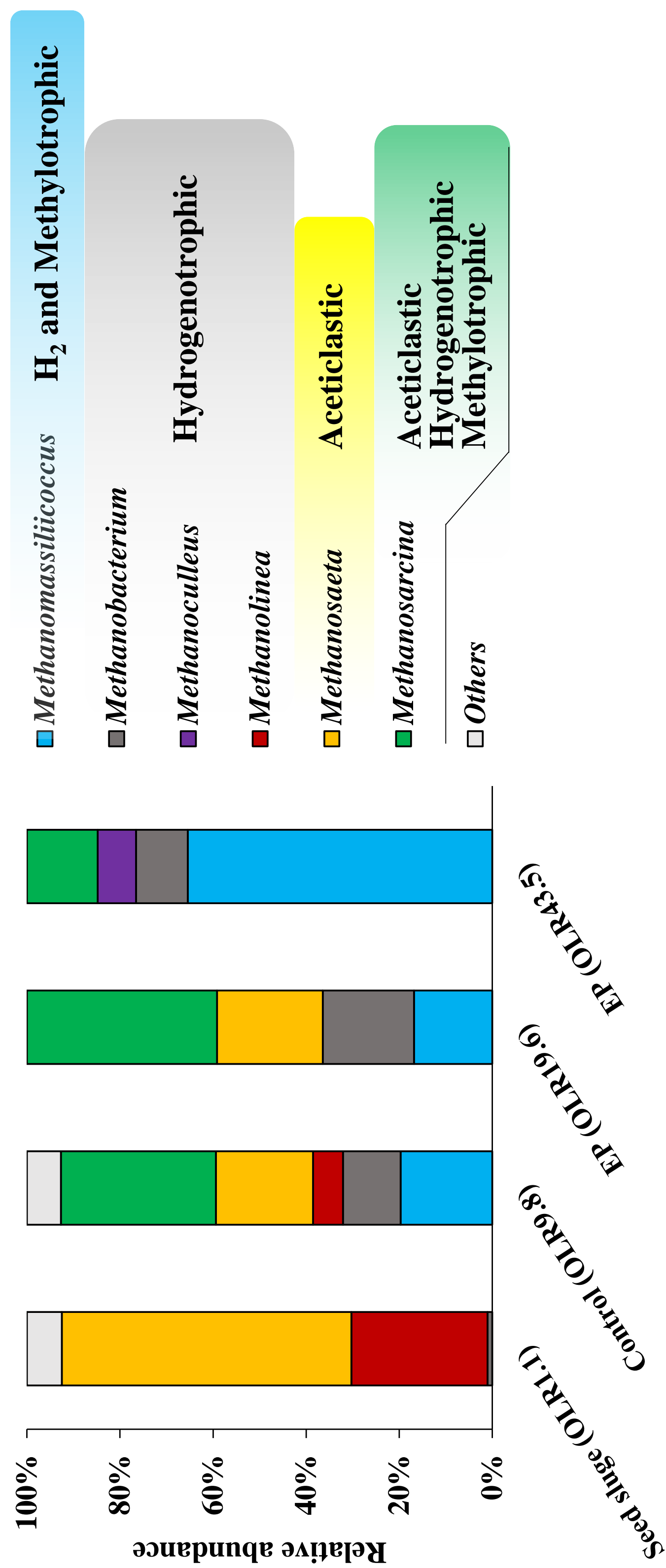

Fig. 4-6. Results of archaea community analysis at different series and OLR under the genus level.

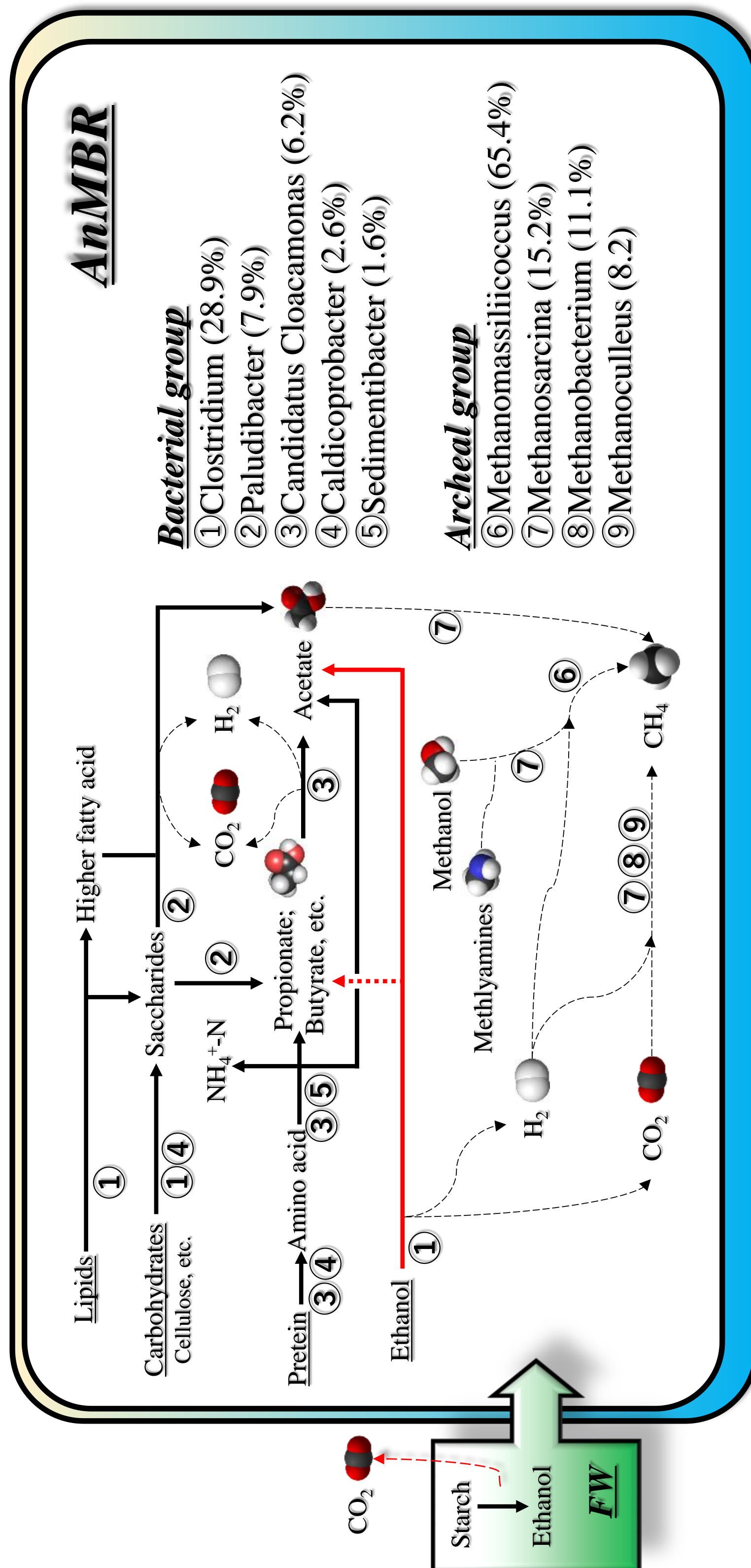

Fig. 4-7. In order to summarize the main metabolic pathways of ethanol pretreated food waste degradation and methanogenesis for high load (OLR 43.5) in the AnMBR, which combined the results of this experiment and previous research.

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Chapter 5. Conclusions and recommendations

In this study, the effectiveness of the ethanol fermentation pretreatment for methanae fermentation was examined. Through previous research and investigation, mastered the basic characteristics of pretreatment of food waste, and gradually improved the experimental methods in this study to achieve the best anaerobic fermentation process of food waste.

In Chapter 2, Ethanol fermentation as a pretreatment process for biomethanation of food waste was conducted. A sequential batch biomethanation experiment was performed for 130 days using AFW that was saccharized and ethanol fermented. A stable state was feasible at least for 56 days of the experiment.

Furthermore, the results described an improvement in the methane content of the biogas, a reduction in sludge generation. The results of the sequential batch experiment followed stoichiometry, and thermodynamics indicated that a biomethanation system that includes pretreatment is a theoretically controllable system. However, due to the slow growth of anaerobic bacteria, it should be considered that solid–liquid separation has to be performed to keep the biomass in the reactor.

In Chapter 3, combining the conclusions and shortcomings of Chapter 2, a new experimental method is tried. The aim of this chapter was operation in the AnMBR system was attempted by using EP for AFW rich in starch.

Ethanol fermented substrate was fed to the AnMBR several times per day. The HRT reduced stepwise from 20 to 5 days to increase the load by raising feeding volume. The

OLR was 6.6 to 26.5 g-COD/L/d. The control series (without pretreatment) was operable to an OLR of up to 8.8 g-COD/L/d, whereas EP series was 26.5 g-COD/L/d. At three times the load of the control series, EP series can still reduce the sludge yield by 27-46%.

By comparing the Gibbs free energy changes for the methane conversion, EP was demonstrated that effective in avoiding accumulation of propionic acid. The methane fermentation process generated a large proportion of acetic acid in VFA generation whereas in the control series it was propionic acid. Therefore, experiments have proved that using AnMBR treatment of EP is feasible, and EP significantly improves AnMBR performance. The use of pretreatment in AnMBR shows that it avoids the serious accumulation of propionic acid and the subsequent decreased pH, and membrane contribute to prevent the runoff of anaerobic microorganisms. The author considered that this method has the potential to operate under higher loads.

In Chapter 4, a higher operating load was at tempted using EP for AFW in an AnMBR system by using a sequential batch experiment, where ethanol fermented substrate was fed to the AnMBR. In the experiments in this chapter, food waste with a moisture content similar to that of actual food waste is used.

The control series (without ethanol fermentation) was operable to an OLR of 13.0 g COD/L/d, while the EP series was operable to an OLR of 43.5 g COD/L/d, 3 times higher than the capability of the control series. In high load operation, EP also proved effective in maintaining a stable methane yield and stable long term operation of the reactor; together, this demonstrates that EP of AFW significantly improves anaerobic digestion performance.

The abundance of varieties of hydrogenotrophic methanogens rate in the AnMBR were

determined to be enhanced using EP for FW through high-throughput sequencing analysis. The large amount of hydrogenotrophic methanogens reduces the partial pressure of hydrogen, thereby promoting the biodegradation of the substrate by bacteria. The hydrogenotrophic methanogens with a high tolerance of ammonium inhibition, and higher ammonium then improves the AD buffer capacity. Therefore, a virtuous cycle was formed in the EP series as an additive that was also responsible for the stable and high operational loading by adoption of ethanol fermentation pretreatment.

The purpose of this study was to explore the potential by adoption of ethanol fermentation pretreatment for methane fermentation of food waste, to obtain "stable high load operation", "high methane concentration", and "high decomposition rate", and to clarify the principle. The results of this study can realize the miniaturization of fermentation reactor. As a kind of practical application proposal, the use of biological anaerobic membrane in this study has achieved the expected goal, providing a reference for further research and engineering applications. Therefore, in order to confirm whether the results of this research can be used in methane fermentation facilities in the future. It will be necessary to verify this with a continuous reactor. However, existing modules are prone to blockage and relatively expensive, which may affect large-scale practical applications. Further work is necessary to improve anaerobic membrane washing and management methods to extend its service life. And explore other methods to maintain the concentration of anaerobic bacteria in the AD reactor.

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