Doctoral Dissertation

Enhanced anaerobic digestion of Korean food waste by bioaugmentation with rumen culture

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Department of Urban and Environmental Engineering
(Environmental Science and Engineering)

Graduate School of UNIST

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Advisor
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ABSTRACT

Anaerobic digestion (AD) is considered a viable method for treating food waste (FW) because it biologically converts organic waste into biogas. However, FW has unique characteristics that complicate its stable AD over the long term, for example, seasonal variations in the production and composition of FW. Korean FW is characterized by a high content of vegetables and fruits (approximately 55%) that are rich in dietary fiber, which can cause difficulties in hydrolysis and, thus, degradation of FW. The amount of FW increases dramatically during the “Kimjang” season that mainly Napa cabbage accounts for approximately 20% of the total FW produced in Korea. This significant seasonal variation in the composition of FW recurs annually, and it could influence the performance and stability for the AD of FW. Nonetheless, few studies have systematically investigated the effect of this variation on the performance of AD and methods to enhance the stability of FW digesters suffering the issue of seasonal variations in FW.

Bioaugmentation is a method for improving the degradation of organic pollutants through the addition of exogenous microorganisms that can degrade the target compounds in situ. Therefore, the selection of appropriate microorganisms that can thrive and retain the desired metabolic properties in a given environment is an important factor in bioaugmentation. Bioaugmentation has been suggested as a promising strategy for enhancing the performance of AD at the microbial community level. Rumen provides a favorable environment for the formation and development of a naturally formed anaerobic microbial community consisting of metabolically versatile microorganisms, and rumen microorganisms are a good source of hydrolytic bacteria capable of decomposing complex matters, including fibers. Additionally, rumen microorganisms contain acidogens and methanogens, and they produce methane as the final product of biodegradation, similar to AD microbial communities. These characteristics make it feasible to use rumen microorganisms as an exogenous microbial source for bioaugmentation of AD processes. In this doctoral research, the bioaugmentation potential of rumen culture for enhancing the biomethanation of Korean FW was examined, with emphases on increase in substrate digestibility and long-term stability of the bioaugmented process.

In study 1, the potential of rumen fluid (RF) as a bioaugmentation source was first examined in batch tests. RF and two cellulolytic *Clostridium* species were tested in different combinations and various seeding ratios to determine the optimal bioaugmentation source and ratio by using simulated Korean FW. Then, a continuous experiment employing the optimal bioaugmentation condition determined in the batch test (10% RF to the inoculated anaerobic sludge on a volatile suspended solids (VSS) basis) was performed using the same substrate. The experimental results indicated that bioaugmentation with RF effectively enhanced the biomethanation of FW in both batch and continuous modes. The microbial community structures, especially bacterial community structures, shifted significantly after the introduction of RF. Therefore, it was found to be possible to alter the composition and function of microbial communities and, thus, to enhance the

biomethanation of FW through bioaugmentation with RF.

In study 2, for comparison, the aforementioned bioaugmentation strategy was applied to single- and two-phase processes for treating real Korean FW. In the two-phase process, the amount of RF to be added to the acidogenic reactor was determined based on the VSS concentration in the reactor (i.e., smaller amount of RF compared to that used in the single-phase process) to test the possibility of reducing the consumption of RF, which is a relatively scarce resource. Both processes were operated at varying organic loading rates (OLRs, 0.5–6.0 g volatile solids (VS)/L·d) without pH control. Both processes showed comparable methanogenic performances at OLRs ≤4.0 g VS/L·d, and the acidogenic reactor maintained stable production of volatile fatty acids (mainly lactate) and ethanol, despite the highly acidic pH ≤3.4. However, the single-phase process achieved stable AD performance with an increased OLR 5.0 g VS/L·d, whereas the two-phase process failed. These results can be ascribed to the provision of a more favorable environment for syntrophic interactions between acidogens and methanogens and the addition of more amount of RF in the single-phase process. Consequently, the single-phase configuration was selected for the subsequent long-term experiment because achieving stable and robust performance is important for AD plants.

Study 3 focuses on the feasibility of bioaugmentation with rumen culture (RC) as a strategy to enhance the biomethanation of FW in batch and long-term continuous experiments. Batch tests were conducted for three inocula (i.e., anaerobic sludge with FW, RC-inoculated RF with FW, mixed culture of anaerobic sludge and RC with FW) with Napa cabbage (i.e., simulated kimjang waste (KW)) and cellulose. The results of the three subculture cycles indicated that the mixed-culture inoculum provided a higher biogas yield than the other inocula, indicating that bioaugmentation with RC has the potential for enhancing the biomethanation of fiber-rich FW. Then, bioaugmentation with RC was examined in the continuous experiment with fluctuations by adding KW into FW (0–20% of the total substrate VS). The results demonstrated that bioaugmentation with RC effectively increased the biomethanation of FW (by 12.3% increase in methane yield compared to the control without bioaugmentation), especially after the addition of KW. Changes to the microbial community structure corresponding to bioaugmentation and adaptation to fluctuations in substrate composition, such as the emergence of hydrolytic/acidogenic bacteria originating from the RC and the dominant shift to hydrogenotrophic methanogenesis, were observed. Importantly, the bioaugmented microbial populations seemingly remained active and helped sustain the enhanced AD performance in the long-term experiment (>38 months). Therefore, the proposed bioaugmentation strategy proved to be effective for improving the robustness and resilience of an FW digester in terms of handling seasonal fluctuations in FW composition and loading.

In conclusion, this study verified that bioaugmentation with RC is a practical tool for enhancing the AD of Korean FW in terms of energy production and process stability. Moreover, long-term effectiveness of the bioaugmentation strategy was demonstrated in the continuous mode with varying fractions of KW (i.e.,

simulated seasonal variations). The findings of this study will be useful for managing AD plants that treat FW and significantly add to the literature on this topic.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Background

1.1. Challenges faced in food waste management

The quantity of food waste (FW) generated is increasing worldwide owing to population and economic growth, and FW has become a major sustainability issue in most countries. Annual FW production in Korea is approximately 5.2 million tons, and it accounts for more than 22% of the total municipal solid waste production [1]. Daily FW production has been increasing since 2014, probably because of increases in the use of delivery services and number of single-person household (Fig. 1-1). FW is rich in readily biodegradable organic compounds and causes serious environmental pollution if not treated properly. The estimated cost of managing the FW generated in Korea is 800 billion won per year. Direct landfilling and ocean dumping of FW and other organic wastes, which were the major disposal routes, have recently been banned in Korea. Consequently, approximately 85% of FW has been recycled as fertilizer or animal feed, but even this practice has proved to be socially problematic. Therefore, alternative methods for proper land-based FW management are required urgently.

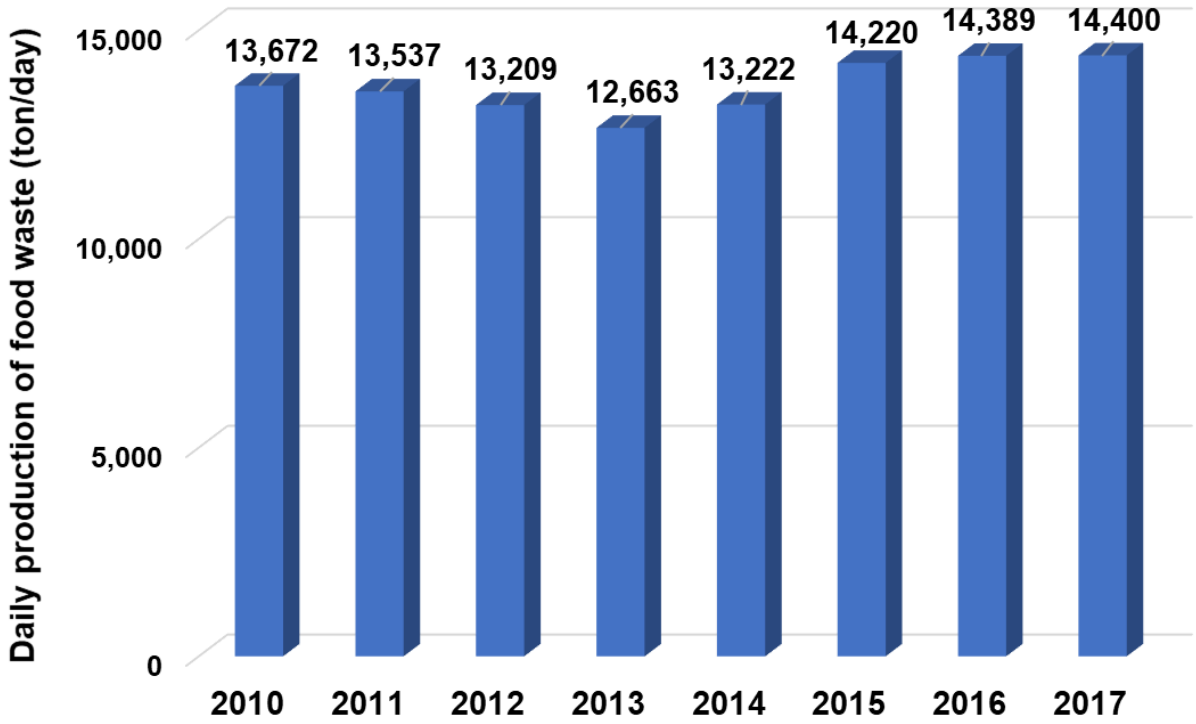


Figure 1-1. Korean FW generation (Ministry of Environment).

Anaerobic digestion (AD) is considered a viable method for treating organic wastes, including FW, because it biologically converts organic waste into methane-rich biogas through four pathways; hydrolysis,

acidogenesis, acetogenesis, and methanogenesis (Fig. 1-2). Methane gas produced using an AD process can be considered a renewable energy source. FW, characterized by high organic content, is a good feedstock for methane production through AD. Although AD has been used for many years to manage various high-strength organic wastes, including FW, several technical and economic challenges associated with the use of AD have not been overcome thus far. An important technical challenge is process control for stabilization and optimization to enhance process efficiency.

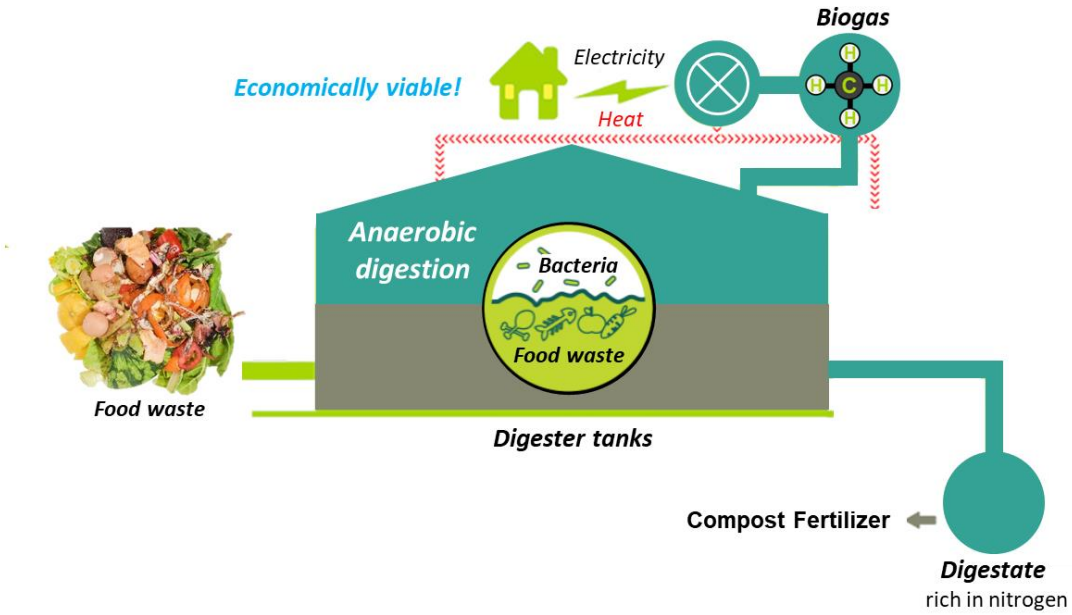


Figure 1-2. AD process.

In AD, biodegradability depends on substrate composition. As a substrate, Korean FW exhibits dynamism in terms of its composition of carbohydrates, lipids, and proteins owing to the diverse sources of FW, such as houses, schools, restaurants, and hospital cafeterias. The composition of FW fluctuates daily or seasonally owing to daily or seasonal changes in food ingredients [2]. Accordingly, the potential methane yield of Korean FW ranges from 0.356 to 0.471 L/g VS_{fed} [3]. From the process control viewpoint, these fluctuations make it difficult to maintain stable process performance in terms of biogas production and biodegradation in AD. Moreover, changes in FW composition can cause the instability in the AD process. Several parameters affect the stability of AD. For instance, the stability of AD is adversely affected by the rapid conversion of easily biodegradable content such as sugars to volatile fatty acids (VFAs) (>1,500 mg/L), which reduces system pH (<6.0) with low buffer capacity (ratio of intermediate alkalinity to partial alkalinity higher than 0.3) and the ratio of total VFAs to total alkalinity ratio (>0.35) [4, 5]. Another example is that high protein

contents in FW generate ammonia and hydrogen sulfide during AD, and high levels of these substances (>150 mg NH_3-N/L and >2% H_2S) inhibit AD, especially methanogenesis. Furthermore, lipid contents in FW either generate long-chain fatty acids or cause foaming in AD. Common long-chain fatty acids such as oleic and stearic acid inhibit methane production in AD when their concentrations exceed 1.0 g/L [6]. Therefore, process stability based on various instability parameters should be considered to achieve stable process performance over the long term. The process instability results obtained herein indicate that process recovery is time consuming and expensive. Therefore, resilience and stability of the AD process for treating FW should be considered in the light of fluctuations in composition.

Another concern pertaining to the AD of FW is the rate-limiting effect of slow hydrolysis of suspended organic materials, which potentially affects process efficiency [7]. Specifically, Korean FW is characterized by high contents of vegetables and fruits (approximately 55%) [8], which increase to even higher levels during “Kimjang” season owing to the preparation of large amounts of kimchi (a traditional Korean fermented vegetable product) in late autumn (Fig. 1-3). A large quantity of vegetable waste is produced during this season, and Napa cabbage, the most common ingredient in kimchi, accounts for more than 20% of the total FW produced in Korea. Napa cabbage contains a high content of dietary fibers, including cellulose, lignin, and non-cellulosic polysaccharides (approximately 26–32% in dry weight and 1–2% in wet weight) [9, 10]. Complex fibers are not easily degraded, and therefore, hydrolysis of such fibers may affect methanogenesis, which, commonly, is the rate-limiting step in AD, and consequently, reduce the overall efficiency of AD. In particular, approximately 10% of Korean FW is accounted for by complex fibers, which can potentially increase biomethanation by 10%. Therefore, efficient hydrolysis of complex organics is the key for optimizing the biomethanation of Korean FW.

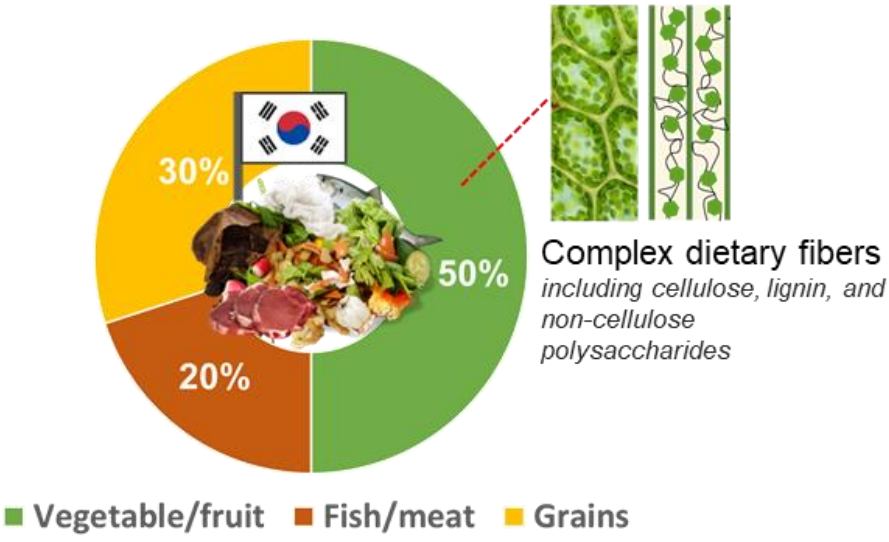


Figure 1-3. Composition of Korean FW [11].

Most studies thus far have focused on the process factors of hydraulic retention time (HRT), organic loading rate (OLR), pH, temperature, and nutrient concentration for improving AD performance. Additionally, studies have attempted pretreatment of FW by using biological, chemical, thermal, mechanical, and ultrasonic methods or to change reactor configurations, such as single- and two-phase reactor, upflow anaerobic reactor, and hybrid reactor, to enhance higher performance [12]. Although these approaches have been applied to the AD process, the understanding of the AD process remains limited to some extent. AD is a biologically complex process involving diverse microbial communities that interact mutually for fermenting organic waste. However, the understanding of the functions of and relationships among microbial communities in AD is limited despite the various efforts to improve the performance of AD. Therefore, the adoption of a biological approach to enhance AD performance is essential for understanding and controlling the AD process.

1.2. Bioaugmentation for enhancement of AD

Many attempts have been made to enhance the hydrolysis rate of FW by using different methods, for example, thermal/thermochemical [13, 14], microaeration [15], microwave [16], and enzymatic [17] pretreatments. Nevertheless, the application of these methods has been limited because they incur additional costs and generate byproducts such as furfural [18]. Compared to physical and chemical pretreatment methods, bioaugmentation is a biological approach to improve AD. This approach involves inducing shifts in the structures and interactions of microbial communities without byproduct inhibition and additional cost. Bioaugmentation, which is the addition of exogenous microorganisms to a bioprocess, is considered a promising strategy for improving the overall process performance at the fundamental level [19] (Fig. 1-4). An important factor in bioaugmentation is the selection of appropriate microorganisms that can survive and retain the requisite properties in a given environment [20]. Despite the tedious enrichment and acclimation steps of the microorganisms to be introduced, several studies have reported successful application of bioaugmentation to the biomethanation of different organic wastes [21-24]. The majority of these studies have attempted to introduce one or a few selected species of microorganisms with specific functional characteristics. However, such an approach may be weak in terms of functional stability and robustness, especially in complex mixed-culture ecosystems [20] because the selected species were mostly grown in pure culture systems and may find it difficult to adapt to new environmental conditions and the associated complex indigenous communities of microbes. This potential limitation can be mitigated by using a complex consortium comprising diverse microbial populations with similar functions (i.e., functional redundancy) in pre-adapted condition or in symbiotic relationships. This approach is less detrimental to the survival or growth of augmented microorganisms. Although bioaugmentation can change the indigenous microbial community through competition or symbiotic relationships, it is difficult to predict whether such changes

would be positive or negative. Even when bioaugmentation improved performance significantly, according to a few studies, the effects were shortly maintained [25]. In addition, it is difficult to replicate the same method under different conditions and to evaluate quantitatively the results of process parameters and microbial community changes. Therefore, for the application of bioaugmentation, it is necessary to understand the anaerobic microbiome and evaluate the process parameters quantitatively. To understand AD at the fundamental level, microbial techniques have been developed and applied to study microbial community structures. These techniques help obtain a deeper understanding of the enormity of microbial community structures at the molecular level. Consequently, microbial community structure analysis and process analysis to determine the effect of bioaugmentation can help us to devise a strategy for enhancing AD performance and understanding AD with bioaugmentation at the fundamental level.

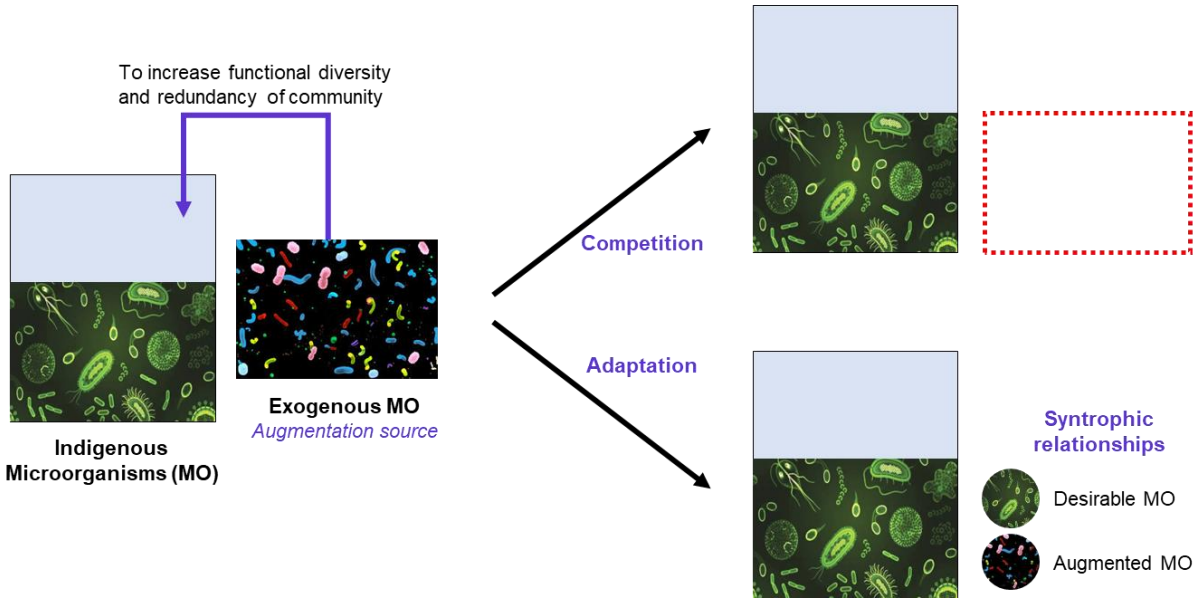


Figure 1-4. Bioaugmentation process.

Rumen microorganisms constitute a stable, naturally formed anaerobic culture that is reported to be versatile in hydrolyzing a wide range of organic compounds, including refractory fibers with complex structures [26]. Rumen microorganisms involve different anaerobic microbial groups, for example, bacteria, archaea, protozoa, and fungi, that interact with each other (Fig. 1-5). In rumen, hydrolytic/acidogenic bacteria initially decompose macromolecules into simple molecules, such as organic acids, H_2, and CO_2. Rumen archaea are mostly hydrogenotrophic methanogens that consume H_2 to generate methane and, hence, to maintain a low hydrogen level in the rumen [27]. Therefore, rumen microflora can be potentially mimicked in the AD of organic wastes such as FW. In particular, rumen bacteria, which have mainly been identified as *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens*, specialize in the degradation of fibrous matter. Because of this ability of rumen bacteria, rumen

fluid (RF) collected from rumen has been introduced to pretreat the substrate or inoculate the culture for enhanced biomethanation of different organic wastes, including FW [28-30]. Studies have compared the biodegradability achieved by using RF and anaerobic sludge or mesophilic acidogens. In [31], it was reported that when fermenting rice straw (RS), VFA production with RF was higher than that with anaerobic sludge inoculum. Particularly, [32] reported that VFA production was higher (71.2%) when using RF than that (59.8%) when using mesophilic acidogens to degrade Korean FW. These results indicate the good potential of RF as an exogenous microbial source for bioaugmentation of AD processes. Moreover, rumen archaea, mainly methanogens, form syntrophic relationships with rumen bacteria. During the fermentation of organic wastes, H_2 is produced by many bacteria through hydrolysis and acidogenesis. If H_2 is accumulated, it can affect the fermentation rate and efficiency by introducing imbalance in reaction equivalents. However, hydrogenotrophic methanogens in rumen archaea can consume H_2 through syntrophic H_2 transfer reactions. Syntrophic H_2 transfer is considered a crucial microbial interaction in anaerobic digesters. Acetate and H_2 are produced as the major sources of methanogenesis through the conversion of longer-chain fatty acids, such as propionate and butyrate, by means of syntrophic interaction. Therefore, if syntrophic relationships are inhibited, the process can become unstable. Thus, the symbiotic microorganisms in RF may potentially be beneficial as a bioaugmentation source for maintaining or recovering process stability.

From the viewpoint of bioaugmentation of large-scale AD processes, however, RF is a scarce resource because of the small amounts of cow rumen available compared to the large quantities of anaerobic sludges in field plants. In addition, no study has investigated RF-based bioaugmentation in single AD systems treating FW and rumen inoculum systems. Thus, to examine the possibility of RF-based bioaugmentation, various tests should be conducted to verify possibility and repeatability with the same source. In this light, a rumen inoculum system is needed for conducting experiments at the laboratory scale and for application at the field scale. In this study, to enhance the efficiency and stability of AD, bioaugmentation with both RF and rumen inoculum system as bioaugmentation sources is examined.

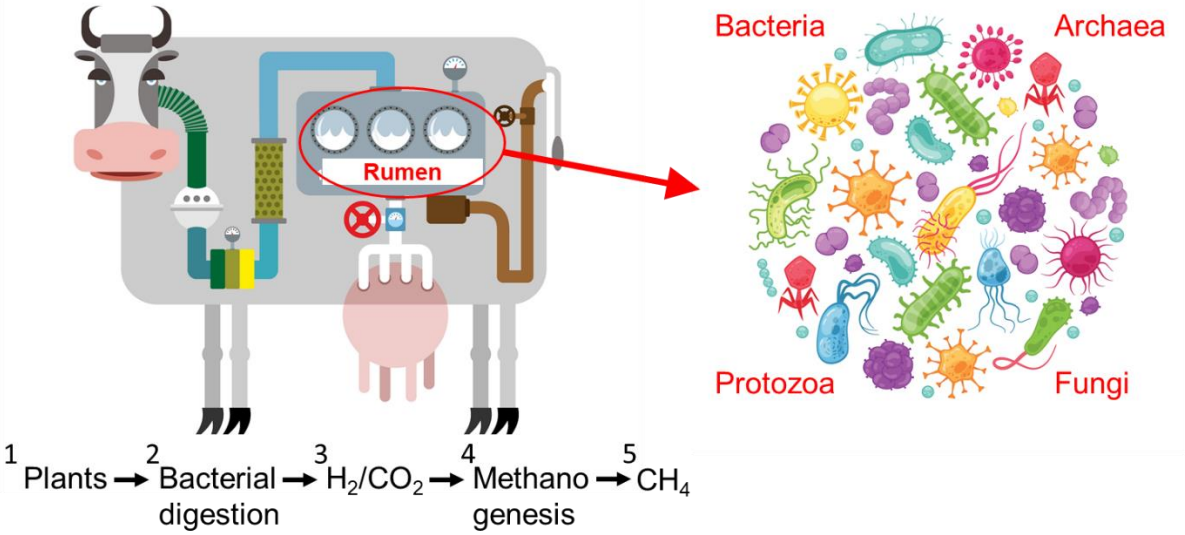


Figure 1-5. Rumen microorganisms.

1.3. Rumen microorganisms

Rumen microorganisms include bacteria, archaea, protozoa, and fungi. Bacteria account for more than half of the digestive action of rumen on plants, and they are classified based on their production and consumption of fiber, starch, and lactate, as well as their ability to digest sugars (http://www.milkproduction.com/Library/Scientific-articles/Animal-health/Rumen-Microbiology/). The major species of fiber-degrading bacteria in rumen are *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens* [33]. Fiber-degrading bacteria in rumen are very sensitive to acid. In environments with pH lower than 6.0, these bacteria do not function well. Starch- and sugar-degrading bacteria such as *Streptococcus bovis*, *Bacteroides ruminicola*, and *Selenomonas ruminantium* account for a significant part of the bacterial population in rumen. Because most cows are fed diets containing more than 30% starch and sugar, these bacteria significantly play a role in degrading starch and sugar. In addition, these bacteria can generate various fermentation products (acetate, propionate, butyrate, lactate, and H_2) under more acidic conditions (pH <5.7). Especially, in environments with pH lower than 5.4, the proportion of lactic acid bacteria increases, and they mainly produce lactate (https://www.daf.qld.gov.au/business-priorities/agriculture/animals/dairy/nutrition-lactating-cows/healthy-rumen-function). Rumen archaea such as hydrogenotrophic methanogen or aceticlastic methanogen (*Methanobacterium ruminantium* or *Methanosarcina*) can utilize H_2 or any accumulated acidic products.

Lignocellulosic biomass, including cellulose, hemicellulose, and lignin, is biologically difficult to degrade through AD, which can reduce the efficiency of AD. RF obtained from the stomachs of cows has specialized

abilities to degrade plants, including lignocellulosic biomass. Thus, it has been employed to pretreat various lignocellulosic biomasses, such as agricultural residues, municipal solid wastes, aquatic plants, and FW (Table 1-1). High rates of cellulose to VFA conversion have been achieved by using RF in batch or sequenced-batch reactor (SBR) tests [34-36]. In [35], a higher rate of VFA production was achieved with RF 100% than that with RF 20% as the pH decreased from 6.92 to 5.46. In [31], anaerobic fermentation of RS with RF at pH 7 was studied. A higher VFA yield of 0.36 g/g VS was achieved compared to the 0.13–0.29 g/g VS yield achieved using anaerobic sludge (AS) as the inoculum. Korean FW contains approximately 50% of cellulosic materials (i.e., vegetables), and the hydrolysis and acidification of Korean FW with RF and mesophilic acidogens (MA) has been tested using a leaching bed (LB) at pH >6.5 [32]. Acidogenesis with VFA production was higher at 71.2% when using RF than the 59.8% when using MA. The production of acidified VFA products increased as the HRT was prolonged from 0.25 d to 1 d. Different RF ratios of 0–10% were tested for treating municipal solid waste by using a LB [37]. The results indicated that large quantities of acidic products were obtained when using 5% and 10% RF. However, the quantities of acidic products obtained using 5% and 10% RF were similar; thus, 5% RF was considered the optimum ratio. The optimum pH range of rumen microorganisms was reported to be 6.0–7.0 for cellulose or protein degradation [32]. This is similar to the bovine rumen of real cows, the pH of which varies substantially from 5.8 to 6.9 according to feeding cycles [38]. In this study, rumen pH and VFA concentration exhibited opposite tendencies. After feeding, the minimum pH was obtained when the VFA concentration was the maximum over a 6-h period. Subsequently, the pH increased as the VFA concentration decreased owing to methanogenesis. Therefore, the pH condition, RF ratio, and HRT condition are important factors for increasing the acidification of lignocellulosic biomass.

Table 1-1. Pretreatments related to hydrolysis and acidogenesis of various substrates by using RF, as described in the literature.

Reactor	Feed	Inoculum	Conditions	Results	References
LB	FW	RF and MA	HRT of 0.25–1 d pH >6.0	High quantity of VFAs with HRT of 1 day, Acidogenesis of 71.2% (RF) and 59.8% (MA)	[32]
LB	MSW	RF 0–10%	pH 4.2–5.9	High quantities of acidic products in 5% RF	[37]
Batch	RS	RF	pH 7, 39°C, 120 h	VFAs yield of 0.36 g/g VS (0.13–0.29 g/g VS in AS)	[31]
Batch	CS	RF	40°C	VFAs yield of 0.59–0.71 g/g VS	[29]
Batch	Cellulose	RF	pH 4.8–7.3, 40°C	Optimum pH values of 6.8 and 7.3	[34]
Batch	Cellulose	RF 100, 20%	35°C	VFA concentrations of 15,000 ppm (100%) and 2,000 ppm (20%)	[35]
SBR	Cellulose	RF	pH >6.25, 39°C, HRT of 0.67 d	50% COD conversion	[36]
Cow	Mixed feed	RF	12-h feed cycle	VFA concentration of 130 mM, minimum pH 5.8	[38]

*Leaching bed = LB; Sequencing batch reactor = SBR; Cow = real cow; Food waste = FW; Municipal solid waste = MSW; Rice straw = RS; Corn stover = CS; Mixed feed = corn silage, alfalfa haylage, dry shell corn, whole cottonseed, soybean meal, dried distiller grains, roasted soybeans, blood meal; Rumen fluid = RF; Mesophilic acidogens = MA; Hydraulic retention time = HRT;

Rumen microorganisms mainly include cellulolytic bacteria, as well as hydrogenotrophic methanogens and aceticlastic methanogens. These methanogens can produce methane gas by utilizing hydrogen or acetic acid resulting from the degradation of lignocellulosic biomass. In [38], it was demonstrated that in the process of rumen digestion of lignocellulosic biomass, methanogenesis can increase the pH and utilize high VFA concentrations. Therefore, rumen microorganisms can potentially serve as the inoculum source for increasing methane production in the AD of lignocellulosic biomass. However, this aspect has rarely been studied for various types of lignocellulosic biomass, for example, cellulose, plant waste, municipal solid waste, and FW (Table 1-2). The effect of inoculum type (AS and RF) and their mixture ratio (AS:RF = 100:0 and 75:25, respectively) were evaluated in terms of methane potential when treating fresh and pretreated crop materials (reed, silage, cucumber, and tomato) in batch tests [39]. Although the methane yields obtained

by using AS and mixture inoculum (AS:RF = 75:25) to treat fresh crop materials (0.22−0.36 L/g VS_{fed}) were similar, the mixture inoculum test improved the methane yields of pretreated silage (by 16%) and reed (by 40%). These results indicated that the solubilization of lignin and hemicellulose through pretreatment could improve the hydrolysis of cellulose by bacteria and, thus, facilitate easier conversion of cellulose to methane gas. To evaluate the degradability of lignocellulosic biomass by using a digester sludge and RF, the AD of aquatic plants (bulrush) and cellulose was studied [40]. The results indicated that methane production in the inoculated DS was higher than that in the inoculated RF. However, the maximum VFA formation rate in RF was higher than that in DS. The differences in product distribution suggest that the stronger hydrolysis activity in RF and stronger methanogenic activity in DS could be employed in a two-stage biological process. A two-stage reactor consisting of an acidogenic phase and methanogenic phase can be expected to enhance overall AD performance by providing an ideal biphasic ecosystem. Owing to such advantages, RF as an acidogenic inoculum has been applied to treat organic wastes, including FW and grass with using AS as a methanogenesis inoculum [41, 42]. Unlike most studies, which have focused on the pretreatment of energy crops and lignocellulosic biomass, in [41], the authors attempted to assess the treatment of FW by using RF in a two-stage process. Rapid acidification with VFA accumulation at pH >6.0 was achieved in an LB by using RF. Consequently, COD removal of 98.6% and CH_4 yield of 0.27 L/g VS_{fed} were obtained in the methanogenic phase of an upflow anaerobic sludge blanket (UASB). Grass fermentation was researched in a two-stage hydrolytic reactor with RF and in a methanogenic reactor with AS [42]. In this study, the pH in the hydrolytic reactor was not controlled, and the effluent pH decreased to 4–4.5. Although this pH level was not optimum for rumen microorganisms, methane production increased owing to enhanced VFA production in the hydrolytic reactor. This finding suggests that a low pH condition along with RF can potentially facilitate substrate acidification. The effect of the RF inoculum ratio on the treatment of municipal solid waste was studied in a continuously stirred tank reactor (CSTR) [43]. RF inoculum ratios of 10% and 15% gave higher biogas yields of 0.51 and 0.55 L/g VS_{fed} compared to 0% and 5%. Co-digestion of cattle manure and organic kitchen waste by using RF inoculum was studied to increase biogas production [44]. It was reported that co-digestion could increase the biogas yields by 24–47% over the control with a single substrate (cattle manure or organic kitchen waste). This indicated that co-digestion could improve the biogas production and RF as the inoculum could play a role in the AD of organic wastes.

Table 1-2. AD of various substrates using RF, as reported in the literature.

Reactor	Feed	Inoculum	Conditions	Results	References
Batch	Reed, silage, cucumber, tomato	AS 100%, 75% RF 0%, 25%	35°C	CH_4 yield with RF 25% increase by 16% for silage and by 40% for reed	[39]
Batch	Cellulose, bulrush	DS 100% RF 100%	35°C and 40°C	Product yields of 362 mg COD/g (RF) and 464 mg COD/g (DS)	[40]
CSTR	MSW	RF 0, 5, 10, 15%	30°C	Biogas yields 0.26, 0.23, 0.51, and 0.55 L/g VS	[43]
Batch	Co-digestion of CM and OKW	RF	30°C	Co-digestion biogas yield increased by 24–47%	[44]
MUSTAC	FW	RF 5% (LB), AS (UASB)	37°C pH >6.5 HRT 1.5 d	CH_4 yield of 0.27 L/g VS and COD removal of 98.6%	[41]
Two-stage CSTR	Grass	RF (first), AS (second)	HRT 2 d	Acidogenic phase (low pH 4–4.5)	[42]

*Continuous stirred tank reactor = CSTR; Multi-sequential batch two-phase anaerobic composting with leaching bed (LB) and upflow anaerobic sludge blanket (UASB) = MUSTAC; Municipal solid waste = MSW; Cattle manure = CM; Organic kitchen waste = OKW; Food waste = FW; Anaerobic sludge = AS; Digester sludge = DS;

1.4. Objectives

The present study aims to verify the potential of rumen culture for the bioaugmentation of biomethanation (efficiency), stability, and resilience of AD of Korean FW (Fig. 1-6). The novelty of this study is the bioaugmentation of AD of Korean FW by using rumen culture. To assess the potential of rumen culture in terms of the bioaugmentation of AD of FW, the following studies were conducted:

Study 1: The potential of RF as a bioaugmentation source was examined in batch tests and continuous tests.

Study 2: To select the optimal configuration for the bioaugmentation strategy, the single- and two-phase processes for treating Korean FW were compared.

Study 3: The feasibility of bioaugmentation with rumen culture obtained from an RF culturing system as a strategy to enhance the biomethanation of FW was determined in batch and long-term continuous experiments. In the continuous tests, it was examined the effect of bioaugmentation on the stability and

sustainability of the AD of Korean FW with consideration of fluctuations in the composition of Korean FW due to KW.

In further studies, for the total treatment of FW, AD effluent will be tested with single-stage partial nitritation and anammox processes with poly(vinyl alcohol) cryogel by using an up-flow dual-bed moving-bed reactor.

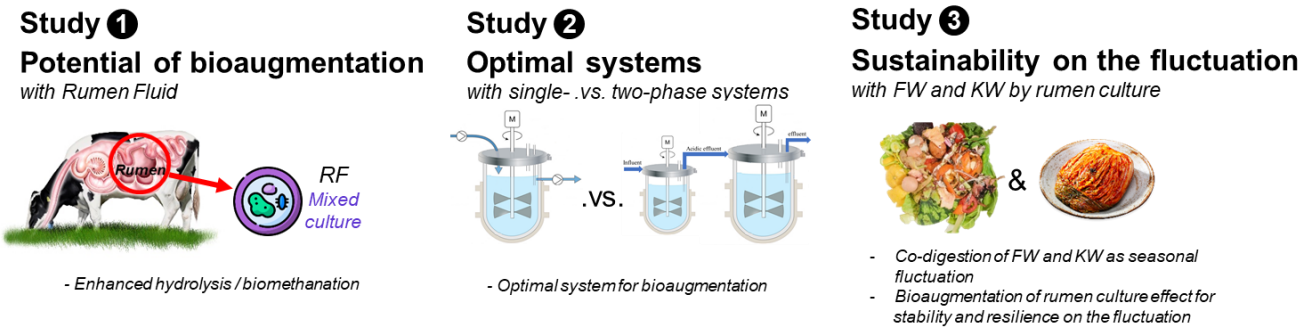


Figure 1-6. Research scheme.

2. [Study 1] Potential of bioaugmentation to improve AD of FW

2.1. Introduction

Korean FW has been gradually increased since 2014 which causes serious environmental pollution. Biomethanation through AD has been gaining increasing attention as a viable means to treat FW owing to its ability to covert organic pollutants into energy-rich biogas. Although AD has long been practiced to deal with various high-strength organic wastes including FW, there still is ample room for improvement in terms of conversion efficiency and reaction rate. In particular, Korean FW composed to large amount of vegetables and fruit wastes which contain higher complex fibers such as cellulose, lignocellulose, and lignin. Fibrous matters are not easily biodegradable which cause slow hydrolysis in AD and consequently reduce the process efficiency in terms of organic removal and biogas production. Therefore, it is essential to improve the degradation of fibrous matters in Korean FW for enhancing AD of FW. Although many studies have been conducted to improve the efficiency in AD of Korean FW, they have mostly been limited to simple physical and chemical process [14, 15] and monitoring process factors or approaches based on the changes of operation conditions i.e., HRT, OLR as the trial-and-error [45, 46].

Bioaugmentation is one of the viable methods to improve the degradation of complex organic matters or to recover AD performance after perturbation by adding exogenous microorganisms with potential to degrade the target compounds in situ [47]. It is biological approach at the level of microbial community in

order to improve AD performance. In bioaugmentation, it is important factor to select proper microorganisms able to survive and retain the requisite properties in the given environment. Most previous studies have been conducted to bacterial-based bioaugmentation for enhancing the hydrolysis [21-24]. On the other hands, archaeal-based bioaugmentation has been limited to bioaugmentation of ammonia tolerant methanogens [48]. Particularly, the majority of previous studies have been conducted to introduce one or a few specialized species. However, such a case has been failed by some limitations in terms of slow growth rate like methanogens and unsuccessful immobilization in continuous system [49]. It indicates that single species may be difficult to retain the functional stability and robustness in complex mixed-culture systems by environmental conditions or competition with indigenous community. If a complex consortium is augmented, therefore, these complex groups with diverse populations may complement this limitation by functional redundancy or syntrophic relationships.

RF has potential for bioaugmentation source with complex consortium. Because rumen microorganisms consisted of bacteria, archaea, protozoa, and fungi are specialized to ferment complex organics such as plants in anaerobic condition like AD. So, these microorganisms with specific bacteria degrading fibrous matters have been utilized to pretreat the complex organics such as lignocellulosic biomass and FW (Table 1-1). In addition, some researches have been applied to inoculum source in AD system because RF included methanogens can contribute to the methanogenesis from the products of hydrolysis and acidogenesis (Table 1-2). Therefore, RF can be potentially considered to bioaugmentation source for enhancing AD performance of Korean FW.

In study 1, to examine the potential of bioaugmentation to enhance the biomethanation of FW, preliminarily, various bioaugmentation sources and augmented ratio were evaluated with three different specific microorganisms in batch mode; *Clostridium acetobutyricum* (CA), *Clostridium cellulolyticum* (CC), and RF. CA and CC as functional single species for cellulosic degradation and RF as functional mixed groups were selected in the experiment to compare the differences of between single and mixed group. From the result of preliminary test, RF showed superior to other species. To examine the feasibility of RF as bioaugmentation source, firstly, the methane potential of bioaugmentation with RF was evaluated in mesophilic batch mode. To determine appropriate augmentation ratio, the experiment was conducted to various augmentation ratio.

After appropriate augmentation ratio was selected from the result of batch tests, the second experiment examines the feasibility of RF as bioaugmentation source in continuous reactor operation. Most field-scale anaerobic digesters employ CSTR running in continuous mode where, in contrast to a batch process, excess biomass is continuously removed out. In such a process, outcompeted or unfavored populations under certain operating conditions are washed out of the system, potentially resulting in a deterioration of performance. On this account, the effect of RF addition was further examined, in terms of both performance enhancement and process stability, in a lab-scale CSTR. To better understand the underlying microbial ecology related to the

effect of the bioaugmentation with RF, variations in microbial community structure were also examined to the reactor samples before and after the bioaugmentation.

2.2. Materials & methods

2.2.1. Substrate and inocula preparation

Simulated FW (SFW) was prepared to batch and CSTR tests based on the actual FW generated in Korea and used as the substrate for AD tests: 16% boiled rice, 8% napa cabbage, 20% potato, 20% onion, 2% white radish, 7% apple, 7% orange, 5% pork, and 15% mackerel on a wet weight basis. The SFW ingredients, except boiled rice, were chopped before being mixed and ground in a household blender. The mixed slurry was adjusted to the desired moisture content (85 ± 3%, w/v) with tap water.

Digested sludge from a full-scale anaerobic co-digester treating sewage sludge and FW was used as inoculum for AD experiments. Basic physicochemical characteristics of the seed sludge and the substrate are given in Table 2-1. CA and CC were subcultured in laboratory. RF, used as bioaugmentation source, was collected using a stomach tube from a healthy cow (Fig. 2-1). Digestate and RF were both sieved (mesh size, 860 μm) to remove coarse particles just prior to their addition to the cultures.

Table 2-1. Physicochemical characteristics of the seed sludge and the substrate.

	Anaerobic sludge	SFW
pH	7.51	4.10
Total solids (g/L)	18.9 ± 0.1	127.0 ± 6.4
Volatile solids (g/L)	11.7 ± 0.0	120.5 ± 6.2
Suspended solids (g/L)	14.5 ± 1.2	-[a]
Volatile suspended solids (g/L)	9.5 ± 0.7	-
Total COD (g/L)[b]	14.6 ± 1.8	120.3 ± 2.0
Soluble COD (g/L)	0.2 ± 0.0	71.7 ± 0.8
Cellulose (g/L)	0.1 ± 0.0	2.7 ± 0.2

[a] Not available.

[b] COD, chemical oxygen demand.

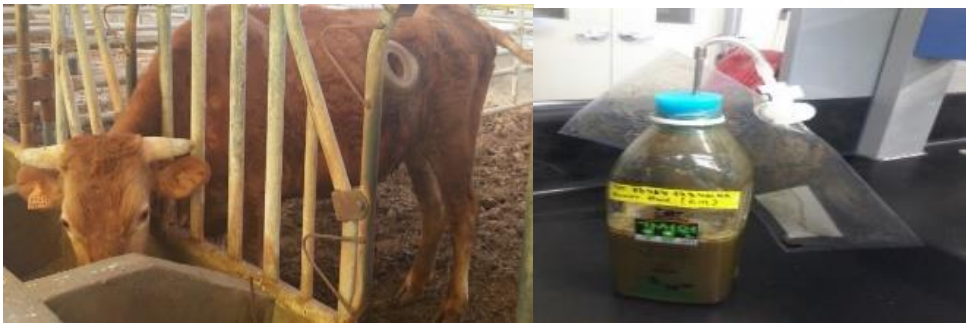


Figure 2-1. RF from cow.

2.2.2. Batch tests

Batch tests were conducted in triplicate using 120-mL serum bottles with a 60-mL working volume. Each bottle was prepared with 2.5 mL model FW (315 mg as volatile solids (VS)) and 50 mL anaerobic sludge (630 mg as VS), filled up with distilled water. First batch tests, thirteen different augmentation conditions with three inocula were tested in parallel with the non-augmented control (Table 2-2).

Table 2-2. Experimental conditions for batch tests 1.

Run	CA (%)	CC (%)	RF (%)	AS	SFW
1	0	0	0		
2	3	0	0		
3	0	3	0		
4	0	0	3		
5	3	3	0		
6	3	0	3		
7	0	3	3	50 mL (640 mg	2.5 mL (315 mg
8	3	3	3	VS)	VS)
9	1.5	1.5	0		
10	1.5	0	1.5		
11	0	1.5	1.5		
12	1	1	1		
13	10	0	0		
14	0	0	10		

%, augmentation ratio to the AS based on volatile suspended solids (VSS).

*CA, *Clostridium acetobutyricum*; CC, *Clostridium cellulolyticum*; RF, Rumen fluid.

Second batch tests, Five different augmentation ratios with RF (i.e., 1, 3, 10, 20, and 30% to the inoculated digestate on the basis of volatile suspended solids (VSS)) were tested in parallel with the non-augmented control (Table 2-3). Each bottle was purged with nitrogen gas and sealed with a rubber stopper. The prepared bottles were incubated at a set temperature of 35℃ with shaking at 100 rpm for 28 days with monitoring of biogas production. The measured biogas volume was corrected to standard temperature and pressure (STP; 0℃ and 1atm) conditions.

Table 2-3. Experimental conditions for batch tests 2 with different augmentation ratios.

Run	AS (mL)	SFW (mL)	RF (%)	DW (mL)	Test volume (mL)
Blank	0	2.5	0	57.5	60
Control	50	2.5	0	7.5	60
Run 1	50	2.5	1	7.3	60
Run 2	50	2.5	3	6.9	60
Run 3	50	2.5	10	5.5	60
Run 4	50	2.5	20	3.5	60
Run 5	50	2.5	30	1.5	60

%, augmentation ratio to the AS based on VSS

2.2.3. Continuous tests using CSTR

A CSTR with a working volume of 4.5L was anaerobically operated in semi-continuous mode with once- or twice-a-day feeding of SFW (12% VS, w/v). The reactor was initially filled with the same anaerobic digestate as for the batch tests and started up with gradually increasing the OLR to 2 g VS/L·d during the first 40 days of operation. RF biomass, prepared in the same way as for the batch tests, was added in a ratio of 10% to the reactor mixed liquor on a VSS basis to the reactor on day 56. The reactor was operated under mesophilic conditions (35 ± 2℃) without pH control throughout the operation.

2.2.4. Molecular fingerprinting and sequencing

DNA extraction was performed using an automated extractor (Exiprogen, Bioneer, Daejeon, Korea) as previously described [50]. A 1-mL aliquot of the effluent sample was repeatedly pelleted and resuspended in distilled water, and a 200-μL portion of the final resuspension was loaded on the extractor. The purified DNA was eluted in 200-μL of elution buffer and used as template for subsequent molecular analyses. Preparation of bacterial and archaeal 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE) of the amplicons were carried out as previously described [50]. The DGGE gels were stained with

SYBR Safe (Molecular Probe, Eugene, OR) and visualized under blue light. DNA sequences of selected bands from the gels were retrieved and analyzed for phylogenetic affiliation against the GenBank and RDP 10 databases as previously described [50]. The RDP classifier was used for taxonomic assignments of the recovered sequences at a bootstrap confidence threshold of 80%. All nucleotide sequences reported in this study have been deposited in the GenBank database: KT366739-KT366748

2.2.5. Cluster analysis

The bacterial and archaeal DGGE fingerprints were each transformed into a binary matrix by scoring the presence or absence of individual bands as 1 or 0. The created matrices were used to visualize the shifts in bacterial and archaeal community structures by cluster analysis using the unweighted pair group method with arithmetic means (UPGMA). Clustering calculation was carried out based on the Sorensen (Bray-Curtis) distance measure, suggested to be most suitable for analyzing microbial community data [51], using PC-ORD 6 software (MjM software, Gleneden Beach, OR).

2.2.6. Analytical methods

Chemical oxygen demand (COD) was colorimetrically measured using HS-COD-MR kit (HUMAS, Daejon, Korea), and solids were analyzed according to the protocols in Standard Methods [52]. Volatile fatty acids (VFAs; C2–C7) were quantified using a 7820A gas chromatograph (Agilent, Palo Alto, CA) with a flame ionization detector and an Innowax column (Agilent). Biogas composition was determined using another 7820A gas chromatograph coupled with a thermal conductivity detector and a ShinCarbon ST column (Restek, Bellefonte, PA). Filtered samples through a membrane filter (0.45-μm pore) were used for the measurement of soluble COD and VFAs. All analyses were replicated at least twice.

2.3. Results & discussion

2.3.1. batch tests

In batch test 1, the methane yield (i.e., methane gas produced per unit mass of substrate fed) tended to increase with the addition of RF regardless of the combination with CA and CC (Fig. 2-2). Especially, the methane yields were increased to 12.8% and 30.1% in Run 4 (RF 3%) and Run 14 (RF 10%) compared to Run 1 (Control), respectively. From the result, RF could attribute to the enhancement of biomethanation from SFW compared to single species of CA and CC. Therefore, to confirm the appropriate augmentation ratio of RF, batch test 2 was investigated.

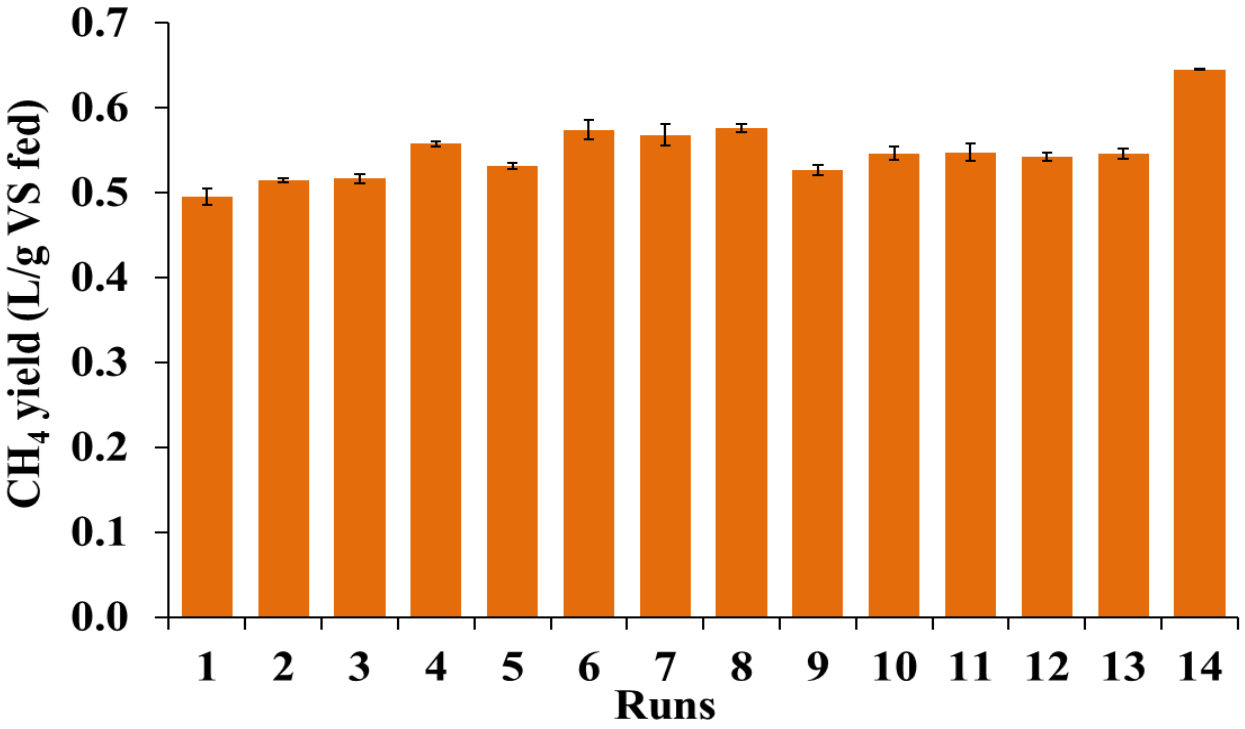


Figure 2-2. Cumulative methane yield in batch test 1.

In batch test 2, biogas production started immediately after the initiation of incubation without a lag phase in all batch runs, except for the SFW-only blank which showed insignificant biogas production. This could be attributed to the use of a high inoculum to substrate ratio (i.e., low food to microorganism ratio), which is beneficial to avoid process imbalance due to rapid acidification of easily biodegradable organics. The cumulative biogas production during the 28-day digestion period was greater in the runs augmented with RF, particularly in Runs 3–5 with higher augmentation ratios (by 10–30%), compared to in the control (Table 2-4). For each run, the batch profile was fitted to a modified Gompertz equation (Eq. 2-1) in order to describe the biogas production kinetic.

$$B_t = B_p \cdot \exp\left[-\exp\left\{\frac{R_m \cdot e}{B_p}(\lambda - t) + 1\right\}\right] \qquad \text{Eq (2-1)}$$

where B_t is the cumulative biogas production (mL) after time t, B_P is the biogas production potential (mL), R_m is the maximum biogas production rate (mL/d), λ is the lag phase length (day), and t is the incubation time (day). All batch runs showed a good fit to the equation with a high regression coefficient ($r^2 > 0.97$). The estimated model parameters are summarized in Table 2-4. Agreeing with the experimental observations, no lag phase was identified for all test runs. The augmented runs showed significantly greater B_P values (by

up to 37%) compared to the control while the *R*m value remained fairly constant (within 5% difference). This indicates that the bioaugmentation with RF had a beneficial effect on the methanation yield of SFW.

Table 2-4. Batch test 2 results and estimated Gompertz model parameters.

	Control	Run 1	Run 2	Run 3	Run 4	Run 5
Cumulative biogas production (mL)	237.4	244.0	245.8	262.2	280.3	301.2
Methane content (%)	57.2	59.7	57.4	59.7	61.3	62.0
Biogas yield (L/g VS_{fed})	0.75	0.77	0.78	0.83	0.89	0.96
R_m (mL/d)[a]	154.3	156.9	160.3	160.0	161.5	158.5
B_P (mL)[b]	218.8	224.4	226.3	240.8	257.9	277.6
λ (day)[c]	–[d]	–	–	–	–	–
R^2	0.975	0.974	0.976	0.977	0.979	0.981

[a] The maximum biogas production rate.

[b] Biogas production potential.

[c] Lag phase length.

[d] Not observed.

The methane yield (i.e., methane gas produced per unit mass of substrate fed) tended to increase with the amount of RF added, from 0.43 L/g VS_{fed} in the control to 0.59 L/g VS_{fed} in Run 5, while the methane content of biogas remained within a relatively narrow range of 57–62% (Fig. 2-3). The improvement in methane yield was particularly evident in Runs 3–5 augmented with higher concentrations of RF. Such enhanced biomethanation could be attributed to improved microbial activity by the introduction of rumen microorganisms. The batch experimental results overall demonstrated the potential of RF bioaugmentation as a means to enhance the biogas production from FW. In the study, 10% bioaugmentation was considered as optimum ratio because Korean FW consisted of about 10% fibrous matters which has potential for the 10% conversion to biomethanation [53-55]. In addition, RF is restricted source collected from cow, thus large amount of RF is not appropriate to apply in field scale system. Therefore, 10% bioaugmentation corresponding to increase over 10% biomethanation is selected to next experiment for bioaugmentation ratio.

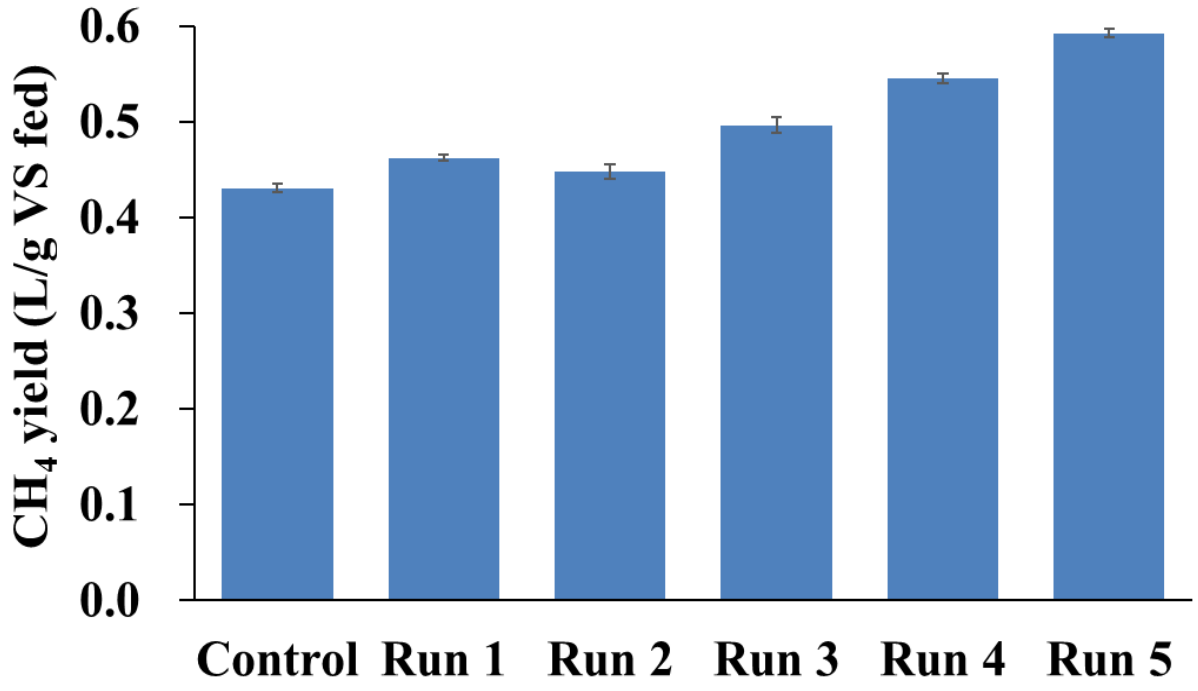


Figure 2-3. Cumulative methane yield in batch test 2 with different augmentation ratios.

2.3.2. Continuous tests with bioaugmentation

Most field-scale anaerobic digesters employ CSTR running in continuous mode where, in contrast to a batch process, excess biomass is continuously removed out. In such a process, outcompeted or unfavored populations under certain operating conditions are washed out of the system, potentially resulting in a deterioration of performance. On this account, the effect of RF addition was further examined, in terms of both performance enhancement and process resilience, in a lab-scale CSTR. Biogas production and VFAs

profiles in the reactor are shown in Fig. 2-4. Biogas production increased with gradually elevating the OLR to 2 g VS/L·d during the start-up period and reached a stable level after about 50 days of operation. RF was added to the reactor after collecting the reactor performance data at the design OLR of 2 g VS/L·d during days 52 to 56 (P1). Interestingly, the biogas production rate (8.9%) and yield (8.5%) showed a statistically significant increase after the bioaugmentation in FB (measured during days 68 to 73 (P2); p <0.05, Student's t-test), meaning that the addition of RF enhanced the biomethanation of SFW. On the other hands, the biogas production rate in FC decreased with the increased VFAs concentrations and lower pH condition (<7.4) compared to FB (pH >7.6). This result indicated that FC might be affected by any inhibitory effect of SFW, while FB with bioaugmentation was likely maintained to process performance in terms of higher biogas production, low residual VFAs, and higher buffering capacity.

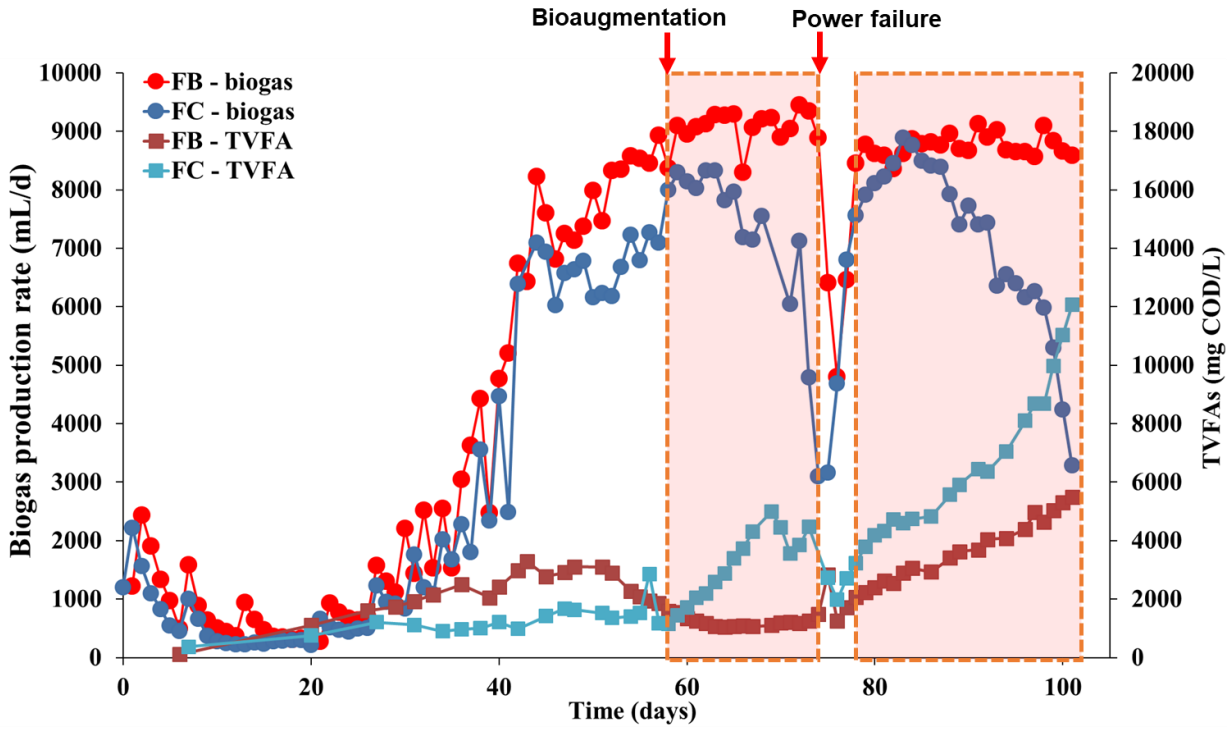


Figure 2-4. Biogas production and VFAs concentration profiles during the CSTR operation.

Unfortunately, an accidental power failure on day 74, the reactor agitation and temperature control were temporarily halted for about 6 hours. The daily biogas production decreased by about 30% in one day after the event, along with sudden increases in the residual acetic and propionic acid concentrations, and so reactor feeding was stopped for performance recovery. Residual VFAs were stabilized to the pre-event level in one day of batch incubation, and the semi-continuous feeding resumed on day 76. In FB, Although FB exhibited resilience performance with increased biogas production and reached a stable level (measured during days 92 to 101 (P3)), residual VFAs continued to accumulate after resuming the feeding. On the other hands, in FC,

the biogas production rate decreased with the accumulation of VFAs after resuming the feeding in P3. The biogas yields measured during P1–P3 were comparable to or higher than those reported previously for AD of FW [56-58].

Given that the pH was maintained between 7 and 8 throughout the experimental period presumably owing to the high alkalinity of the feed, the process deterioration in FC is attributable to a kinetic uncoupling between acid producers and consumers rather than reactor souring. Moreover, the unstable methanation performance along with the accumulation of VFAs suggests that methanogens rather than acidogens were more adversely affected by the unintended failures in temperature control on days 74. Such an effect may have aggravated the imbalance between acidogenesis and methanogenesis, caused by the significant difference in growth rates of faster-growing acidogens and slower-growing methanogens [59], in the reactor.

2.3.3. Microbial community structure

DGGE was performed to analyze the variations in microbial community structure among the reactor samples before and after the bioaugmentation with RF (Fig. 2-5). The bacterial DGGE profiles of FB on days 56 and 84 apparently differed from each other (Fig. 2-5A). This implies that the bacterial community structure in the reactor was significantly affected by the addition of RF. Cluster analysis also revealed that the bacterial community structure in FB before adding RF (day 56) was remotely related to the ones after adding RF (days 84, 92, 101, and 118) that are closely clustered together (Sorensen distance, D_S <0.20) in the bacterial cluster dendrogram (Fig. 2-5C). These suggest that the altered bacterial community structure by the bioaugmentation remained relatively stable until day 118. Supportively, several bands presumably derived from RF (e.g., B3 to 5) were not detected on day 56 but on days 84 and afterwards. This may indicate that some rumen bacteria adapted to and thrived in the experimental reactor anaerobically digesting SFW.

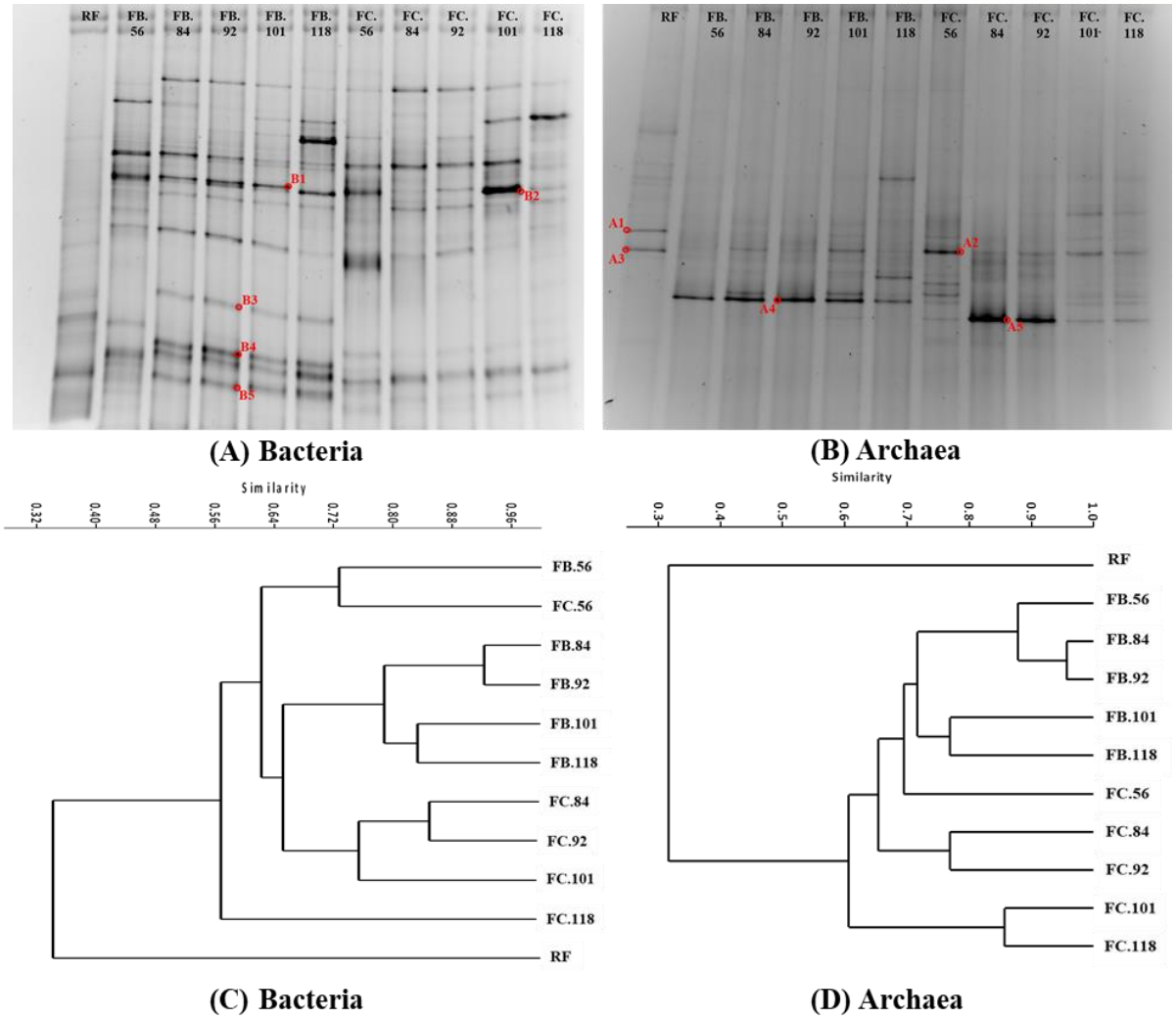


Figure 2-5. Bacterial (A) and archaeal (B) 16S rRNA gene DGGE profiles and cluster dendrograms of bacterial (C) and archaeal (D) community structures based on the DGGE profiles.

Among five bacterial sequences retrieved from the DGGE gel (i.e., B1 to 5), B1 and 2 occurred in all reactor samples but not in the RF. In particular, B1 might be major bands in FB. These bands were assigned to the order *Clostridiales* whose members are frequently found in fermentation environments (Table 2-5). Band B2 was closely related (>97% sequence similarity) to saccharolytic *Clostridium populeti* capable of fermenting glucose and cellulose to H_2, CO_2, and various organic acids [61]. Therefore, these bacterial groups were likely performed to degrade organic matter containing cellulose in FW. On the other hand, the remaining bands (i.e., B3 to 5) were observed in the post-bioaugmentation samples and RF only. This suggests that the bacteria corresponding to these bands likely originated from RF and remained in the reactor. Bands B3 and 4 were both assigned to the genus *Syntrophomonas* which can oxidize long-chain fatty acids syntrophically with a H_2-scavenging partner under anaerobic conditions [61]. The populations related to these bands presumably participated in the degradation of fatty acids with methanogens as syntrophic partner

in the reactor. In addition, *Syntrophomonas* genus as acetogenic syntrophic bacteria has been commonly observed in treating lignocellulosic biomass and rumen consortia [62, 63]. Band B5 was assigned to the genus *Lutispora* able to ferment proteins and amino acids to VFAs with the formation of H_2S as a byproduct [64] as well as to degrade cellulose as known cellulolytic organisms [65]. Thus, it implies that B5 play a role for the degradation of fibrous matters of FW in FB reactor.

In contrast to the bacterial community, no visible structural variation was observed in the archaeal community before and after the bioaugmentation with RF (Fig. 2-5B and D). This may be ascribed to the much less diverse nature of methanogens than of acidogens [66]. The cluster dendrogram shows that the day 101 community profile in FB are more distantly related (D_S >0.25) among the profiles analyzed from the reactor samples, with the day 56, 84, and 92 profiles being more closely clustered ($D_S \leq 0.12$). Although not clear, this seems to be related to the continued accumulation of VFAs, particularly those with a chain length of C_3 or longer (Fig. 2-4).

Bands A1 and 3, the dominant archaeal bands in RF, were both not closely related to known species, but to environmental clones from cow or termite guts (Table 2-5). Band A2, observed in all reactor samples as a minor band, was closely related to hydrogenotrophic *Methanospirillum stamsii* isolated from syntrophic methanogenic granules [67]. Bands A4 and 5 were both assigned to the aceticlastic genus *Methanosarcina*. *Methanosarcina* species are metabolically versatile with the ability to use H_2/CO_2 and methyl compounds for methanogenesis as well as to grow on acetic acid [68]. The growth of *Methanosarcina* species is reportedly more favored at a relatively high residual VFAs concentration, particularly acetic acid [59].

Table 2-5. Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands.

Bands	Closest relatives	Accession number	Similarity (%)	Classification[a]
Bacteria				
B1	clone NLAE-zl-C589	JQ608288	92.6	*Clostridiales*
	Clostridium populeti	NR026103	92.3	
B2	*Clostridium populeti*	NR024993	97.8	*Clostridium XlVa*
B3	clone B5C10	AB997588	99.8	*Syntrophomonas*
	Syntrophomonas wolfei subsp. saponavida	NR115849	93.0	
B4	clone B5C10	AB997588	100.0	*Syntrophomonas*
	Syntrophomonas wolfei subsp. saponavida	NR115849	93.2	
B5	clone BL15	EU586220	99.8	*Lutispora*
	Lutispora thermophila	NR041236	95.9	*Lutispora*
Archaea				
A1	clone C62-31	HQ413068	99.6	*Euryarchaeota*
	Candidatus Methanoplasma termitum	CP010070	95.2	
A2	*Methanospirillum stamsii*	NR117705	98.2	*Methanospirillum*
A3	clone: G-32	AB906250	98.5	*Methanobrevibacter*
	Methanobrevibacter olleyae	NR043024	96.7	
A4	*Methanosarcina acetivorans*	NR074110	98.2	*Methanosarcina*
A5	*Methanosarcina siciliae*	JX943603	99.6	*Methanosarcina*

[a] The lowest rank assigned by the RDP Classifier at a bootstrap cutoff of 80%

The molecular analysis results overall suggest that the bioaugmentation with RF significantly affected the structure of bacterial community rather than of archaeal community. The alteration in the bacterial community structure by the introduction of rumen bacteria was likely related to the enhanced methanation performance (Fig. 2-4 and 2-5). Although further research on the long-term stability in continuous mode is required, our observations suggest the potential of the bioaugmentation with RF as an approach for enhanced AD of FW.

2.4. Summary

The effect of bioaugmentation with RF on the biomethanation of FW was assessed in batch and

continuous modes. Adding RF had a beneficial effect on biogas yield in batch tests, and a 10% augmentation ratio on a VSS basis, which showed a 10.4% increase in biogas yield compared to the control, was applied to the subsequent CSTR experiment. Both biogas production rate and yield increased after the addition of RF (P2) by 8.9% and 8.5%, respectively, compared to those in P1. Accidental power failure caused adverse effects on the methanation activity, resulting in an accumulation of VFAs and thus a serious process deterioration in FC. By contrast, FB with the addition of RF exhibited resilient performance in terms of the rapid recovery of biogas production against accidental power failure. The bacterial community structure in the CSTR changed significantly before and after adding RF, whereas the archaeal community structure changed little throughout the experiment. *Syntrophomonas* and *Lutispora* populations, which originated from RF, likely contributed to the hydrolysis and acidogenesis in the reactor. The alteration of structure and activity of the bacterial community by the introduction of rumen bacteria was potentially beneficial for the enhanced methanation of FW. Although the bioaugmentation with RF could enhance the biomethanation from FW, it is still limited effect in short-term period and single system. Therefore, further study would be determined to appropriate system and long-term effect of bioaugmentation.

3. [Study 2] Optimal systems for bioaugmentation

3.1. Introduction

AD has been widely operated to manage various organic waste such as FW. However, the AD of FW has practical limitations related to the characteristics of FW such as low pH, low alkalinity, and low trace element content [69]. Low pH and alkalinity of FW can lower the buffering capacity and thus the process stability of a digester, particularly under high OLR conditions. Deficiency of trace elements is also reported as a common cause of process upset in anaerobic FW digestion processes [70].

Conventional AD plants treating FW have typically employed a single-phase CSTR configuration. In a single-phase system, all reaction steps of the AD pathway, from hydrolysis to methanogenesis, occur in one reactor operated under optimal conditions for methanogens, i.e., neutral pH and long HRT (usually more than 20–30 days). This means that acidogens, which have different physiological and growth characteristics from methanogens, are under suboptimal conditions in such a reactor [59]. Moreover, single-stage AD process cannot be optimized to hydrolysis of complex matters such as lignocellulosic feedstock [39]. A two-phase AD system, consisting of two reactors in series operating under different conditions, was proposed in an attempt to resolve this limitation [71]. The first reactor (i.e., acidogenic phase) is often operated under moderately acidic conditions (pH 5–6) at a short HRT <5 days to form a favorable environment for the hydrolysis and acidogenesis by the growth of acidogens and prevent methanogenic activity, while the second reactor (i.e., methanogenic phase) is run under optimal conditions for methanogens as for the single-phase

system. This two-phase configuration is anticipated to enhance the overall performance as far as an ideal biphasic ecosystem is maintained. Owing to such advantages, it has been proposed to be advantageous over single-phase configuration and widely applied in the pilot- and field-scale processes treating various organic wastes including FW, sewage sludge, animal manure, and their mixtures [72-74]. However, the significance of its effect is debatable because AD involves complex syntrophic relationships between acidogens and methanogens [75].

Reactor pH is a major factor in determining the fermentation type [76]. FW is readily fermented and prone to acidification; thus, it is often required to control the pH of the acidogenic phase to avoid highly acidic conditions that may disrupt both acidogenic and methanogenic activities. The pH control is generally achieved by adding alkaline compounds such as NaOH, $NaHCO_3$, and KOH into the reactor. However, the use of a buffer solution results in additional operating costs, and salts from alkaline compounds, such as Na^+ and K^+, that can negatively affect microbial activity [77]. These limitations should be considered when operating AD processes, particularly those with two-phase configuration. Minimizing or avoiding the use of a buffer solution could be a direct way to mitigate such limitations. However, if pH is not controlled, the acidogenic pH can quickly drop to <4, which is well below the optimal range for acidogenesis, when treating readily biodegradable substances such as FW [78, 79]. This means that acidogens will be under suboptimal pH conditions, which is inconsistent with the original intention of the two-phase configuration. In addition, acidic products with low pH have an inhibitory effect on the methanogenesis by directly and indirectly effect of high acids and lowering buffer capacity [80]. However, the acidification in low pH condition could be performed by lactate-type fermentation of carbohydrates treating organic wastes [81]. Although previous study neutralized the pH of the acidogenic phase effluent, two-phase AD was successfully operated with stable biogas production despite of high acidic products with mainly lactate and acetate. Therefore, it can potentially be considered to two-phase AD of FW with uncontrolled pH for reducing the operational cost and avoiding the harmful effect of salts in buffer solution.

According to the results of study 1, bioaugmentation with RF could enhance the biomethanation accomplished with the bacterial community structures as acidogenesis. It suggests that the hydrolysis and acidogenesis activity by bioaugmentation can be important to enhance the biomethanation. Two-phase system with specialized acidogenic phase can be considered to optimum process for improving the effect of bioaugmentation with RF. In addition, by adding the RF into acidogenic phase, it can reduce the amount of RF for bioaugmentation. Because RF from cow is restricted source to apply the large amount in field scale, it is needed to reduce absolute dosage of RF in order to apply the bioaugmentation in real system. In addition, most two-phase systems have been operated with the control of pH >5 in acidogenic phase. However, it is not reported to compare the biomethanation efficiency for single- and two-phase CSTR with bioaugmentation of RF under uncontrolled pH. Therefore, in this study 2, single- and two-phase CSTR are examined to compare the proper operating condition in terms of HRT and OLR and the feasibility of

bioaugmentation with RF with uncontrolled pH.

3.2. Materials & methods

3.2.1. Substrate and inocula preparation

The real FW was collected from cafeteria in UNIST and consisted of mainly cooked rice and smaller amounts of flour products, soup, vegetables and meat. The collected FW was ground into a slurry using a household blender and passed through a 3 mm mesh. The prepared FW slurry was adjusted to a VS content of 10% (w/v) with tap water and stored at 4°C until use. The average physicochemical characteristics of the FW substrate are summarized in Table 3-1.

Table 3-1. Physicochemical characteristics of FW.

Parameter	Unit	Value[a]
pH		4.2 (1.9)
Total COD	g/L	133.3 (14.9)
Soluble COD	g/L	58.7 (8.5)
Total solids	g/L	103.4 (5.9)
Volatile solids	g/L	97.0 (5.9)
Total nitrogen	g/L	2.6 (0.5)
Total phosphorus	g/L	0.5 (0.1)
Total carbohydrates	g/L	61.4 (11.4)
Carbon	% dw[b]	48.1 (2.8)
Hydrogen	% dw	7.1 (0.3)
Oxygen	% dw	37.3 (2.0)
Nitrogen	% dw	3.7 (1.2)
Sulfur	% dw	0.1 (0.1)
C/N ratio		13.9 (3.3)
Alkalinity	mg/L as $CaCO_3$	31.0 (27.0)
Fe	mg/L	1.987 (2.088)
Ni	mg/L	0.158 (0.248))
Co	mg/L	0.002 (0.002)
Al	mg/L	0.817 (0.189)
Cr	mg/L	0.013 (0.009)
Cu	mg/L	0.141 (0.112)
Mn	mg/L	1.081 (0.599)
Zn	mg/L	1.163 (0.956)
Mo	mg/L	0.398 (0.652)
W	mg/L	0.021 (0.011)

[a] Determined from four different batches of FW prepared at different points during the course of the experiment. Standard deviations are in parentheses.

[b] dw, dry weight.

Anaerobic sludge from a full-scale anaerobic co-digester treating sewage sludge and FW was used as inoculum for AD experiments. RF, used as bioaugmentation source, was collected using a stomach tube from a healthy cow in Daegu University (Fig. 3-1). Anaerobic sludge and RF were both sieved (mesh size, 860 μm) to remove coarse particles just prior to their addition to the cultures.

Figure 3-1. RF from Daegu University.

3.2.2. Single- and two-phase processes operation

A single-phase and a two-phase semi-continuous AD processes treating FW (10% VS, w/v) were operated in parallel at stepwise increasing OLRs from 0.5 to 6.0 g VS/L·d by reducing the HRT from 100 to 16.7 days (Table 3-2). A CSTR with a working volume of 2 L was used as the single-phase process (Rs) (Fig. 3-2). The two-phase process consisted of an acidogenic (Ra) and a methanogenic (Rm) CSTR with a working volume of 0.5 and 2 L, respectively. Rs and Rm were initially filled up with anaerobic sludge while Ra was with 0.4 L of anaerobic sludge (80%, v/v) and 0.1 L of FW. For bioaugmentation with RF, Rs was added with 10% of RF at day 36 and Ra was added with 10% of RF at 0 day based on the reactor sludge VSS concentration. Both single-and two-phase processes were started up at a low OLR of 0.5 g VS/L·d. In the two-phase process, the overall OLR was altered by changing the HRT of Rm accordingly while operating Ra at a fixed HRT of 4 days throughout the experiment. All reactors were operated without pH control and maintained at 35 ± 1°C. Trace elements (100 mg Fe/L as $FeCl_3 \cdot 6H_2O$, 2 mg Co/L as $CoCl_2 \cdot 6H_2O$, and 1 mg Ni/L as $NiCl_2 \cdot 6H_2O$ in the substrate) were supplemented to Rs and Rm every other day from days 227 and 101, respectively, and on wards to support the growth of methanogens [70]. Biogas production was continuously measured by a MilliGascounter (Ritter) connected to the reactor headspace. The pH in each reactor was continuously monitored using a pH electrode installed in the reactor.

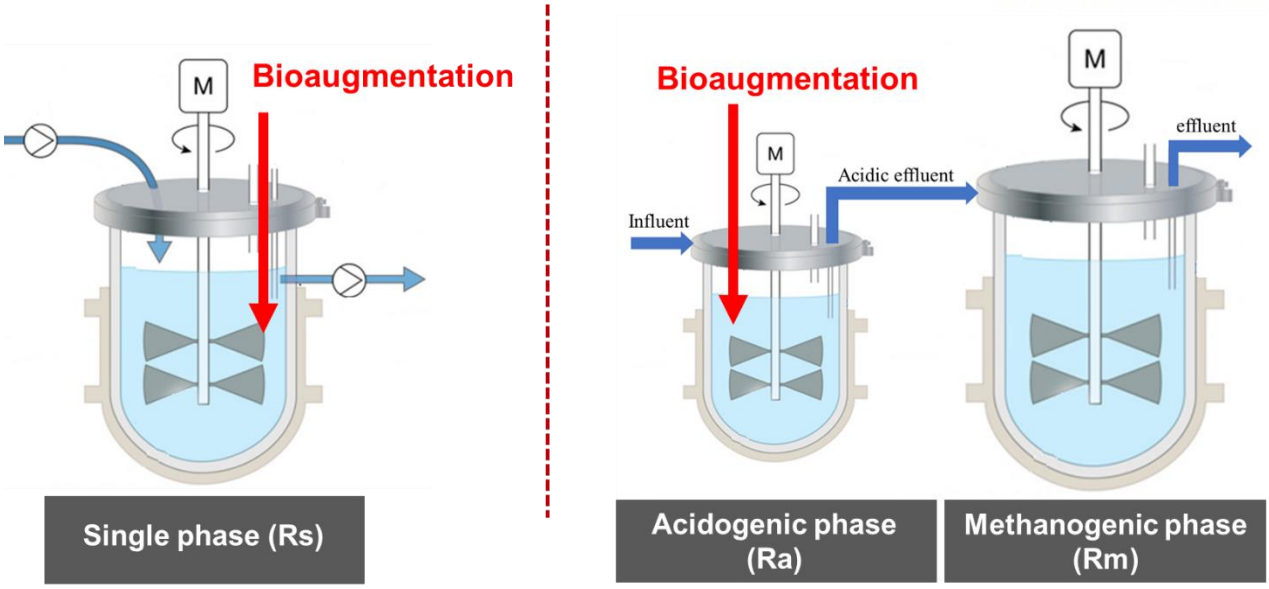


Figure 3-2. Reactor configurations of single- and two-phase processes.

Table 3-2. Operating conditions of the single- and two-phase processes.

	Phase	Period (day)	OLR (g VS/L·d)	HRT (day)	Trace element supplementation
Single-phase process	Start-up	0–28	0.5	200	No
	1	29–58	1.0	100	No
	2	59–107	2.0	50	No
	3	108–142	3.0	33.3	No
	Recovery	143–225	0.0–1.5	200	No
	4	226–248	1.0	100	Yes
	5	249–298	2.0	50	Yes
	6	299–341	3.0	33.3	Yes
	7	342–387	4.0	25	Yes
	8	388–488	5.0	20	Yes
	9	489–545	6.0	16.7	Yes
Two-phase process	Start-up	0–16	0.5	200	No
	1	17–31	1.0	100	No
	2	32–81	2.0	50	No
	3	82–177	3.0	33.3	Yes
	4	178–216	4.0	25	Yes
	5	217–300	5.0	20	Yes

3.2.3. Analytical methods

COD was colorimetrically analyzed using HS-COD-MR kit (HUMAS), and solids were measured according to the protocols in Standard Methods [52]. VFAs (C_2–C_7) and ethanol were measured using a 7820A gas chromatograph (Agilent) equipped with a flame ionization detector and an Innowax column (Agilent). Lactate was quantified using a 1200 series high-performance liquid chromatography (Agilent) coupled with a diode array detector and an Acclaim OA column (Dionex). Samples for soluble COD, VFAs, and lactate measurements were prepared by filtration through a membrane filter (pore size, 0.45 μm). A 7820A gas chromatograph equipped with a thermal conductivity detector and a ShinCarbon ST column (Restek) was used to determine biogas composition. Two ICS-1100 ion chromatographs (Thermo Scientific)

equipped with IonPac AS14 and IonPac CS12A columns were used to measure anions and cations. Sample pH was measured using a pH meter (ORION 3-Star, Thermo Scientific). Alkalinity was determined using ORION Total Alkalinity Test Kit (Thermo Scientific). C, H, O, N, and S contents were determined on a dry weight basis using a Flash 2000 elemental analyzer (Thermo Scientific). Trace elements were measured using an inductively coupled plasma-optical emission spectrometer (700-ES, Varian). All analyses were replicated at least twice.

3.3. Results & discussion

3.3.1. Performance of the single-phase process

Changes in the performance of Rs during the experiment for over 18 months with varying OLRs are shown in Fig. 3-3. Rs was operated at 0.5 g VS/L·d OLR during the start-up period of 28 days and then subjected to increasing OLRs up to 6.0 g VS/L·d. The biogas production rate increased, while the residual VFAs levels remained near zero, with increasing OLRs to 2.0 g VS/L·d (Figs. 3-3 and 3-4). At 2.0 g VS/L·d OLR, the biogas production gradually increased. However, the reactor performance deteriorated significantly and failed to reach the steady state when the OLR was increased to 3.0 g VS/L·d. In this phase, a sharp decrease in biogas production was observed along with a marked accumulation of VFAs, resulting in an unfavorably high VFAs-to-alkalinity ratio of over 0.4 (Fig. 3-4) [82]. This is a sign of the imbalance between acidogenesis and methanogenesis. It is also notable that the free ammonia (FA) concentration increased above 100 mg NH_3-N/L, which could cause significant inhibition of methanogenic activity [83]. Despite the process imbalance, the pH remained above 7.3. This may be, at least in part, attributable to the high accumulation of ammonium (Table 3-3) that has a buffering capacity [84]. Therefore, the process deterioration at 3.0 g VS/L·d OLR seems to be due more to kinetic uncoupling between acidogens and methanogens rather than by souring of the reactor [85]. AD of FW as the sole substrate is prone to process imbalance or even failure by the limitation of trace element seven at relatively low OLRs [70]. The lack of trace elements may be a reason for the observed process deterioration given the low concentrations of trace elements in the substrate (Table 3-1).

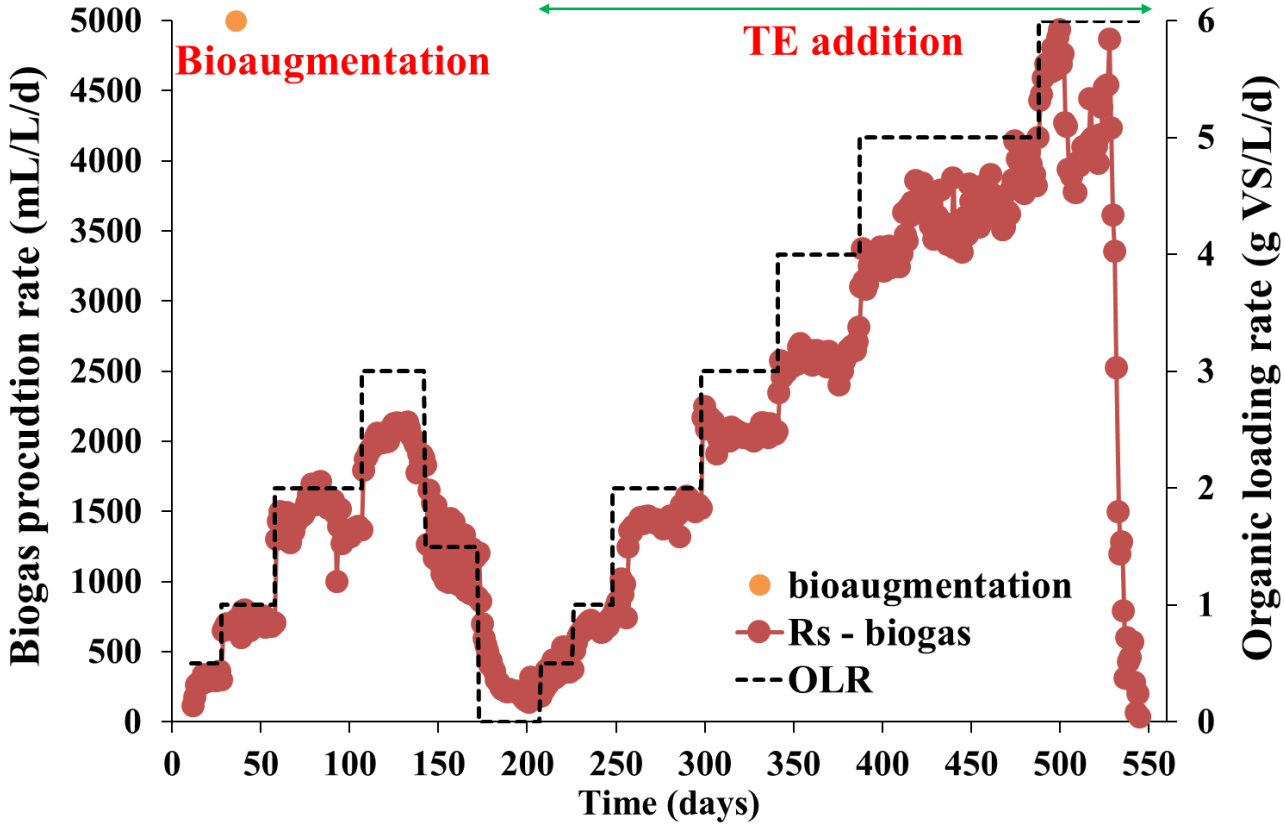


Figure 3-3. Biogas production profiles in single-phase process. Biogas production rate was normalized with unit reactor volume of 2 L.

Rs was stabilized by interrupting the feeding for 35 days from day 173 and the residual VFAs decreased to the bottom. Continuous feeding was then resumed at a low OLR of 0.5 g VS/L·d as for the start-up. After the recovery phase of 82 days (days 143–225), Rs were operated at increasing OLRs from 1.0 to 6.0 g VS/L·d by adding trace elements (Phases 4–9; Table 3-2) to prevent the lack of trace elements in the reactor. Interestingly, after the addition of trace elements, the reactors did not experience process deterioration and showed stable performance until it failed at an OLR of 6.0 g VS/L·d. This means that the process imbalance in Phase 3 was likely due to the lack of trace elements and that additional trace elements were required for the stable operation of Rs at an OLR of 3.0 g VS/L·d or higher. During Phases 4–8, the pH (7.4–7.7) and the VFAs/alkalinity ratio (<0.1) were maintained at fairly stable and favorable levels. Correspondingly, efficient substrate removal (VS removal, 74.7–85.7%) and conversion to methane (methane yield, 0.32–0.47 L/g VS_{fed}) were achieved (Table 3-3). The methane yields during the phases were comparable to those observed at similar OLRs for single-phase AD systems treating FW: 0.38–0.49 L/g VS_{fed} at 1.0–5.5 g VS/L·d [70, 86]. Accordingly, the methane production rate increased steadily with increasing OLR in Phases 4–8. Interestingly, the FA concentration showed a decreasing trend after the addition of trace elements, despite the increase in the OLR and thus the NLR was increased. A similar observation was reported by [87]. The reason

for this phenomenon is yet unclear although a previous study suggested that trace elements may affect the microbial utilization of nitrogen in the AD [88]. Benefits of trace element supplementation have often been reported in AD processes treating different substrates lacking in trace elements [70, 88, 89].

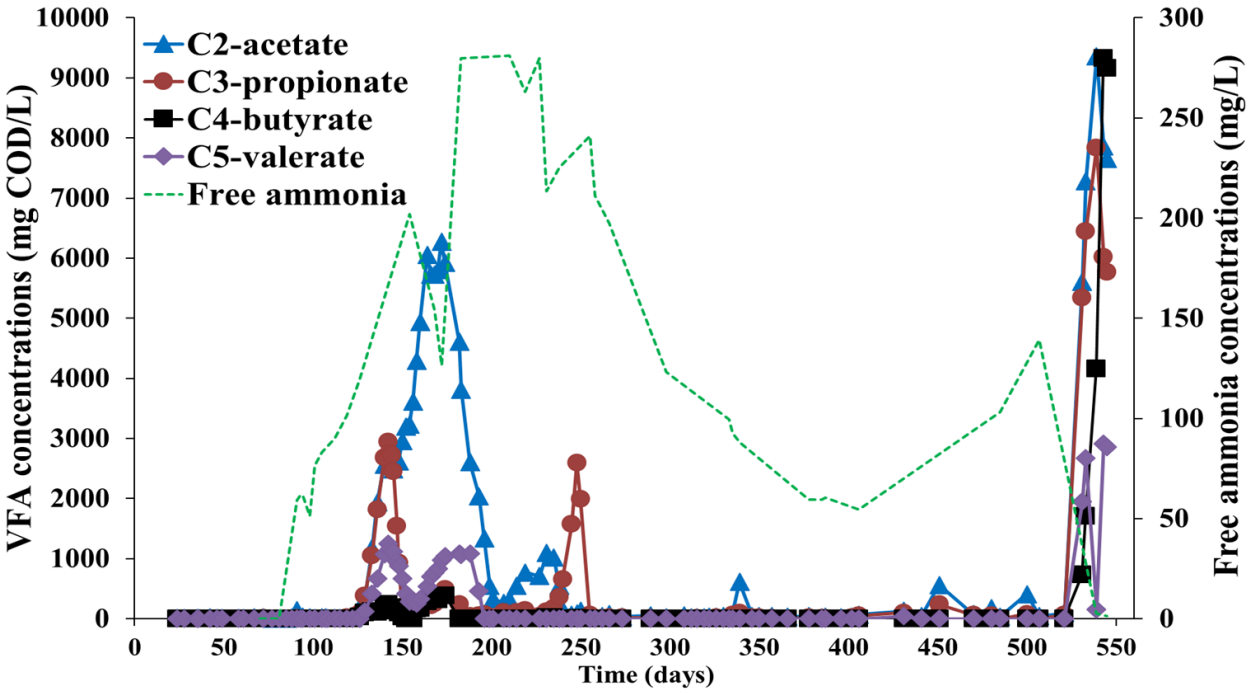


Figure 3-4. VFAs and FA profiles in single-phase process.

Rs achieved a stable steady-state operation with maximum methane yield (0.47 L/g VS_{fed}) and production rate (2.3 L/L·d) at a high OLR of 5.0 g VS/L·d (Phase 8; Table 3-3). However, a further increase in OLR to 6.0 g VS/L·d (Phase 9) led to an immediate reduction in methane yield, followed by a rapid drop in the methane production rate and a drastic increase in the FA concentration to a highly toxic level (>200 mg NH_3-N/L). After approximately two turnovers of the HRT (ca. 34 days) in Phase 9, methane production rapidly decreased to an insignificant level (<0.2 L/d) along with a significant accumulation of VFAs (>30 g COD/L), butyrate in particular. Consequently, the pH (<5.1) and VFAs/alkalinity ratio (>4.9) became very unfavorable for an efficient AD. With the accumulation of VFAs, the FA concentration decreased rapidly to zero. This is attributed to the low pH in the reactor, given that ammonia (pKa at 35°C, 8.95) is present entirely as ammonium ion at acidic pH [90]. These results suggest that acidogenesis and methanogenesis were significantly imbalanced by organic and hydraulic overloading in Phase 9 (OLR, 6.0 g VS/L·d; HRT, 16.7 days). Washout of biomass, particularly slow-growing methanogens and syntrophic VFAs degraders, can cause a loss of methanogenic activity and make the overall process unstable. Given that an HRT of 16.7 days is fairly short for a complete anaerobic degradation of complex organic compounds in a CSTR, biomass washout substantially affected the process deterioration. Additionally, the process deterioration was preceded

by a buildup of FA. This suggests that FA could be a causative factor in the deterioration of reactor performance. The lack of other trace elements, for example, Se, which is reportedly beneficial for operating an AD process at a high OLR [70, 87], might have also affected, in part, the process deterioration in Phase 9.

3.3.2. Performance of the two-phase processes

The two-phase processes (Ra-Rm) were operated at an OLR of 0.5 g VS/L·d for the initial 16 days for start-up and then with stepwise increases in OLR up to 5.0 g VS/L·d (Phases 1–5) over a total of ten months. After the start-up period, Ra maintained high fermentation activity while producing no gas other than carbon dioxide throughout the experiment without pH control (Fig. 3-5). The pH in acidogenic phase remained highly acidic (3.3–3.4) and the major fermentation products were lactate, ethanol, and acetate (in the order of high to low concentrations), in agreement with the general understanding that more reduced compounds, for example, lactate and ethanol, are formed as fermentation products under low pH conditions [76, 91]. A previous study on the phase-separated AD of Korean FW also reported lactates as dominant acidogenic products at a similar pH range to that in Ra [91]. Lactic acid bacteria are able to regulate intracellular pH and, consequently, more acid-tolerant than other fermentative bacteria [92]. Some of them can grow even at pH as low as 3.0, and low pH selection has been used to enrich lactic acid bacteria or promote lactate production in mixed cultures [93]. Moreover, rumen microorganisms also include lactate producing bacteria. They can play a role for degrading starch or sugar in low pH condition. Therefore, Ra functioned effectively and stably as the process for hydrolysis and acidogenesis of FW with bioaugmentation of rumen culture even under uncontrolled pH conditions, although highly acidic pH may potentially inhibit the activity of hydrolytic bacteria [94].

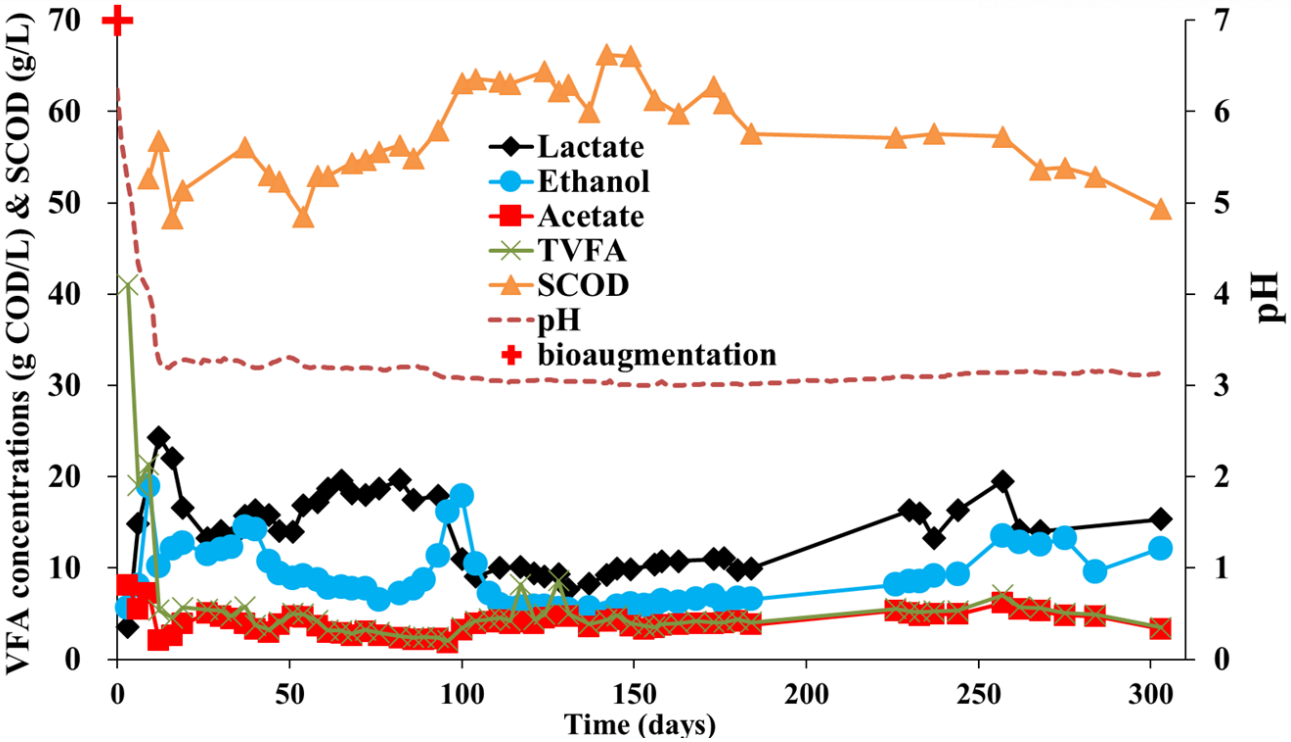


Figure 3-5. Acidogenic products in the two-phase acidogenic process.

The biogas production profiles were shown during two-phase AD experiments in Fig. 3-6. The pH in Rm was maintained between 7 and 8 throughout the experiment until the process failure in Phase 5. This indicates that methanogenic phase reactors had sufficient buffering capacity to deal with the acidic influent (i.e., the effluent from Ra) at OLRs up to 4.0 g VS/L·d. This may be attributed to in the substrate and possibly in part to the residual alkalinity from the seed sludge. Rm maintained a stable biomethanation performance until it reached an OLR of 3.0 g VS/L·d, which caused process deterioration in Rm as in Rs (Fig. 3-6). After entering Phase 3, the biogas production decreased along with the rapid accumulation of VFAs, mostly propionate which was produced from the fermentation of lactate in Ra effluent. To avoid the consequences of trace element deficiency, Rm was supplemented with trace elements on day 101 in Phase 3. The methane yield recovered to a steady level after the addition of trace elements along with the resolution of accumulated VFAs (<0.1 g COD/L). Therefore, the process imbalance observed in Phase 3 was ascribed to the lack of trace elements. It has been previously reported that trace elements can serve as cofactors for important metalloenzymes involved in the AD pathway [95-97]. At a further elevated OLR of 4.0 g VS/L·d, methane yield (0.44 L/g VS_{fed}) and methane production rate (1.5 L/L·d) increased (Phase 4) without any inhibition. Consequently, during Phases 1–4, both pH (7.3–7.5) and VFAs/alkalinity ratio (<0.01) were maintained fairly stable, with very low levels of residual VFAs (<0.1 g COD/L), except the fluctuation in Phase 3, besides, stable performance with VS removal (78.8–84.5%) and methane yield (0.38–0.47 L/g VS_{fed}) (Table 3-3).

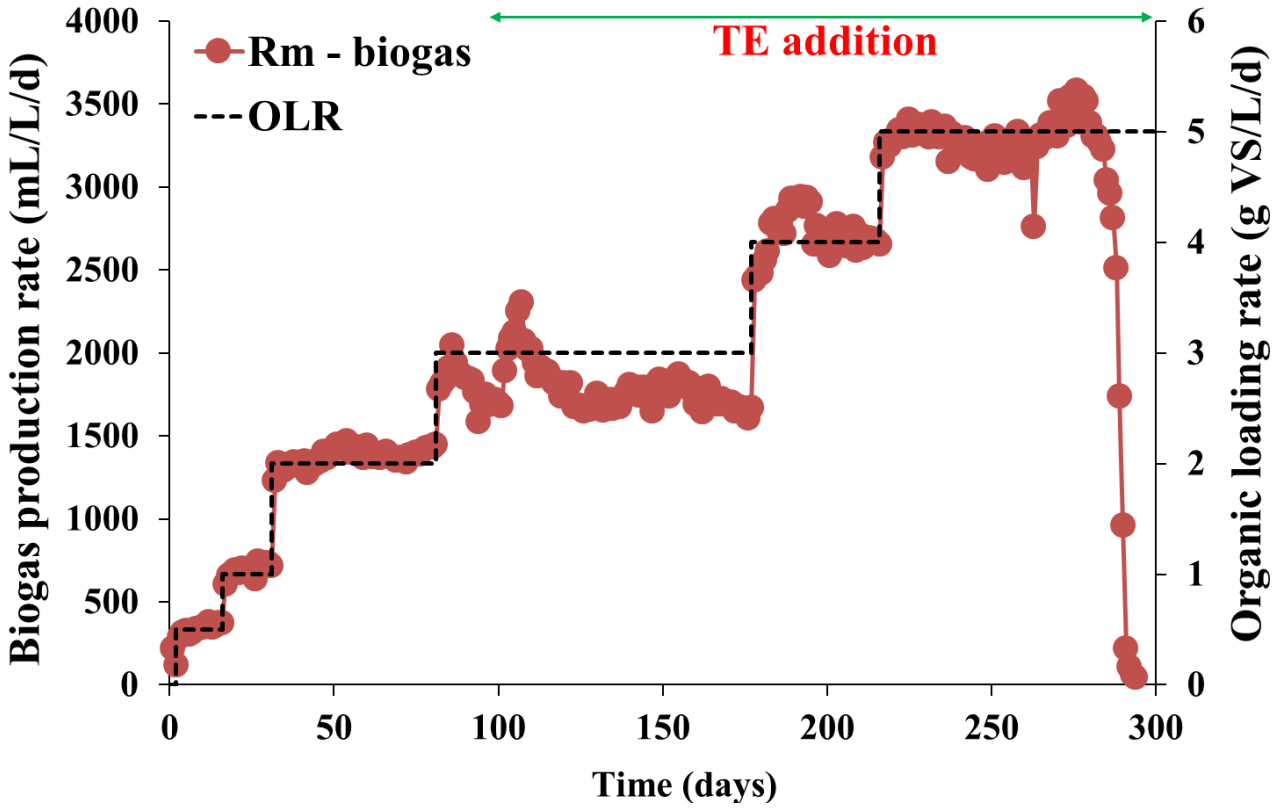


Figure 3-6. Biogas production profiles in two-phase methanogenic process. Biogas production rate was normalized with unit reactor volume of 2.5 L.

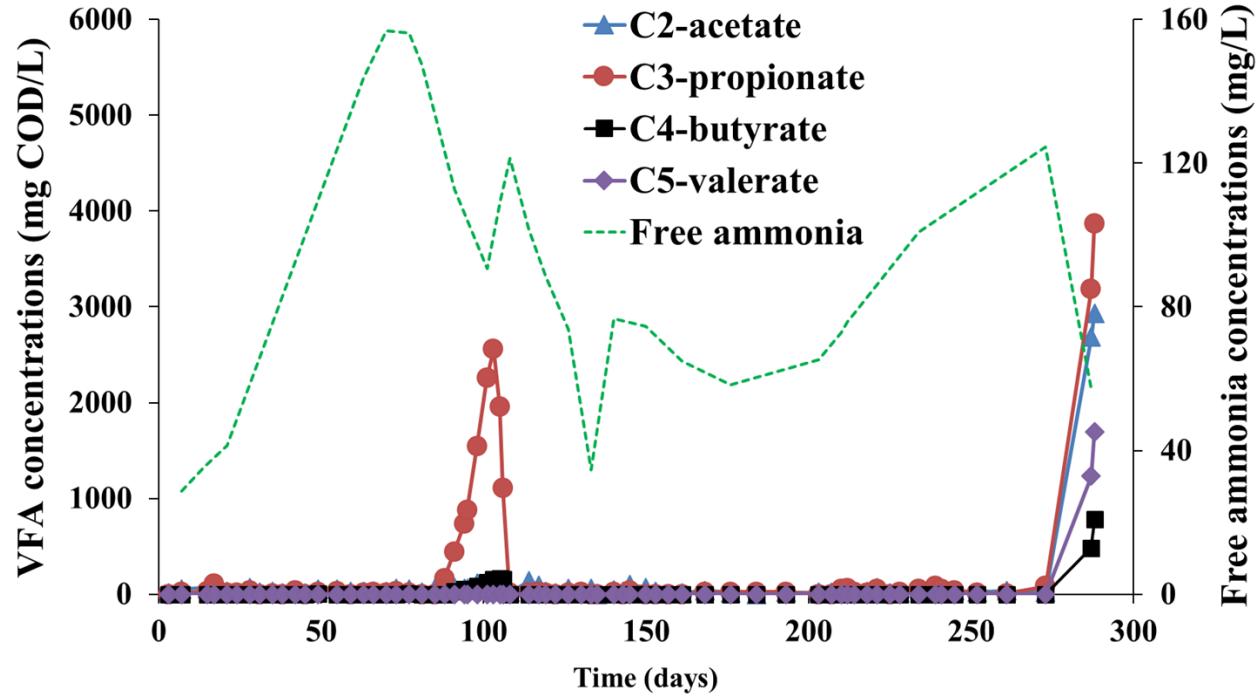


Figure 3-7. VFAs and FA profiles in two-phase methanogenic process.

Rm showed stable methane production while maintaining very low residual VFA levels for the first 70 days (ca. 3.5 turnovers of the HRT) of Phase 5, but then, its performance decreased abruptly with decreasing biogas production and accumulation of VFAs. It is interesting that propionate was the most contributing acid to the process imbalance, in contrast to Rs where butyrate was (Fig. 3-6). These seem to be related to the high concentration of lactate in the effluent from Ra fed to Rm because lactate is readily converted to propionate and acetate in anaerobic systems [98, 99]. These results suggest that the process deterioration at 5.0 g VS/L·d OLR is likely associated with the accumulation of propionate, toxic to methanogens and often used as an indicator of process imbalance [45, 100, 101]. Syntrophic oxidation of propionate is necessary for its complete mineralization under anaerobic conditions [59]. Given that syntrophic propionate degraders grow very slowly only in syntropy with hydrogen/electron-consuming partners, biomass washout seems to have contributed to propionate accumulation and thus to process deterioration. In addition, high acidic influent from Ra and the accumulation of propionate may affect to the buffering capacity in Rm. Therefore, two-phase system with uncontrolled pH was not suitable for stable performance at higher OLRs by acidic inhibition. Although the overall HRT for the two-phase system in Phase 5 is 20 days, HRT that is allowed for methanogens to actively grow is only 16 days in Rm given that Ra (HRT, 4 days) remained highly acidic (pH <3.5) throughout the experiment. This is consistent with the observation of the reactor failure in Rs at an OLR of 6.0 g VS/L·d, which corresponds to an HRT of 16.7 days (Fig. 3-5). In addition, as in Rs, process deterioration was preceded by a buildup of FA in Rm.

Table 3-3. Steady-state performance data at experimental phase.

	Rs									Rm				
OLR (g VS/L·d)	1.0	2.0	3.0	1.0	2.0	3.0	4.0	5.0	6.0	1.0	2.0	3.0	4.0	5.0
Phase	1	2	3[a]	4	5	6	7	8	9[a]	1	2	3	4	5[a]
Biogas production rate (L/L·d)	0.7 (0.0)[b]	1.6 (0.1)	–	0.9 (0.1)	1.5 (0.0)	2.1 (0.0)	2.7 (0.1)	4.0 (0.1)	–	0.7 (0.0)	1.4 (0.0)	1.7 (0.0)	2.7 (0.0)	–
Biogas yield (L/g VS_{fed})	0.711 (0.027)	0.789 (0.037)	–	0.937 (0.083)	0.770 (0.019)	0.785 (0.013)	0.739 (0.017)	0.813 (0.022)	–	0.804 (0.013)	0.771 (0.008)	0.703 (0.026)	0.770 (0.013)	–
Methane production rate (L/L·d)	0.4 (0.0)	0.9 (0.0)	–	0.6 (0.0)	0.8 (0.0)	1.2 (0.0)	1.6 (0.1)	2.3 (0.0)	–	0.4 (0.0)	0.8 (0.0)	0.9 (0.1)	1.5 (0.0)	–
Methane yield (L/g VS_{fed})	0.409 (0.016)	0.463 (0.021)	–	0.312 (0.000)	0.412 (0.004)	0.450 (0.009)	0.443 (0.020)	0.466 (0.000)	–	0.472 (0.011)	0.444 (0.005)	0.384 (0.028)	0.440 (0.011)	–
VFAs (g COD/L)	0.0 (0.0)	0.0 (0.0)	7.0 (0.0)	0.5 (0.1)	0.1 (0.1)	0.3 (0.3)	0.1 (0.0)	0.2 (0.1)	30.0	0.1 (0.1)	0.0 (0.0)	0.2 (0.0)	0.1 (0.0)	8.5
Soluble COD (g/L)	1.0 (0.1)	1.1 (0.3)	8.2 (0.1)	3.5 (0.4)	3.1 (0.0)	2.7 (0.1)	1.6 (0.0)	1.6 (0.3)	39.0	0.6 (0.0)	0.9 (0.0)	1.0 (0.1)	1.1 (0.1)	8.3
COD removal (%)	80.4 (0.8)	74.8 (1.5)	70.1 (0.9)	85.3 (2.3)	84.5 (2.4)	76.8 (1.7)	76.4 (3.0)	71.4 (0.6)	25.0	84.7 (0.6)	81.7 (4.4)	82.5 (2.0)	76.0 (2.3)	77.0
VS removal (%)	81.5 (0.2)	78.8 (0.4)	76.2 (0.0)	85.7 (0.0)	85.2 (0.6)	79.6 (0.0)	74.9 (0.7)	74.7 (0.4)	66.7	84.5 (0.1)	82.3 (0.1)	81.4 (0.6)	78.8 (0.9)	–
FA (mg NH_3-N/L)	–	52 (14)	103 (0)	118 (2)	92 (3)	65 (2)	44 (1)	106 (0)	3	–	152 (6)	69 (9)	73 (6)	94

[a] The reactor failed to reach a steady state. Data were collected at the end of the experimental phase.

[b] Standard deviations are in parentheses.

[c] Not determined

3.4. Summary

Under uncontrolled pH condition, Ra could produce acidic products with bioaugmentation and then Rm was performed to similar and lower methane production rate with Rs without acidic or VFAs inhibition. However, the two-phase system failed at an OLR of 5.0 g VS/L·d, while the single-phase system achieved a steady performance at the same OLR and then higher biogas productivity. Although Ra of the two-phase system could reduce the dosage of RF compared to Rs, Rs was more resilience at higher OLRs which was necessary to operate biological process. In addition, the installation and operation costs for two-phase system are more required than single-phase system, otherwise biogas productivity are lower than single-phase system. However, this result was different from previous studies to compare the single- and two-phase system treating FW at higher OLRs condition. In previous studies, it was reported that two-phase system was more stable and biogas production than single-phase system [57, 102, 103]. Because two-phase system provides optimum environmental conditions for each phase. Therefore, bioaugmentation with RF may more affect to the process performance in single-phase system compared to two-phase system at uncontrolled pH condition. Because RF contained acidifying bacteria and methanogen can provide a favorable effect for both acidogenesis and methanogenesis in single-phase system. This effect of bioaugmentation with RF may be advantage for maintaining resilience and enhanced performance in single-phase system with more diverse microbial community. Consequently, these results suggest that a single-phase configuration with high resilience and low cost is more appropriate than a two-phase system for the AD of FW using bioaugmentation under high OLRs conditions without pH control.

4. [Study 3] Sustainability and stability effect against fluctuations in Korean food waste with kimjang waste under bioaugmentation with rumen culture

4.1. Introduction

AD is widely used for FW management because of the ability of AD to mineralize organic pollutants into CH_4 and CO_2. FW, characterized by high organic content, is a good feedstock for realizing high biogas yields through AD. Nevertheless, the AD of FW is often fraught with the challenges of low process efficiency and poor process stability. The composition of FW in terms of carbohydrates, lipids, and proteins varies depending on the source of generation of FW, such as houses, restaurants, and industries. Such composition changes have various inhibitory effects, for example, VFA accumulation with a pH drop and reduced buffer capacity owing to the presence of an easily biodegradable fraction, high levels of FA owing to high protein content, and high levels of long-chain fatty acids or foaming problem owing to the presence of lipids. These effects can reduce process efficiency and cause process instability, mainly through the inhibition of

methanogenesis as the end reaction of AD. Moreover, in Korea, FW consists mainly of vegetable and fruit wastes (more than 50%), and its composition varies on daily and seasonal bases [8]. Especially, in the "Kimjang" season in late autumn, the preparation of large amounts of kimchi leads to a severe increase of the amount of kimjang waste (KW), which accounts for approximately 20% of the total Korean FW generation in that period. This increase in FW generation causes overloading of the AD process for treating surplus wastes. If the treatment capacity of the process is exceeded, excess VFAs from easily biodegradable organics accumulate, leading to a decrease in pH drop and process imbalance between acidogenesis and methanogenesis. The possible eventual outcomes include process instability and failure. Additionally, KW containing mainly Napa cabbage, which has a large content of complex fibers, is not easily biodegradable, which affects hydrolysis efficiency. Therefore, for the AD of Korean FW, it is crucial to maintain process stability and resilience against fluctuations in the composition and generation of FW and improve the efficiency of hydrolysis of the complex organic matters in FW.

Bioaugmentation, which involves introducing exogenous microorganisms with desired metabolic functions into a microbial system, is considered a viable approach for improving the biodegradability of fibers. It can be performed by adding one or more known species or a mixed consortium of diverse species. The latter can be more advantageous from the viewpoint of maintaining robustness of the augmented system, given that bioaugmentation is essentially a method for exogenously increasing the functional diversity and redundancy of a microbial community [25]. In addition, the strategy of a using a pre-adapted culture as opposed to a pure culture offers is beneficial for bioaugmentation because it does not concern the growth and survival of the augmented source in the new environmental condition.

Natural fiber-degrading systems, such as rumen, can serve as a mixed-consortium source for bioaugmentation to promote the degradation of fibers. Rumen microorganisms constitute a robust, naturally formed anaerobic consortium that is effective for hydrolyzing and fermenting diverse organic substances, including complex fibrous compounds [104]. Rumen microorganisms interact with each other to degrade and convert complex macromolecules into simple molecules, for example, organic acids and H_2/CO_2, and finally into CH_4. This allows for the maintenance of a low hydrogen partial pressure in the rumen for efficient AD of the feed [27]. Therefore, rumen microorganisms may have potential for improving the AD of FW in terms of the hydrolysis of complex matters and maintaining the process stability through methanogenesis with H_2 and acetate as intermediates of hydrolysis and acidogenesis, respectively. Because of this ability of rumen microorganisms, RF and microorganisms originated from rumen microflora have been introduced to pretreat the substrate or inoculate the culture for enhanced biomethanation of different organic wastes, including FW [28-30]. Previous studies have reported increased biodegradability when using RF than that when using anaerobic microorganisms in anaerobic digesters [31, 41]. These results indicate the good potential of RF as a bioaugmentation source in AD. Most studies thus far have focused solely on the pretreatment effect. However, the feasibility of using RF for bioaugmentation of the overall performance of AD in treating FW or

for sustaining stable performance levels against process fluctuations has rarely been studied. Even though RF is beneficial as a bioaugmentation source for improving the performance of AD, its application to full-scale systems is difficult because cow rumen is scarce compared to anaerobic sludge. As one option, an inoculum culturing system can be used to solve the scale up problem by securing large amounts of rumen microorganisms. In addition, the use of pre-adapted rumen cultures under FW-fermenting conditions helps maintain specific functional activities and retains the augmented microorganisms in the indigenous system. Therefore, the culturing system can provide one-time or sustainable supplementation of the bioaugmentation source for enhancing efficiency or resilience.

In studies 1 and 2, the feasibility of bioaugmentation with RF for the AD of FW was confirmed with enhanced biomethanation in the short-term. However, there remained questions about an alternative method given the scarcity of RF, process stability, process resilience against seasonal fluctuations in Korean FW, especially during Kimjang season. Therefore, in study 3, to determine the potential of rumen culture (RC) obtained from an RF culturing system and enhancement of the anaerobic degradability of KW, firstly, it was examined to use three different inocula for the biomethanation of KW and cellulose: anaerobic sludge, RC, and mixed culture of anaerobic sludge and RC in lab-scale bioreactors of treating FW. Then, a continuous single-phase process was implemented to determine its stability and sustainability against fluctuations in FW with co-digestion of KW under bioaugmentation with RC as the original strategy.

4.2. Materials & methods

4.2.1. Subculture batch tests

KW was prepared by grinding mainly uncooked Napa cabbage waste collected from a cafeteria at UNIST with a household blender. Cellulose powder (medium fibers) was purchased from Sigma-Aldrich. Digestates from three different lab-scale continuous anaerobic digesters fed with FW, i.e., one inoculated with anaerobic sludge from a full-scale AD plant co-digesting FW and primary sewage sludge (Rs), one inoculated with RF collected through a rumen fistula from a cow (Rr), and one inoculated with anaerobic sludge and augmented with rumen culture from Rr (Rm), were used as inocula for the AD tests. Collected digestates were sieved (mesh size, 860 μm) to remove coarse particles and starved under anaerobic conditions (14 days at 35°C) prior to inoculation to minimize the endogenous biogas production.

Six substrate-inoculum combinations (2 substrates × 3 inocula) were tested for biogas production in batch mode in parallel with five controls (2 without inoculum and 3 without substrate). Each run was triplicated, and a total of 33 bottles were prepared. All test bottles were serially subcultured for three cycles at 35°C for 30 days per cycle. The detailed experimental conditions for anaerobic subculture tests are described in Table 4-1. Subcultures were prepared in 120-mL serum bottles and flushed with nitrogen gas to remove oxygen in

the headspace before being gas-tight sealed with rubber stoppers. The amounts of inoculum and substrate added to a test bottle were adjusted according to the available amount of inoculum, i.e., digestate from the preceding subculture cycle, over subcultures to maintain the substrate-to-inoculum (S/I) ratio (on a VS basis) at similar levels. The remaining volume in the test bottle was filled with distilled water as necessary. Biogas production from each bottle was periodically measured using a gas-tight syringe and corrected to standard temperature and pressure (0°C and 1 bar).

Table 4-1. Experimental conditions for subculture tests.

	Cycle 1		Cycle 2		Cycle 3	
Substrate	KW	CL	KW	CL	KW	CL
Inoculum[a]	600	600	203	203	84	84
CL[a]	0	346	0	106	0	42
KW[a]	510	0	138	0	60	0
Test volume[b]	80	80	50	50	50	50
S/I ratio[c]	0.85	0.58	0.68	0.52	0.71	0.5

[a] mg VS

[b] mL

[c] Substrate-to-inoculum ratio determined on a VS basis.

4.2.2. Substrates, inoculum, and rumen culture in continuous tests

FW was collected from a cafeteria at UNIST and ground using a household blender. Seasonal KW was prepared by grinding uncooked Napa cabbage with a household blender. The prepared FW was adjusted to a VS content of 10% (w/v) with tap water for only feeding FW. For co-digestion, the fractions of FW and KW (FKW) were adjusted to 9:1 (10% co-digestion with KW) and 8:2 (20% co-digestion with KW) based on the substrate VS of 100 g/L, respectively. The physicochemical characteristics of the substrates are summarized in Table 4-2. The prepared substrates were stored at 4°C before use in subsequent experiments. Anaerobic sludge from a full-scale AD plant co-digesting sewage sludge and FW were inoculated in the AD experiments. Rumen culture (RC), used as the bioaugmentation source, was collected from a laboratory-scale AD reactor inoculated with RF treating FW (Fig. 4-1). It was semi-continuously fed with FW. Anaerobic sludge and RC were both sieved (mesh size, 860 μm) to remove coarse particles and used in the experiments.

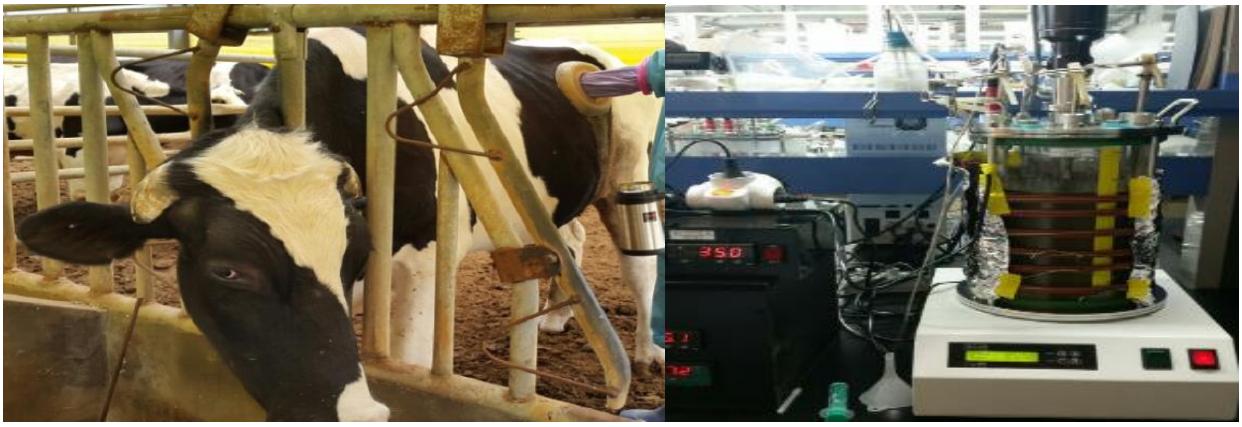


Figure 4-1. Laboratory-scale RF culturing system.

Table 4-2. Physicochemical characteristics of substrates.

	P1 (210–331)[a]	P2 (332–443)		P2 (444–499)		P3 (500–624)		P3 (625–736)		P3 (737–800)		P3 (801–920)		P4 (921–1000)	P5 (1001–1145)	
	FW	FW	FKW	FW	FKW	FW	FKW	FW	FKW	FW	FKW	FW	FKW	FW	FW	FKW
TCOD (g/L)	123.1	142.3	153.2	116.7	115.6	130.6	129.3	102.0	110.7	171.5	180.7	218.2	193.1	175.6	148.1	164.7
SCOD (g/L)	70.4	66.7	62.4	50.2	62.4	49.2	-	60.7	63.5	64.3	63.7	66.6	63.3	64.7	53.5	61.0
TVFA (g COD/L)	3.4	2.4	1.5	1.3	1.6	0.7	-	3.9	3.7	2.5	2.8	3.5	3.0	3.6	1.6	2.4
TS (g/L)	105.7	107.1	108.1	89.6	92.3	96.9	96.9	95.7	100.6	106.7	110.0	110.0	115.7	108.9	103.3	108.0
VS (g/L)	99.5	103.5	102.7	84.5	86.9	90.5	88.8	88.7	91.7	100.0	99.9	102.6	105.9	100.9	96.8	99.2
TC (g/L)	60.6	68.4	72.5	51.5	72.5	19.4	21.3	60.9	47.5	57.7	80.6	108.9	159.6	60.9	77.3	65.5
Crude fat (g/L)	18.6	23.1	15.3	4.6	4.4	9.3	17.9	6.0	6.8	11.4	13.0	19.8	20.0	12.1	15.5	12.8
Protein (g/L)	16.2	12.5	11.6	8.3	8.3	8.8	8.3	35.7	37.0	19.5	42.7	43.3	32.8	31.9	33.1	28.2
Crude fiber (g/L)	2.1	2.6	3.7	2.3	2.7	3.7	5.1	4.9	6.2	4.5	7.5	4.4	6.7	4.6	3.3	5.3
C/N ratio	21.3	30.7	25.7	11.7	16.3	9.9	8.5	11.9	11.0	11.8	10.7	12.3	11.4	12.8	16.0	13.4

[a] Periods (days).

*FKW = food waste and kimjang waste; TCOD = total chemical oxygen demand (COD); SCOD = soluble COD; TVFA = total volatile fatty acid; TS = total solids; VS = volatile solids; TC = total carbohydrates; C/N ratio = carbon to nitrogen ratio.

4.2.3. Continuous test operation

A CSTR with a working volume of 2 L was used in the following four configurations: co-digestion reactor without bioaugmentation (CR), co-digestion reactor with RC bioaugmentation (CB), FW digestion reactor without bioaugmentation (FR), and FW digestion reactor with RC bioaugmentation (FB). The reactors were initially inoculated with the same anaerobic sludge, fed with only FW (10% VS), and started up by gradually increasing the OLR to 2.5 g VS/L·d (HRT 40 days) during the first 106 days of operation. On day 7, RC from the laboratory-scale AD reactor was added in a ratio of 10% to the mixed liquor in each reactor (CB and FB) on a VSS basis. To determine the effect of seasonal fluctuations, CR and CB were initially fed with 10% co-digestion on day 331, which was changed to 20% co-digestion on day 500. Thereafter, only FW was fed on day 920, and finally, CR and CB with 20% co-digestion were fed on day 1000 (Fig. 4-2). Each phase was operated for at least for two turnovers.

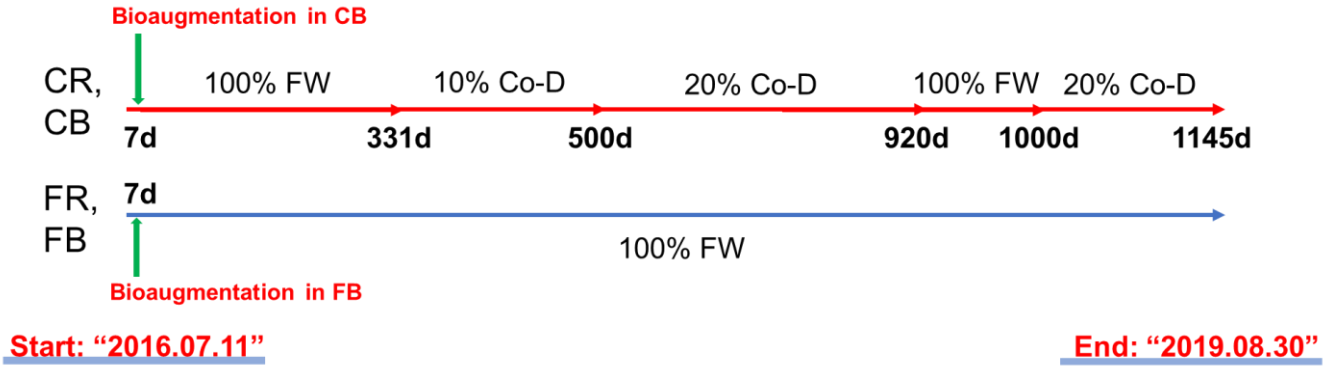


Figure 4-2. Overall experimental flow.

All reactors were operated under mesophilic conditions (35 ± 1°C) without pH control. Trace elements (100 mg Fe/L as $FeCl_3 \cdot 6H_2O$, 2 mg Co/L as $CoCl_2 \cdot 6H_2O$, and 1 mg Ni/L as $NiCl_2 \cdot 6H_2O$ in the substrate) were supplemented to the reactors every other day throughout the operation to support the growth of methanogens.

4.2.4. Molecular fingerprinting and sequencing

For subculture test, digestate was collected from a randomly selected bottle of each triplicate culture at the end of each subculture cycle and analyzed, along with the inocula, for microbial community structure. Total DNA was extracted from the inocula and digestate samples using an automated nucleic acid extractor (Exiprogen, Bioneer, Daejeon, Korea) as previously described [105].

The purified DNA was eluted in 200 μL of elution buffer and stored at –20°C until use. Archaeal and bacterial 16S rRNA genes were amplified by touch-down polymerase chain reaction (PCR) using ARC787F/1059R and BAC338F/805R primer sets with GC clamps attached, respectively, and analyzed by DGGE as previously described [105]. The DGGE gels were stained with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA) and visualized under blue light transillumination. Selected bands were cut out of the gel and eluted in 40 μL of sterile PCR-grade water. An aliquot of each elution was amplified by PCR using the same primer sets as for DGGE but without the GC clamp. The obtained amplicons were cloned (pGEM-T Easy vector (Promega, Madison, WI, USA)) and sequenced. The retrieved 16S rRNA gene sequences were compared against the GenBank and RDP databases for phylogenetic affiliation. Taxonomic assignment of the retrieved sequences was performed using the RDP classifier at a bootstrap confidence threshold of 80%. The nucleotide sequences reported in this study have been deposited in the GenBank database: MH478173–478184.

For continuous test, reactor samples were DNA extracted using an automated nucleic acid extractor (Exiprogen, Bioneer, Daejeon, Korea). The amplicon sequencing was performed according to the manufacturer's instructions (Illumina, CA, USA). Oligomers containing the Illumina overhang adapter sequence as well as the following 16S rDNA region specific sequence were used as amplicon primers: V3–4 region for bacteria, 518F (5'- CCAGCAGCCGCGGTAATACG -3') and 805R (5'-GACTACCAGGGTATCTAATCC -3') [106, 107] and V4-5 region for archaea, 787F (5'-ATTAGATACCCSBGTAGTCC -3') and 1059R (5'-GCCATGCACCWCCTCT -3') [107]. Sample DNAs were PCR-amplified with the annealing temperature set to 58°C. The purified amplicons were PCR-indexed using the Illumina Nextera XT index kit. Taq DNA polymerase kit (Solgent, South Korea) was used for the library construction. The purified library was quantified, pooled and combined with the PhiX control (Illumina). The library was paired-end (150 bp x 2) sequenced using the iSeq 100 platform. The paired reads were first merged and then processed to remove short or low-quality sequences and potential chimeras. More than 50000 filtered reads were obtained from each sample. Operational taxonomic units (OTUs) were defined at 97% sequence-identity cutoff using the VSEARCH algorithm [108]. Taxonomic assignment was conducted using the RDP classifier on-line (https://rdp.cme.msu.edu/classifier/).

4.2.5. Statistical analyses of microbial community data

For subculture test, a matrix each was generated from the archaeal and bacterial DGGE gel images based on the relative intensity (normalized to total band intensity) and position of each band in each lane analyzed using TotalLab 1D image-processing software (TotalLab, Newcastle, UK). For

continuous test, the relative abundance of individual OTUs in total bacterial and archaeal sequences was calculated for each sample and a quantitative matrix was generated for bacteria and archaea, based on the relative abundance data. Cluster analysis was performed on the matrix using the unweighted pair group method with arithmetic means (UPGMA) algorithm to visualize the relatedness between the analyzed bacterial and archaeal community structures. Non-metric multidimensional scaling (NMS) was conducted on the obtained matrices to visualize the direction and magnitude of changes in the archaeal and bacterial community structures. Calculations for clustering and ordination were performed based on the Sorensen distance measure [109] using the PAST software ver. 3.16 (https://folk.uio.no/ohammer/past/) and PC-ORD 6 software (MjM software, Gleneden Beach, OR, USA), respectively.

4.2.6. Analytical methods

Solids were analyzed through the protocols in Standard Methods. COD, total nitrogen (TN), and total phosphate (TP) were colorimetrically measured using HS-COD-MR kit, HS-TN(CA)-H kit, and HS-TP-H (HUMAS, Daejon, Korea), respectively. VFAs (C_2-C_7) were determined using a 7820A Gas Chromatograph (Agilent) equipped with a flame ionization detector and an Innowax column (Agilent). Samples for soluble COD and VFAs were filtered through a membrane filter 0.45 μm. Biogas composition was determined using a 490 Micro Gas Chromatograph (Agilent) equipped with a thermal conductivity detector (TCD). Anions and cations which were prepared by filtration through a filter (pore size, 0.22 μm) were analyzed two ICS-1100 Ion Chromatographs (Thermo Scientific) equipped with IonPac AS14 and IonPac CS12A columns. Alkalinity was measured using ORION Total Alkalinity Test Kit (Thermo Scientific). C, H, O, N, and S contents were determined on a dry weight basis using a Flash 2000 Elemental Analyzer (Thermo Scientific). Trace elements were analyzed using an inductively coupled plasma-optical emission spectrometer (700-ES, Varian). Crude fat was measured using Fat Analyzer (ST 255 Soxtec, FOSS). Total Kjeldahl nitrogen was determined using a Protein Analyzer (Kjeltec 8200, FOSS). Crude fiber was analyzed using a FiberCap with hotplate 2022 (FOSS). All analyses were replicated at least twice.

4.3. Results & discussion

4.3.1. Subculture batch results

No significant biogas production was found in the substrate-only control, and the biogas production from each run was corrected by subtracting that of its inoculum-only control. Biogas

production started immediately after the initiation of incubation without a lag phase in the runs with KW, while a lag phase of up to 6 days was observed in those with CL, in the subcultures (Fig. 4-3). This suggests that all inocula required an adaptation time to be able to utilize CL regardless of the inoculum source, and KW contains a considerable amount of more readily utilizable organic compounds than CL. Interestingly, a stagnant phase occurred during the mid-incubation period in the KW runs, particularly in the first cycle, but not in the runs with CL. The biphasic biogas production pattern likely reflects the sequential utilization of more and less easily biodegradable organic components of KW. Acidification, i.e., accumulation of acids with fermentation of easily biodegradable matter, can inhibit methanogenesis and also be a reason for a stagnant phase [94]. Several studies have reported the inhibition of methanogenesis by acidification in AD processes treating vegetable wastes [51, 56, 110]. However, given that the mixed-liquor pH was higher than 7.2 at the end of each subculture cycle for all runs and the S/I ratio was in an appropriate range for the biogas potential assay [111, 112], the temporary stagnation of biogas production was unlikely to be caused by acidification. CL should first be hydrolyzed by extracellular enzymes to be used for subsequent acidogenesis and methanogenesis [113]. The results suggest that cellulolytic activity was low in the inocula but increased with subculturing, as seen by the decrease in lag time with cycles.

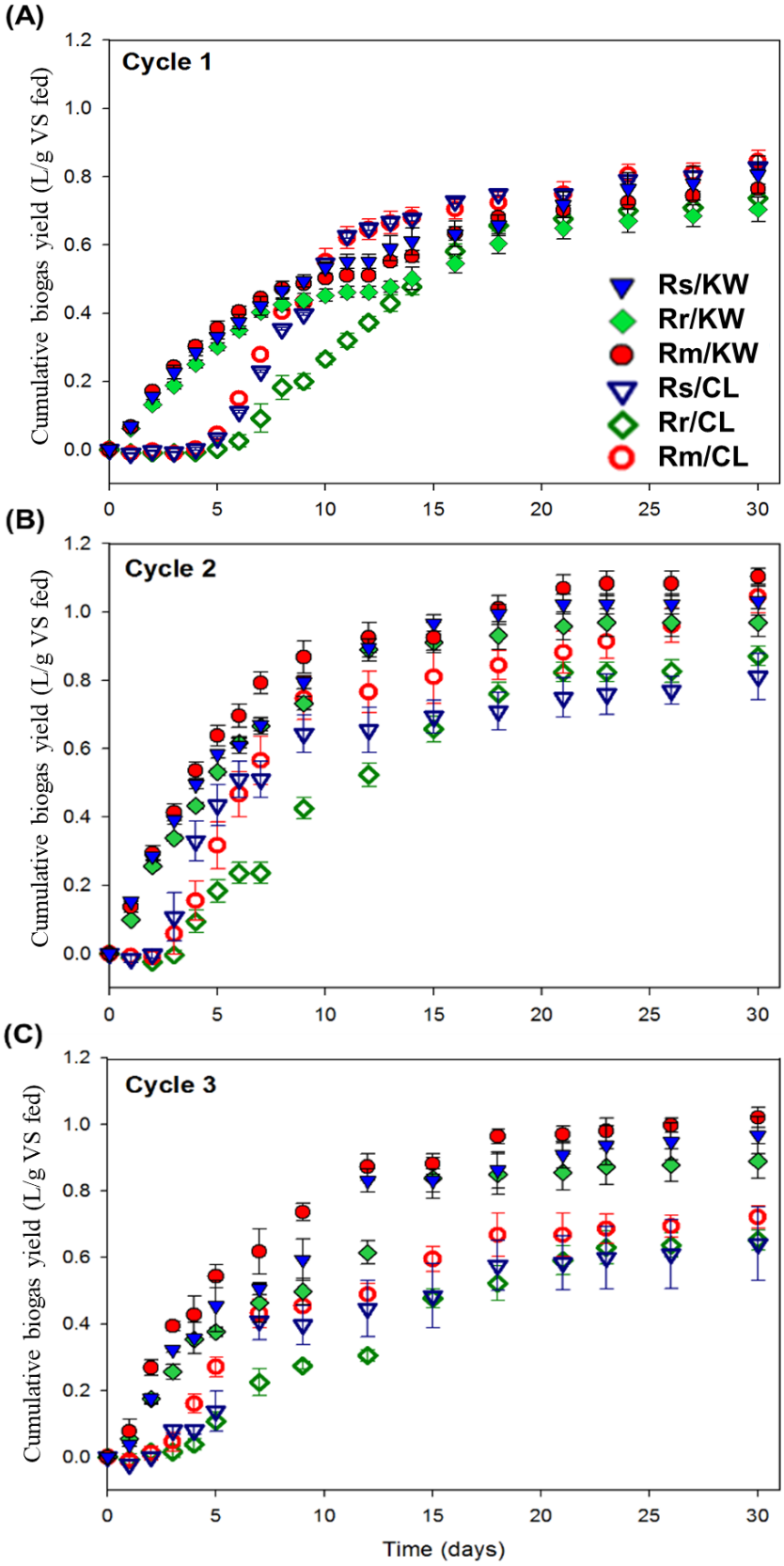


Figure 4-3. Cumulative biogas yield during subcultures. Curves are labeled with the corresponding inoculum sources and substrates.

Despite the lag phase of 5–6 days, the CL runs reached comparable or greater cumulative biogas yield than the KW runs after 30 days of incubation in Cycle 1. This may be explained in part by the need for the inocula to adapt to be able to grow on the less biodegradable fibers of KW after the depletion of readily utilizable organic matter. The KW runs were subjected to such metabolic stress after 10 days of incubation, and the time allowed for digesting less biodegradable components (approximately 15 days) might not be sufficient for their complete utilization. Another possibility may be the high content of complex fibers with low biodegradability in KW [9, 10], which can limit the utilization of KW. However, it appears that the former is more likely than the latter, given that the KW runs showed greater biogas yields than the CL runs in Cycles 2 and 3. The methane content was maintained fairly constant at 50–60% in the KW runs over subculture cycles, while it remained at lower levels (≤50%) in the CL runs. This may be attributed to the different characteristics of the substrates.

The cumulative biogas production profiles of the subculture tests were fitted to a modified Gompertz equation (Eq. 4-1) to describe the biogas production kinetics:

$$B_t = B_P \cdot \exp\left[-\exp\left\{\frac{R_{\max} \cdot e}{B_P}(\lambda - t) + 1\right\}\right] \qquad \text{Eq. (4-1)}$$

where B_t is the cumulative biogas yield (L/g VS_{fed}) at time t, B_p is the biogas yield potential (L/g VS_{fed}), R_{max} is the maximum biogas production rate (L/g VS_{fed}·d), λ is the lag phase length (day), and t is the incubation time (day). All runs showed a good fit to the equation with a high regression coefficient (r^2 >0.96). The estimated model parameters are presented in Table 4-3.

Table 4-3. Modified Gompertz parameters estimated from the subculture experiments.

Inoculum	Rs			Rr			Rm		
Subculture cycle	C1	C2	C3	C1	C2	C3	C1	C2	C3
KW tests									
Biogas yield[a]	0.81	1.03	0.97	0.70	0.97	0.89	0.76	1.10	1.02
B_P[b]	0.77	1.02	0.94	0.68	0.96	0.90	0.73	1.04	0.98
R_{max}[c]	0.14	0.27	0.21	0.11	0.28	0.17	0.13	0.33	0.25
λ[d]	–[e]	–	–	–	–	–	–	–	–
r^2	0.98	0.99	0.98	0.96	0.99	0.98	0.96	0.98	0.98
CL tests									
Biogas yield	0.83	0.81	0.64	0.74	0.87	0.65	0.85	1.05	0.72
B_P	0.78	0.73	0.59	0.73	0.86	0.68	0.78	0.91	0.68
R_{max}	0.30	0.35	0.17	0.17	0.18	0.11	0.28	0.36	0.19
λ	5.00	1.90	2.34	5.95	3.03	3.01	4.51	2.74	1.79
r^2	1.00	0.98	0.97	1.00	0.99	0.99	0.99	0.98	0.98

[a] The observed biogas yield (L/g VS_{fed}).

[b] B_P, the biogas yield potential (L/g VS_{fed}).

[c] R_{max}, the maximum biogas production rate (L/g VS_{fed}·d).

[d] λ, the lag phase length (day).

[e] Not detected.

Consistently with the experimental observations, a lag phase was identified in all runs with KW but not in the runs with CL. The estimated lag length decreased greatly in the subsequent subcultures compared to the initial culture. This suggests that the cellulolytic activity of the inoculated microbial consortia increased while adapting to the culture conditions using CL as the sole carbon source with cycles. It is interesting to note that after the initial adaptation to new substrates (i.e., KW and CL) during Cycle 1, the runs inoculated with Rm showed superior performance, in terms of biogas yield (by 5.2–14.6% based on the observed yields) as well as production rate (by 17.9–47.1% in R_{max}), to the runs inoculated with the other inocula for both substrates. It was also higher than the results of previous studies with 0.450 L/g VS_{fed} from Chinese cabbage, 0.620 L/g VS_{fed} from kimchi factory waste silage [114], and 0.256 L CH_4/g VS_{fed} from Chinese cabbage[115]. This indicates that the Rm microbial consortium responded most favorably and readily to the sudden substrate changes from FW to KW or CL. This appears to be related to the higher microbial diversity of Rm than of the other inoculum sources, given the inoculation and bioaugmentation history of the source digesters (see Subsection 4.2.1.). More diverse microbial communities would be expected to have higher chances of being functionally more versatile and redundant, which can be beneficial in adapting to changes in the

environment. The experimental results suggest that the bioaugmentation with RC had a positive effect on the metabolic capability of the Rm microbial consortium, particularly the fiber-degrading activity. It is notable that the differences in performance according to the inoculum source were more pronounced in reaction rate than biogas yield. This indicates that the beneficial effect of using the bioaugmented Rm inoculum was primarily on the hydrolysis and fermentation, which limit the overall reaction rate in the AD of complex fibers, rather than the methanogenesis.

All subculture runs showed the highest biogas production in Cycle 2, except the CL run inoculated with Rs, where the biogas yield in Cycle 2 was slightly lower but comparable to that in Cycle 1. Interestingly, an apparent reduction in biogas yield was observed between Cycles 2 and 3 in all runs despite the serial subculture in batch mode would provide a stable environment for microbial adaptation and growth [66]. This may be attributed to the decrease in the absolute amount of inoculum added to an assay over subculture cycles (Table 4-3). The inoculum size decreased by approximately 60–65% each cycle because the digestate from a test run after a cycle was used as inocula for the test subculture and the inoculum-only control bottles in the next cycle. Given that inoculum size is a crucial factor affecting microbial growth and activity in AD processes [116, 117], this may have adversely affected the methanogenic performance in Cycle 3.

4.3.2. Microbial community structure of subculture tests

The microbial community structures in the test runs at the end of each subculture cycle and the inocula were analyzed by DGGE and sequencing. Since the DGGE gels were run separately according to the substrate used for the subculture tests, two gel images each were produced for archaea and bacteria. The images were aligned using the DNA samples of the inocula, which were loaded on both gels, as position markers to compare band patterns between the gels. Three archaeal (A1 to 3) and nine bacterial (B1 to 9) bands were selected and cut out of the gels for sequencing analysis (Figs. 4-4 and 4-5). The phylogenetic affiliations of the retrieved sequences are summarized in Table 4-4.

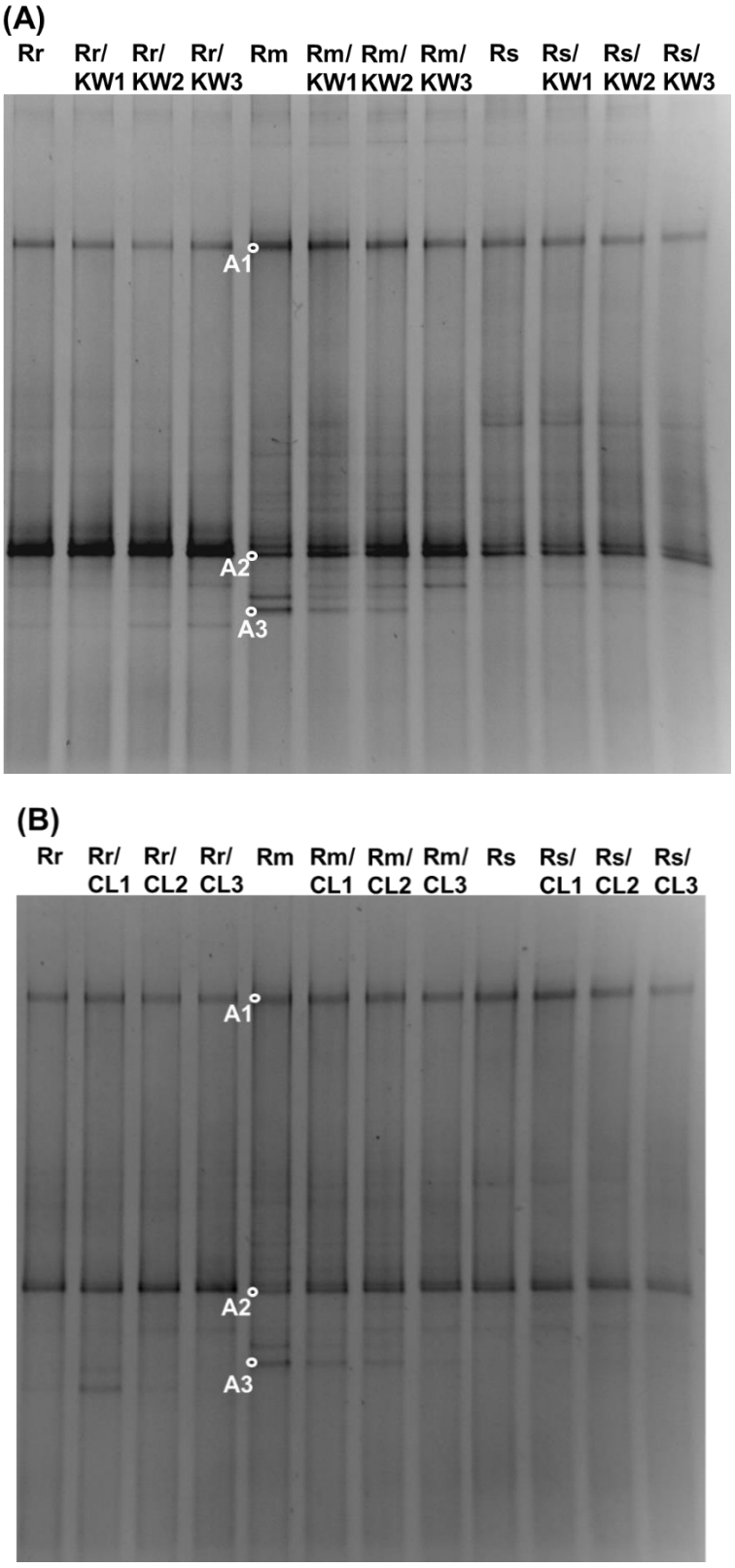


Figure 4-4. Archaeal DGGE fingerprints of the anaerobic subcultures with KW (A) and CL (B). Lanes are labeled with the inocula and subculture cycles.

All archaeal sequences were assigned to methanogen genera, agreeing with the general understanding that archaea in AD environments are mostly methanogens. A1 was assigned to hydrogenotrophic *Methanolinea*, while A2 and 3 were assigned to aceticlastic *Methanosaeta* and *Methanosarcina*, respectively. A1 and 2 appeared as predominant bands in all lanes, indicating that the corresponding *Methanolinea* and *Methanosaeta* populations were likely the major methanogens throughout the subculture regardless of inoculum or substrate. These suggest that methanogenesis occurred through both hydrogenotrophic and aceticlastic pathways in the subcultures and inoculum sources. *Methanolinea* and *Methanosaeta* have frequently been found in various AD processes treating different types of waste [79, 118, 119]. Given that *Methanolinea* species are hydrogenotrophic but require acetate for growth [120], the organism represented by A1 likely contributed not only to the scavenging of hydrogen but also partly to the consumption of acetate. *Methanosaeta* species are strictly aceticlastic and known to play a key role in stabilizing an AD system to maintain low levels of residual acetate and other VFAs [59]. The organism corresponding to A2 seems to be primarily responsible for this role in the subcultures. The *Methanosarcina* population corresponding to A3 appeared in the subcultures inoculated with Rm and Rr at cycle 1. *Methanosarcina* species are metabolically versatile and able to utilize H_2/CO_2 and simple methyl compounds other than acetate, and their growth is reportedly favored at relatively high concentrations of residual VFAs [59]. It indicates that the acidogenesis producing VFAs in Rm is probably enhanced by symbiotic relationship between microorganisms of anaerobic sludge and RC. Consecutively, high produced VFAs may favorably affect to grow the *Methanosarcina* in Rm. A point to note is that A3 showed the highest band intensity in the Rm inoculum and gradually disappeared with subculture cycles for both substrates. This suggests that Rm was presumably under more favorable conditions for *Methanosarcina* to grow (i.e., greater amounts of residual fermentation intermediates) compared to the other inoculum sources.

Table 4-4. Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands.

Band	Closest relatives	Accession number	Similarity (%)	Classification[a]
Archaea				
A1	*Methanolinea tarda*	NR028163	97.4	*Methanolinea*
A2	*Methanosaeta harundinacea*	NR043203	98.2	*Methanosaeta*
A3	*Methanosarcina spelaei*	NR148337	99.6	*Methanosarcina*
Bacteria				
B1	Uncultured bacterium clone 39909	MF769179	100.0	*Prolixibacteraceae*
	Prolixibacter denitrificans	NR137212	86.8	
B2	Uncultured bacterium clone CloningB5+C09	AB997663	100.0	*Bacteroidales*
	Natronoflexus pectinivorans	NR108635	87.7	
B3	Uncultured bacterium clone JKB083	LN624310	100.0	*Bacteroidales*
	Tangfeifania diversioriginum	NR134211	89.6	
B4	Uncultured bacterium clone TC(4)9	KJ734920	99.8	*Bacteroidetes*
	Solitalea canadensis	KF528160	88.4	
B5	Uncultured bacterium clone dgD-107	AB264072	98.0	*Lachnospiraceae*
	Lachnospira multipara	NR104758	96.6	
B6	*Petrimonas sulfuriphila*	LT558828	99.8	*Petrimonas*
B7	Uncultured bacterium clone CloningB3A07	AB997288	99.8	*Ruminococcaceae*
	Saccharofermentans acetigenes	NR115340	87.4	
B8	Uncultured bacterium clone QEDN5CD04	CU926267	100.0	*Firmicutes*
	Desulfotomaculum alcoholivorax	NR042970	86.6	
B9	*Atopobium* sp. canine oral taxon 418	KF030213	99.3	*Coriobacteriaceae*

[a] The lowest rank assigned by the RDP Classifier at a bootstrap cutoff of 80%.

The bacterial sequences retrieved from the DGGE bands were assigned to three phyla *Bacteroidetes* (B1, 2, 3, 4, and 6), *Firmicutes* (B5, 7, and 8), and *Actinobacteria* (B9), commonly present in AD environments (Fig. 4-5 and Table 4-4). The *Bacteroidetes*-related bands appeared in all

lanes although their intensities varied greatly, particularly according to the inoculum source. B1 was observed with a strong intensity in all lanes, indicating that the corresponding bacterium was commonly abundant in all inocula and able to grow well regardless of substrate. B2 and 3 appeared as more prominent bands in the subcultures inoculated with Rm or Rs, and so did B4 in the Rr-inoculated subcultures. Although their roles are unclear, the *Bacteroidetes*-related bacteria were likely involved in the hydrolysis of cellulose and other fibers given that *Bacteroidetes* species have been reported to play a key role in decomposing cellulosic matter in AD processes [121]. B6, the only one classified at the genus level, was closely related to *Petrimonas sulfuriphila* capable of utilizing glucose and cellobiose to produce acetate, H_2/CO_2, and H_2S [122]. B5 and 7 were assigned to the families *Lachnospiraceae* and *Ruminococcaceae*, respectively, belonging to the order *Clostridiales*. Members of the families are present in abundance in mammalian guts and can degrade various fibrous matter including recalcitrant compounds [123]. Although below the cutoff, B5 showed a considerable similarity of 96.6% to a pectin-hydrolyzing rumen bacterial species *Lachnospira multipara* [124]. Given that B7 was observed only in the subcultures inoculated with Rs or Rm, the bacterium represented by this band likely originated from the anaerobic sludge used to inoculate Rs and Rm (see Subsection 2.1). B9 was closely related to an *Atopobium* species whose relatives are commonly found in rumen and gut microbial consortia and able to ferment cellulose [125, 126]. This band appeared with significantly higher intensity in the Rr-inoculated subcultures than in the Rm- and Rs-inoculated ones. The bacterium corresponding to B9 was likely to be a major cellulose degrader in Rr and the Rr-inoculated subcultures. Although most bacterial sequences were poorly related to known species, our results were in accordance with the finding that cellulose-degrading bacteria belong mainly to the phyla *Bacteroidetes* and *Firmicutes* in the human gut [126].

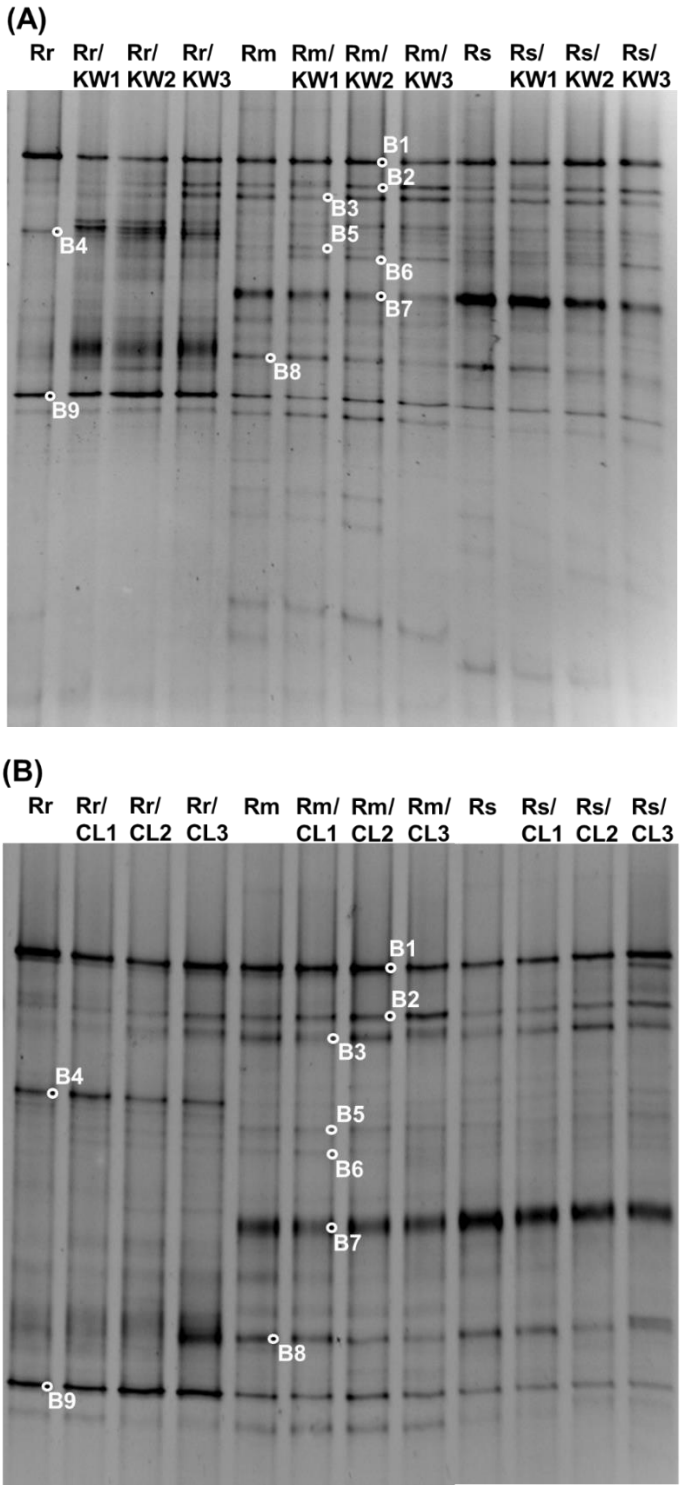


Figure 4-5. Bacterial DGGE fingerprints of the anaerobic subcultures with KW (A) and CL (B). Lanes are labeled with the inocula and subculture cycles.

Bacterial DGGE profiles were much more complex and dynamic then archaeal DGGE profiles in all subculture runs. This reflects that archaeal communities generally have less diverse structures than

bacterial communities in AD processes, largely due to the very narrow substrate spectrum of methanogens [66, 127]. Figure 4-6 shows the NMS plots describing the changes over subcultures in the archaeal and bacterial community structures. NMS is an ordination method which can reduce a DGGE profile generated from a microbial community (i.e., a DGGE lane) into a point in an ordination space so that communities with similar structures are closely located in the space. Both the plots showed acceptable stress (<20) and sufficiently low instability (10^{-4}) values, indicating that the ordination results provide a reliable picture of the changes in the microbial community structures in the subcultures [109]. The cumulative r^2 for the ordination axes was 0.947 and 0.832 in the archaeal and bacterial NMS plots, respectively. This means that 94.7% and 83.2% of the total variance in the analyzed archaeal and bacterial community structures, respectively, can be explained by the obtained NMS plots. It is clearly shown in the NMS plots that both archaeal and bacterial community profiles are clustered according to the substrate and inoculum source rather than to the subculture.

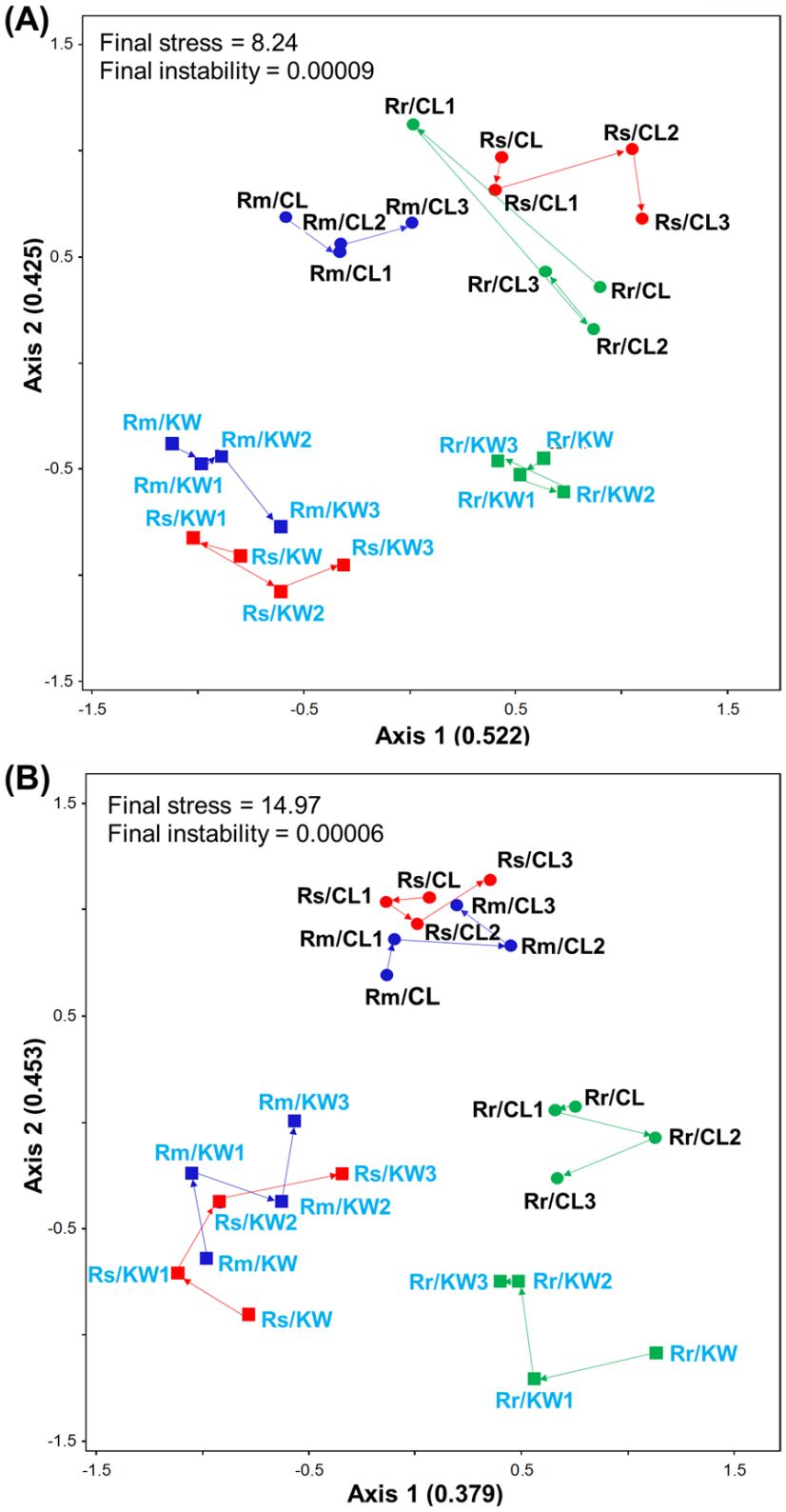


Figure 4-6. NMS plots showing changes in the archaeal (A) and bacterial (B) community structures. Points are labeled with the corresponding inoculum sources followed by the substrates and subculture cycles. Arrows indicate the shifts in community structure with subculture cycles in each run.

This suggests that both substrate and inoculum characteristics likely had a significant influence on the development of microbial community structures in the subcultures, which agrees with previous findings in AD processes [66, 128]. Meanwhile, the changes in microbial community structure over subcultures were relatively minor. The archaeal and bacterial community structures of the subcultures inoculated with the same inoculum were clearly separated according to the substrate. Meanwhile, among the community profiles of the subcultures with the same substrate, those of the Rs and Rm subcultures were located close together, with those of the Rr subcultures being grouped separately. This would be expected given that Rm and Rs were initially inoculated with the same anaerobic sludge, although Rm was later augmented with RC. It is worth noting that the archaeal community structures showed a clearer separation between the subcultures inoculated with different inocula compared to the bacterial community structures. This indicates that the bioaugmentation of Rm with RC likely had a more significant effect on the archaeal community structure than the bacterial community structure in the digester. This possibility can be associated with the less diverse nature of archaeal communities than bacterial communities in AD environments [127], because a small change in band pattern (i.e., appearance or disappearance of one or a few populations) can result in a significant structural change in simple communities. It may also be attributed in part to the distinct methanogen community structure in the rumen, often characterized by the high abundance of hydrogenotrophs, from those in typical anaerobic digesters [27].

Consequently, for both substrates, the subcultures inoculated with Rm showed superior biogas yield and production rate to those inoculated with the other inoculum sources. Both archaeal and bacterial community structures in the subcultures were significantly influenced by the substrate characteristics and the inoculum source. The overall results demonstrated that the RC augmented to Rm likely maintained the fiber-degrading activity and enhanced the AD of the fiber-rich substrates (KW) over repeated subcultures. Given that the inocula were sourced from three anaerobic FW digesters operated for more than ten turnovers of the working volume, the experimental results further suggest the possibility of using mixed culture digestates from bioaugmented or co-inoculated digesters, like Rm in this study, as a microbial source for bioaugmentation. This may help avoid the difficulties in collecting large amounts of RF or culture for augmenting digesters. The outcomes of this study may help with more efficient treatment of large amounts of fruits and vegetables in the AD of Korean FW. Therefore, long term test in continuous mode was examined with co-digestion of FW and KW using bioaugmentation (see 4.3.3.).

4.3.3. Results of process performance with various parameters in continuous test

4.3.3.1. Biogas production performance

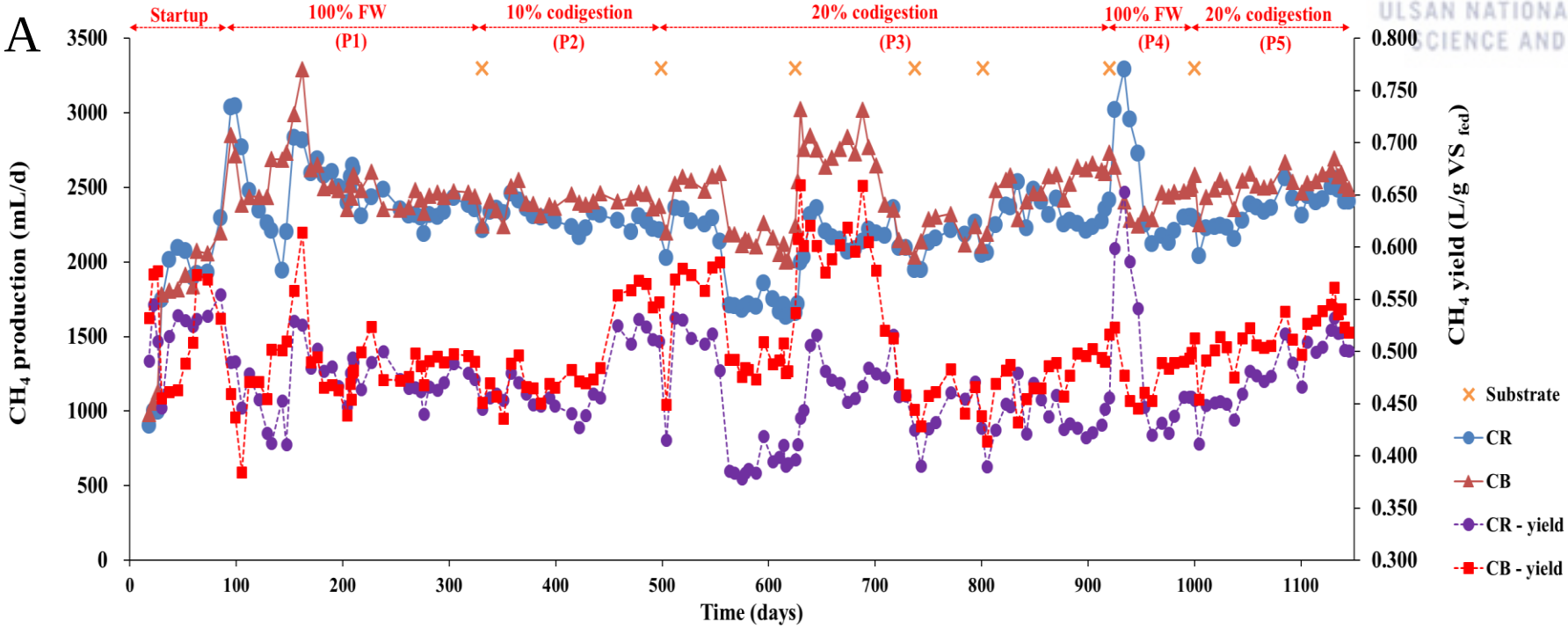
Variations in the methane production performance of the four reactor configurations during the experiment conducted over 3 years with changing FW composition and co-digestion ratio are shown in Fig. 4-7. The reactors were operated by increasing the OLR from 1.0 to 2.0 g VS/L·d during the start-up period of 106 days and then to OLR 2.5 g VS/L·d. The 10% (w/w) bioaugmentation with RC was applied to CB and FB on day 7. Methane production increased gradually with increasing OLR in the start-up period. The methane production rates of the four reactors were stabilized and were similar in P1 with the 100% FW substrate over five turnovers. After 10% co-digestion of CR and CB in P2, methane production in CB gradually increased to more than that in CR. The methane yield in CB increased by 7.7% compared to that in CR in the steady state (days 471–497). By contrast, the differences between FR and FB were not statistically significant. In this phase, the mixture of FW and KW (FKW) contained higher TC and crude fiber, and its C/N ratio was higher than that of FW alone (Table 4-2). This implies that the augmented RC in CB played a role in the fermentation of complex matters such as dietary fibers and, consequently, improved methane production. To reflect the real generation of KW in Korea, 20% co-digestion with KW was applied in P3 after day 500. Although methane production in CB was stable and higher than that in CR for approximately 50 days, methane production in both reactors decreased and remained at certain levels. Remarkably, the methane yield from CR (0.393 ± 0.012 L/g VS_{fed}) was lower than that from CB (0.492 ± 0.017 L/g VS_{fed}) during days 560–625. During days 560–600, FB, similar to CB, sustained stable and higher methane production compared to FR, and methane production decreased rapidly after day 560. In this period, substrate compositions changed to greater extents than before in terms of lower C/N ratio of FW (9.9) and FKW (8.5), higher crude fiber contents in FW (3.7 g/L) and FKW (5.1 g/L), and higher crude fat contents in FW (9.3 g/L) and FKW (17.9 g/L). Especially, FKW with a low C/N ratio and a high fiber content may adversely affect ammonia inhibition or reduce the conversion efficiency owing to higher fiber contents in CR and CB. After changing the substrates on day 625, methane production recovered rapidly in both reactors. Especially, the methane yield (0.609 ± 0.026 L/g VS_{fed}) in CB was significantly higher than that in CR (0.467 ± 0.028 L/g VS_{fed}) for approximately 70 days. On the contrary, FR exhibited a stable level of methane production similar to that before changing FW, but the methane yield in FB was higher than that in FR. After changing the substrates again, the methane yield (0.500 ± 0.014 L/g VS_{fed}) in CB was stable and higher than that in CR (0.443 ± 0.013 L/g VS_{fed}) in the steady state (days 913–920) (Table 4-5). Moreover, the methane yield in FB (0.471 ± 0.008 L/g VS_{fed}) increased significantly compared to that in FC (0.415 ± 0.009 L/g VS_{fed}). These results indicate that substrate fluctuation might affect process performance in terms of methane production. Even if such an inhibition related to substrates affected methane production, bioaugmentation with RC (CB and FB) may have facilitated more sustainable and resilient process performance compared to that of

the systems with no bioaugmentation.

To investigate the effect of fluctuation, the CR and CB reactors were fed with only FW in P4 and then again subjected to 20% co-digestion in P5. Methane production in CR increased initially because of surplus biomethanation with the conversion of the residual VFAs from P3 and then declined after consumption of the residual VFAs (Fig. 4-8B). In the steady state (days 995–1000) in P4, the methane yield (0.520 ± 0.010 L/g VS_{fed}) in CB increased by 12.3% compared to that in CR, while the methane yields of three reactors (CR, FR, and FB) were similar at 0.450–0.456 L/g VS_{fed}. In P5, with a return to co-digestion in CR and CB, methane production in CB was continuously higher than that in CR. Methane production in FR and FB were maintained at similar levels. In this period, each reactor was operated stably with relatively higher C/N ratios of FW (16.0) and FKW (13.4) compared to those of the substrates in P3. Thus, when the appropriate substrate was fed, the differences in process performance among the reactors were smaller, and reactor performance was stable, especially in FR and FB in P5 compared that in P3. This implies that the effect of bioaugmentation in CB with co-digestion of KW is sustainable despite fluctuations in the substrates with co-digestion and FW alone. By contrast, FB with bioaugmentation treating FW potentially sustained a stable performance with substrate inhibition in P3, but it had a similar performance of FC without bioaugmentation when the appropriate substrate was in place. This indicates that the co-digestion of KW containing higher fibrous matter likely facilitated retention of the functional diversity augmented microorganisms, and the retained microorganisms played a role in enhancing the sustainability of the activity in CB.

Overall, the methane production rate in CB was the highest among all reactors (CB, CR, FR, and FB) in steady state in each phase (P2, P3, P4, and P5) (Table 4-5). The differences between the methane production rate in CB and those in the other reactors were statistically significant according to Student's t-test with $p < 0.05$. Particularly, the methane yield in CB by 12.3% than that in CR in P2–5. In addition, it was higher than the values reported in the literature, which were 0.356–0.478 L CH_4/g VS_{fed} from Korean FW in batch tests [1, 84, 129, 130]. Moreover, the methane yields of 0.447–0.516 L CH_4/g VS_{fed} in FB were higher than those reported in the literature. This result suggests that the strategy of bioaugmentation with RC for treating FW and co-digestion of KW possibly enhanced the performance of AD. In addition, unlike the similar performances of all reactors in P1, in P4, the performance with 100% FW was superior in CB than the performance of the other reactors after co-digestion. This result implies that the effect of bioaugmentation can potentially be sustained to stimulate RC through KW co-digestion.

A



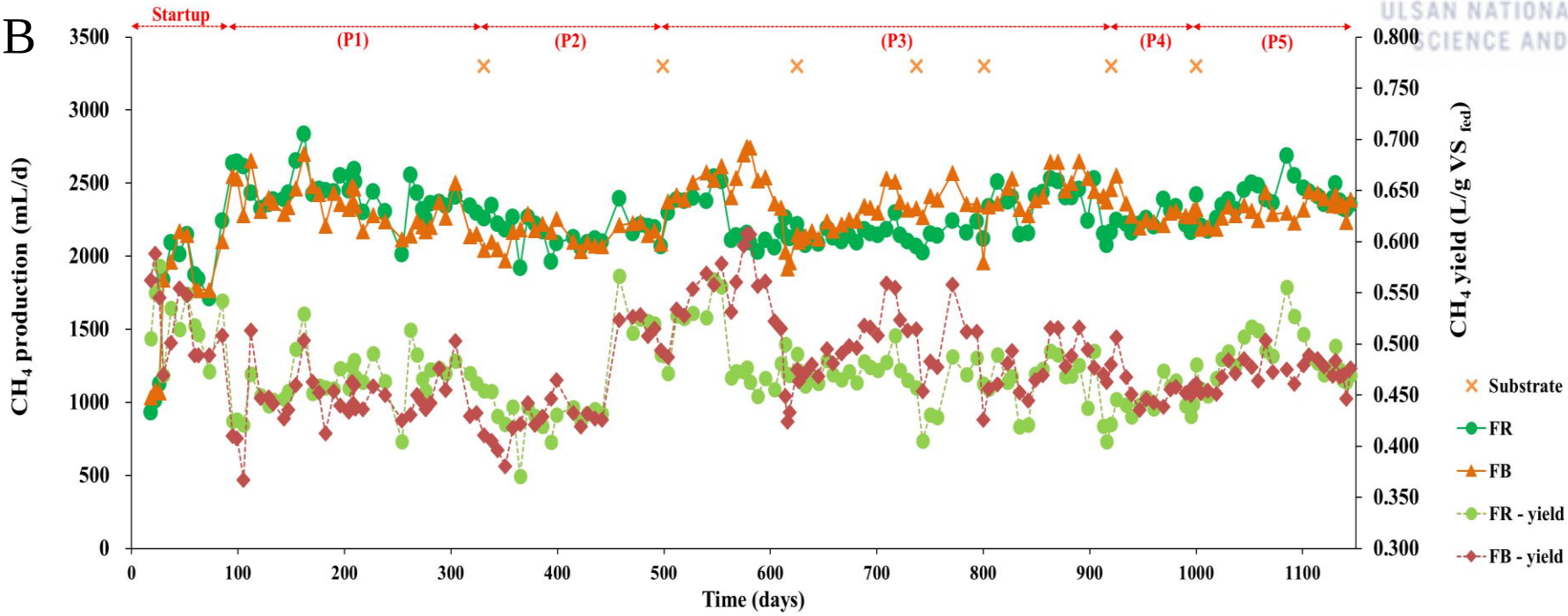


Figure 4-7. Methane production and yield during the experimental periods in (A) CR and CB and (B) FR and FB.

4.3.3.2. Process stability

Although the reactors were operated stably with low VFA concentrations and stable methane production in general, during days 500–625, the performances of the reactors decreased slightly, probably because of changes in substrate composition owing to changes in changing FW and the co-digestion ratio, except for FB (Table 4-2) (Figs. 4-7, 4-8A, and 4-9A). In this period, total accumulated VFA (TVFA) was more than 9 g COD/L (mainly acetate, propionate, and iso-valerate), and COD removal efficiency decreased to <80% in CR, CB, and FR (Figs. 4-8A and 4-9A). In particular, CR had a stronger influence on methane production, TVFA accumulation (<14.7 g COD/L; mainly acetate, propionate, and i-valerate at 8.4, 1.8, and 3.3 g COD/L, respectively), and COD removal efficiency (>70%) than CB. Similarly, FB exhibited relatively stable performance with higher methane yields (0.526 ± 0.064 L/g VS_{fed}), COD removal efficiency (>83%), and low level of TVFA (<0.1 g COD/L) compared to FR in this period. The performance differences between reactors may be ascribed to substrate characteristics or the bioaugmentation effect. First, FW and FKW in P3 exhibited lower C/N ratios, higher crude fat contents, and higher fiber contents than before. The reported optimum C/N ratio without any adverse effect on AD performance was 25−30 [84]. Moreover, the methane production yields increased as the C/N ratio increased from 5.6 to 16.2 when using the mixture of waste-activated sludge and FW [84]. According to these studies, the C/N ratios of 9.9 and 8.5 in FW and FKW, respectively, in this study are considerably lower than the optimum range of values; particularly, FKW is lower than FW (Table 4-2). The resulting lower methane production and accumulated TVFA at pH >7.9 seem to have caused the imbalance between acidogenesis and methanogenesis, rather than souring of the reactors. However, FB was not influenced by changes in substrate composition and exhibited higher methane production and low concentrations of TVFA compared to those of FR. This suggests that bioaugmentation with RC may provide stable performance even under inhibitory conditions. In addition, CB was less inhibited and offered higher methane yields (0.492 L/g VS_{fed}) and lower TVFA concentrations (<9 g COD/L) than CR (0.393 L/g VS_{fed} and >12 g COD/L). VFAs, mainly acetate, propionate, butyrate, and i-valerate, were accumulated at significant concentrations, and especially, i-valerate accumulation was the most toxic among the VFAs in CR [131]. Propionate and i-valerate are more thermodynamically unfavorable than acetate and butyrate. This implies that CR with higher VFAs was affected to a greater extent by a kinetic uncoupling between acidogens and methanogens. Moreover, this result was likely caused by the effect of bioaugmentation due to RC stimulated by co-digestion of KW, which was composed fibrous matters and carbohydrates, or interspecies H_2 transfer of hydrogenotrophic methanogens in RC. In study 1, the effect of bioaugmentation on treating FW was demonstrated to be stable and superior in batch and continuous tests, accompanied by a shift in the microbial community structure.

After FW and FKW were changed on day 625, methane production stabilized and TVFA decreased rapidly in CR, CB, and FR; then, CB exhibited a higher methane production rate over the next 80 days than the others. This result reflected that substrate compositions related to low C/N ratio or higher fiber and fat

content were likely to affect the AD performance. Moreover, the enhanced methane production in CB indicated that co-digestion of KW might have stimulated the RC, and consequently, probably enhanced AD performance. After FW and FKW were changed on day 800, methane production yield in CB increased gradually to a significantly higher level (7–24%) than those in the others in the steady state in P3 (Student's t-test, p <0.05). The methane yield in FB was relatively higher than that in FR in P3. Interestingly, the performance in CR decreased slightly, with low methane production and accumulated TVFA of up to 12 g COD/L. This result reflects that the 20% co-digestion condition, which included a greater amount of KW, likely destabilized AD performance, but bioaugmentation with RC contributed to stabilizing and enhancing AD performance. In P4, to test seasonal substrate fluctuations and sustainable effects of bioaugmentation, CR and CB were fed 100% FW. Initially, methane production in CR increased to a greater extent than those in the other reactors (Fig. 4-7A) because the accumulated TVFA in CR was utilized by methanogenesis under the stable condition. In P5, 20% co-digestion was reapplied to CR and CB. In this period, process imbalance related to the accumulation of VFAs and decreased methane production were not observed. This indicates that the system probably adapted to 20% co-digestion with fluctuations or differences in substrate composition, which may be the cause of differences in process performance between P3 and P5.

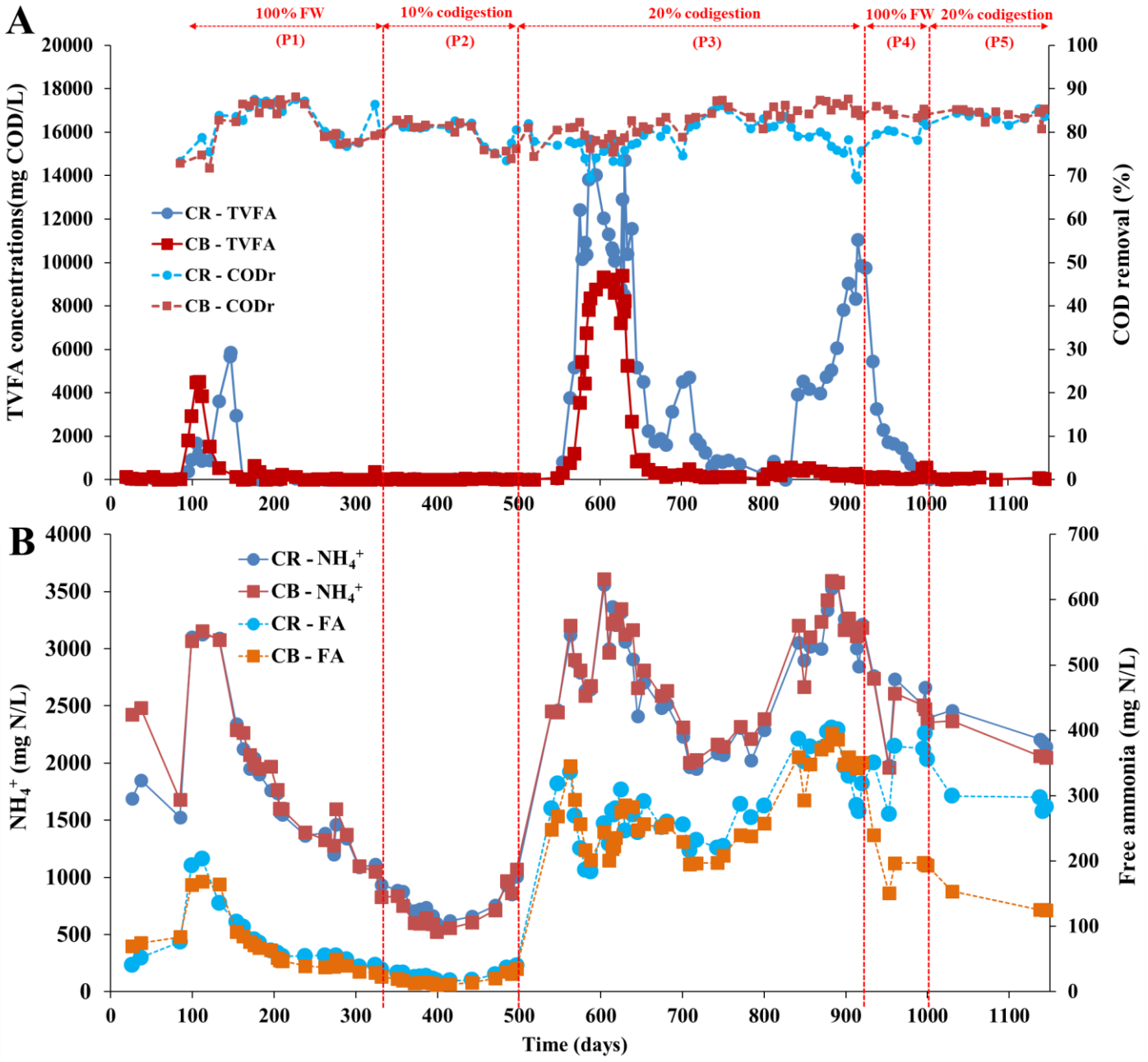


Figure 4-8. Process parameters of TVFA, COD removal efficiency (A), ammonium, and FA concentrations (B) in CR and CB reactors.

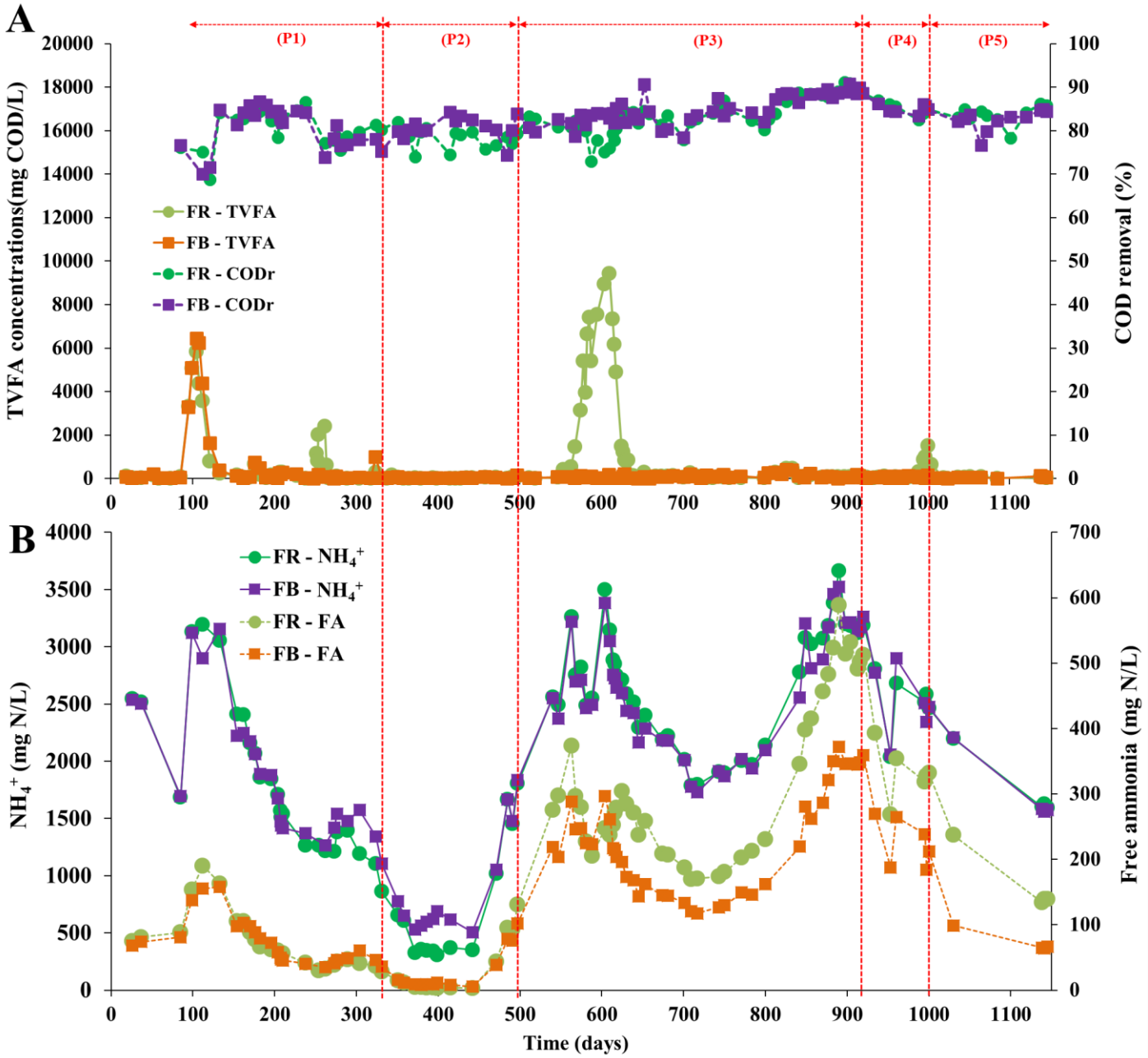


Figure 4-9. Process parameters of TVFA, COD removal efficiency (A), ammonium, and FA concentrations (B) in FR and FB reactors.

Figs. 4-8B and 4-9B show the effluent ammonium ion (NH_4^+-N) and FA concentrations in the experiments. These concentrations depend on pH as two forms in the liquid phase. When increasing pH, the form of ammonium ion transferred to FA form (pKa at 35°C, 8.95) [90]. Ammonium and FA concentrations higher than 3 g NH_4^+-N/L and 0.15 g NH_3-N/L, respectively, are generally known to significantly inhibit methanogenesis in AD, and FA especially has a stronger inhibitory effect than ammonium [49]. In this study, before P3, the FA concentration was considerably lower than its inhibitory threshold. However, during days

500−625 in P3, the FA concentration increased drastically to a highly toxic level (>200 mg NH_3-N/L), along with significant TVFA accumulation in CR, CB, and FR (Figs. 4-8 and 4-9). This was attributed to the low C/N ratio of FW (9.9) and FKW (8.5) (Table 4-2). Although methane production was decreased as well, it remained at steady levels in CR, CB, and FR. This state was described previously as the so-called "inhibited steady-state," and it is considered a suboptimal condition [48]. Similarly, the inhibited steady-state in AD has been reported under high levels of ammonia. However, FB continued to exhibit stable performance with low VFA concentration and stable methane production in this period. After the substrates were changed on day 625, the FA concentration decreased marginally as the substrate C/N ratio increased relatively (FW 11.9 and FKW 11.0), but the FA concentration remained elevated (>130 mg NH_3-N/L). In this period, accumulated VFA declined rapidly in CR, CB, and FR, and the highest methane production was achieved in CB, while other reactors exhibiting low levels of methane production compared to that in CB (Fig. 4-7). This indicated that AD performance was adversely affected by the high FA concentration resulting from the low C/N ratio of the substrate, and AD performance was recovered as the FA concentration decreased relatively. Moreover, as the FA concentrations increased to >300 mg NH_3-N/L over days 800−920, the reactors exhibited stable performance without VFA accumulation, except for CR. This result suggested that the system had adapted to the toxicity of FA for long-term operation. Similarly, studies in the literature have reported the combined effects of VFAs and ammonia on microbial community structures, along with functional pathways [132, 133]. In previous studies, syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis was the dominant pathway when ammonia levels were high. Therefore, the present study demonstrated the possibility of microbial adaptation on high FA levels caused by fluctuation in substrate composition. Especially, bioaugmentation with RC can potentially increase robustness to ammonia toxicity in CB and FB. However, the inhibited steady-state was likely to be maintained in CR owing to the accumulated VFAs, probably because of the adverse synergistic effect of low C/N ratio and KW with higher refractory matter such as crude fiber compared to that in FR. After changing the feed condition to 100% FW with a C/N ratio of 12.8 from day 921 in CR and CB, TVFA decreased rapidly with increasing methane production in CR, and the other reactors performed stably with reduced FA concentrations. This result indicated that the C/N ratio of the substrate was important from the viewpoint of achieving stable AD performance. In addition, based on a comparison of CR and FR, the co-digestion of KW under a low C/N ratio and high fiber content may have affected AD performance to a greater extent than the treatment of FW alone. Once again, CR and CB were operated with 20% co-digestion (C/N ratios of FW = 16.0 and FKW = 13.4) from day 1001. In this period, the FA concentrations in CR and FR were higher than those in CB and FB, respectively, because the pH levels of 8.1 and 7.9 in CR and FR were higher than the pH levels of 7.8 and 7.6 in CB and FB, respectively, which could have facilitated the transformation of a greater amount of ammonium into FA according to the equilibrium relationship. The FA concentrations in CB, FR, and FB were relatively lower than the inhibition threshold concentration of 150 mg NH_3-N/L owing to the higher C/N ratio than that before P3 (days 500–

625). Although the FA concentration in CR remained high (>280 mg NH_3-N/L), the reactor performance was maintained stably with a low VFA level and stable methane production compared to those before P3 with 20% co-digestion. This result indicates that CR adapted to the toxicity of FA over long-term operation, and CR may have potential for microbial adaptation.

Table 4-5. Overall results of average values at steady state.

	P1 (FW) 324–331 d				P2 (10% Co-D) 471–497 d				P3 (20% Co-D) 913–920 d				P4 (FW) 995–1000 d				P5 (20% Co-D) 1138–1145 d			
	CR	CB	FR	FB	CR	CB	FR	FB	CR	CB	FR	FB	CR	CB	FR	FB	CR	CB	FR	FB
CH_4 (L/d)	2.6	2.5	2.5	2.4	2.2	2.4	2.2	2.2	2.3	2.6	2.1	2.4	2.3	2.5	2.3	2.3	2.4	2.5	2.3	2.3
CH_4 yield (L/g VS_{fed})	0.477	0.465	0.471	0.447	0.514	0.556	0.517	0.516	0.443	0.500	0.415	0.471	0.456	0.502	0.450	0.456	0.507	0.527	0.466	0.464
CH_4 yield (L/g $VS_{removed}$)	0.544	0.560	0.554	0.510	0.642	0.696	0.629	0.618	0.551	0.591	0.480	0.545	0.566	0.636	0.571	0.575	0.629	0.659	0.580	0.587
H_2S (ppm)	1016	1067	1035	898	1482	1471	1792	1727	6535	5140	3333	3279	2631	2640	2998	2830	4232	4098	1973	2180
TVFA (g COD/L)	0.1	0.1	0.2	0.2	0.0	0.0	0.0	0.0	9.7	0.2	0.1	0.1	0.3	0.5	1.1	0.1	0.1	0.1	0.1	0.1
SCOD (g/L)	2.0	2.4	1.7	1.6	0.7	0.8	1.0	1.2	13.2	2.4	1.5	1.2	3.3	2.4	3.3	1.3	1.7	1.6	1.9	2.2
TAN (g-N/L)	1.0	0.9	1.0	1.2	0.8	0.8	1.4	1.4	3.0	3.2	3.2	3.2	2.5	2.4	2.5	2.4	2.2	2.1	1.6	1.6
FA (mg NH_3-N/L	38	26	33	41	32	26	77	64	294	347	502	351	374	195	326	212	286	125	138	65
CODr (%)	83.1	79.4	80.8	76.7	76.7	75.2	77.9	79.6	71.6	84.4	89.3	89.0	82.6	84.5	84.7	85.6	84.7	83.5	85.9	84.7
VSr (%)	82.7	82.7	79.9	80.1	77.7	77.4	78.7	80.0	80.5	84.6	86.5	86.4	77.4	78.9	78.9	79.4	80.6	80.2	80.3	79.0
TC (g/L)	2.5	2.7	2.4	2.9	4.8	4.8	4.0	3.4	3.3	2.6	2.9	2.7	2.2	2.0	1.6	1.8	2.7	2.7	2.9	3.1

4.3.4. Microbial community structure in continuous tests

The reactor samples for microbial community analysis were collected on days 8, 331, 497, 581, 681, 920, 1000, and 1145 from CR and CB and on days 8, 331, 581, 681, 920, and 1145 from FR and FB; seed sludge (AS), and RC, respectively. A total of 1,700,789 bacterial and 1,947,940 archaeal reads were obtained from the reactors by means of NGS. The number of bacterial and archaeal operational taxonomic units (OTUs) were 1,318 and 45, respectively. A total of 25 bacterial phyla were identified from the retrieved 16S rRNA gene sequences. *Actinobacteria*, *Bacteroidetes*, *Cloacimonetes*, and *Firmicutes* were commonly found as the major phyla in all reactor samples (Fig. 4-10). *Bacteroidetes* was the most abundant phylum accounting for 18.7–57.7% of the total bacterial reads, followed by *Firmicutes*, *Cloacimonetes*, and *Actinobacteria*.

A total of 7 archaeal families were identified, and *Methanotrichaceae* and *Methanobacteriaceae* were the major families in the samples (Fig. 4-11). *Methanotrichaceae* accounted for more than 96% of AS and were present dominantly in the reactor samples collected on days 8 and 331. *Methanotrichaceae*-related sequences were assigned to the strictly aceticlastic methanogen. These results suggest that the primary route for methanogenesis is likely to be the aceticlastic pathway in all reactors in the start-up (day 8) and stabilized periods (day 331) when treating FW. However, the presence of hydrogenotrophic *Methanobacteriaceae* family increased as the presence of *Methanotrichaceae* declined in CR and CB on day 497 for 10% co-digestion of KW. Moreover, a pattern of decrease in *Methanotrichaceae* was observed in the sample obtained on day 581; simultaneously, hydrogenotrophic methanogens of *Methanobacteriaceae*, *Methanoregulaceae*, or *Methanospirillaceae* increased in all reactors. These results indicated that the methanogenic pathway shifted from the aceticlastic to the hydrogenotrophic pathway in this period. Interestingly, these changes occurred when the reactors were affected by the low C/N ratio of the substrate along with FA concentrations higher than 300 mg NH_3-N/L. High FA concentrations were reported to have a stronger inhibitory effect on aceticlastic methanogens than on hydrogenotrophic methanogens due to ammonia stress, and consequently, the methanogenic pathway shifted to hydrogenotrophic methanogenesis [133].

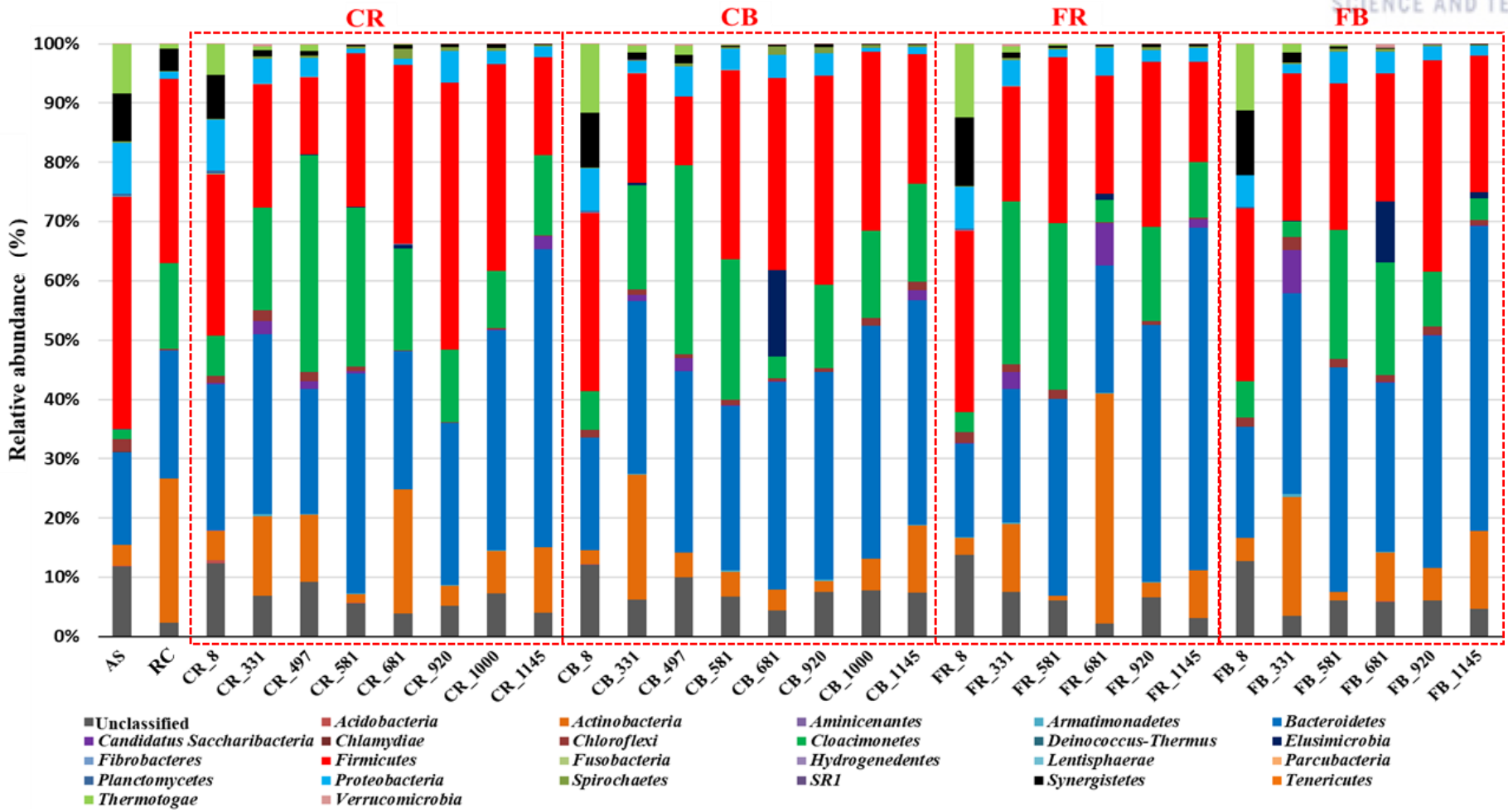


Figure 4-10. Bacterial community structures characterized at the phylum level.

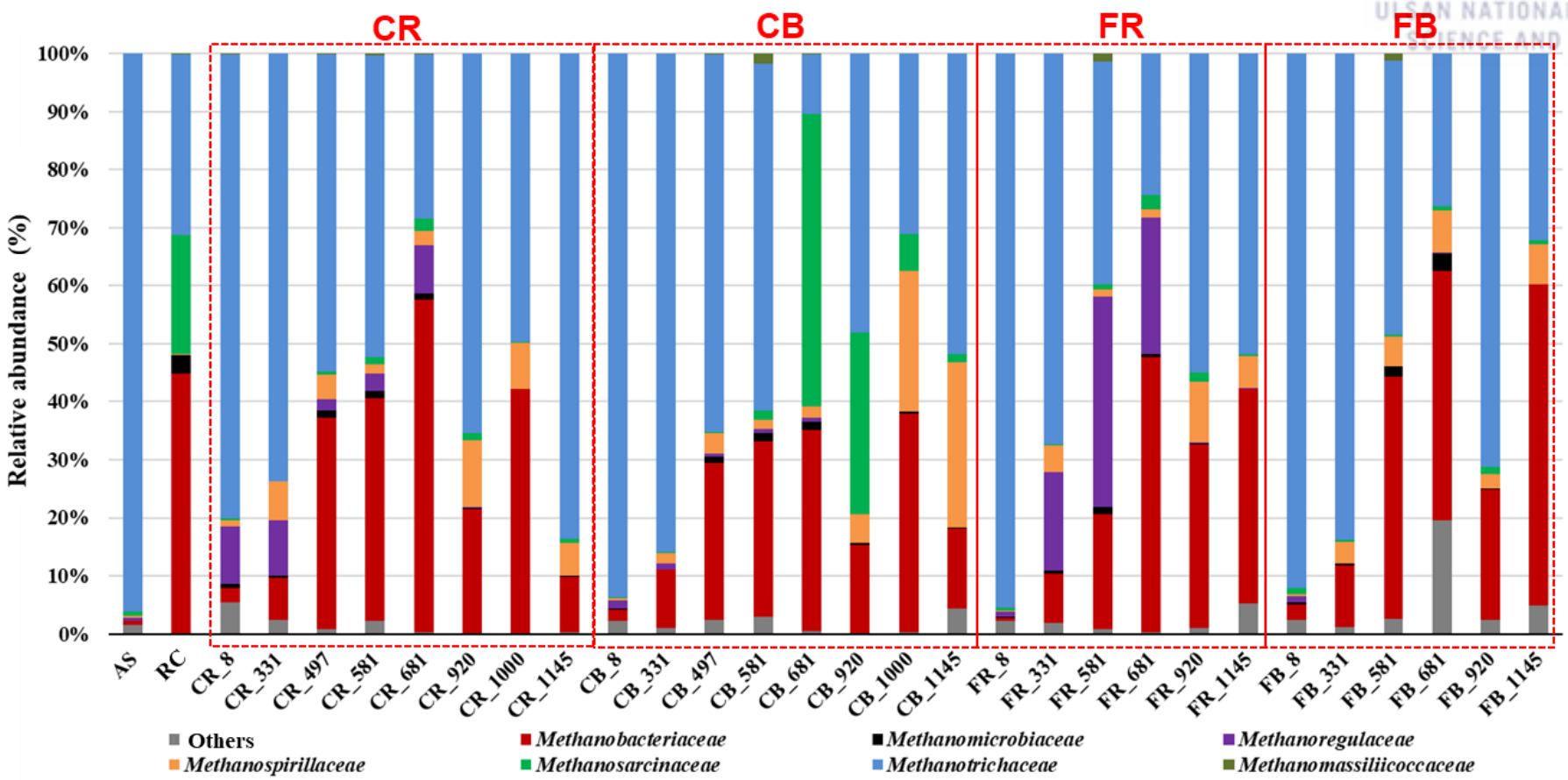


Figure 4-11. Archaeal community structures characterized at the family level. Others include unclassified sequences at the desired taxonomic levels and operational taxonomic units less than 1% relative abundance in all archaeal libraries.

The recovered sequences were clustered into 1,318 bacterial and 45 archaeal OTUs, and the major OTUs (>3% relative abundance in at least one library) are summarized in Tables 4-6 and 4-7. Among the 37 major bacterial OTUs (OTUs B1 to B37), 15 were assigned to known genera (Table 4-6). OTU B1 was assigned to the genus *Candidatus* Cloacamonas, which can syntrophically ferment amino acids and sugars to produce hydrogen [134, 135]. This OTU could probably have formed a syntrophic relationship with hydrogenotrophic methanogens for interspecies electron transfer. Therefore, the prevalence of OTU B1 in most samples, especially on day 581, suggests that the OTU could have fermented higher amino acids from low C/N ratio substrates, and the produced H_2 could have been utilized syntrophically for hydrogenotrophic methanogenesis. In particular, the OTU was predominant in CR and CB with 10% co-digestion on day 497; moreover, OTUs B5 and 23 found in increased proportions. Additionally, OTU B1 was dominant in RC, indicating that the OTU was retained in the bioprocess. Although OTUs B5 and 23 were not related to known species (<97% similarity), B5 assigned to *Bacteroidetes* is commonly found in AD environments responsible for acidogenic fermentation. Therefore, these acidogenic bacteria may have contributed to the degradation of various organic matters in FKW. OTUs B3, 4, 7, 8, and 16 were usually found to be abundant after P3 under high levels of FA. OTU B3 was assigned to the family *Porphyromonadaceae*, which was found to be closely related to *Petrimonas sulfuriphila* with a similarity 97.6%. This OTU is a sulfate reducer that produces acetate and H_2/CO_2 from glucose and lactate [122]. OTU B8 assigned to *Olsenella uli* (similarity 99.3%) can produce lactate from carbohydrates [136]. In particular, OTUs B3 and 8 were found simultaneously in the samples, indicating that these OTUs were syntrophically related to the production and degradation of lactate in the system. OTUs B4 and 16 were assigned to phyla *Bacteroidetes* and *Firmicutes*, respectively. These phyla, which are involved in hydrolysis and acidogenesis, are commonly found in AD process environments. OTU B7, which was assigned to the *Ruminococcaceae* family, is commonly present in the gut and is capable of utilizing complex carbohydrates [26, 137]. These results suggest that these OTUs were potentially involved in the hydrolyzation of carbohydrates and protein in FW and FKW. OTU B9 was abundant only on day 681 in CB (14.6%) and FB (10.2%), and it was assigned to *Candidatus* Endomicrobium (similarity 97.2%). This OTU is an intracellular symbiont from the termite gut, and it is associated with the symbiont cellulolytic protist *Trichonympha*. It can ferment glucose to lactate, acetate, and H_2/CO_2, and it may have formed a syntrophic relationship with hydrogenotrophic methanogens for interspecies electron transfer [138, 139]. Therefore, the OTUs present in CB and FB may have been derived from RC and involved in the hydrolyzation of cellulosic fibers. Although OTU B20 assigned to phylum *Bacteroidetes* is not a closely related known species, it was abundant in RC, CB, and FB. This indicates that the OTU was possibly derived from RC and retained its activity related to hydrolysis and acidogenesis. Interestingly, OTU B28 assigned to *Actinomyces europaeus* with a similarity of 99.7% is relatively abundant in RC and FB samples under high FA concentrations. This implies that the OTU in FB may have been derived from RC. This OTU can ferment carbohydrates to organic acids, including acetate and lactate. Therefore, diverse bacteria related to hydrolysis and acidogenesis and possibly derived from RC were likely

to have play a role in the fermentation of FW and FKW.

Eight major archaeal OTUs (OTUs A1–A5 and A7–9) were classified at the genus level (Table 4-7). By contrast, OTU A6 was classified at the family level, and OTU A10 remained unclassified even at the phylum level. OTU A10 was most closely related to an uncultured *Methanosarcinales* archaeon clone belonging to aceticlastic methanogens. OTUs A1, A2, and A8, assigned to the aceticlastic genus *Methanothrix*, accounted for more than 67% of the total archaeal reads before the application of co-digestion (days 8 and 331); especially, AS accounted for 96.1% of the total archaeal reads. Therefore, it is likely that aceticlastic methanogenesis was the main methanogenesis route in all reactors in the start-up and P1 periods.

Notably, after day 581, the dominance shifted between aceticlastic methanogens and hydrogenotrophic methanogens, from *Methanothrix* to *Methanobacterium*, *Methanoregulaceae*, and *Methanospirillum*. These changes were accompanied by the accumulation of VFAs and increase in FA concentration at low C/N ratios of the substrates. Particularly, OTU A3 related to *Methanobacterium* constituted the largest part of the archaeal community because aceticlastic methanogens are generally less tolerant to ammonium toxicity than hydrogenotrophic methanogens [133]. In addition, the dominant genus *Methanothrix* is adversely affected by high VFA concentrations. By contrast, hydrogenotrophic methanogens are robust to ammonia toxicity owing to syntrophic acetate oxidation. The dominant hydrogenotrophic methanogens at high ammonium levels were reported in a laboratory- and full-scale AD study [132]. Interestingly, the presence of OTU A3 related to *Methanobacterium* was much more in FB (41.5%) than in FR (19.8%) on day 581, and FB reactor maintained stable performance without accumulation of VFAs compared to the FR reactor. Moreover, *Methanobacterium* was dominant in the RC samples, which may have effected greater changes in the microbial community structure with hydrogenotrophic methanogenesis in FB than in FC. *Methanobacterium* has been observed to be dominant in RC, and it can play a role in mitigating the hydrogen partial pressure build up associated with the accumulation of VFAs [140]. *Methanobacterium* has also been reported as the dominant methanogen at FA concentrations higher than 0.44 g NH_3-N/L, it could have grown relatively faster than other methanogens [132]. When the performance of reactors was recovered on day 681 with decreasing VFA concentration, *Methanobacterium* populations increased in all reactors. This implies that in this study, *Methanobacterium* played a role in maintaining stable reactor performance under ammonia stress. However, interestingly, when the presence of OTU A1, which is related to aceticlastic methanogens, weakened in the other reactors, OTU A4, which was assigned to the aceticlastic genus *Methanosarcina*, was dominant only in CB, even when methane production levels were high (Fig. 4-7). A relative abundance (20.1%) of *Methanosarcina* was observed in RC as well, indicating that the *Methanosarcina* in CB was derived from RC. As a facultative aceticlastic methanogen that is favorable atrelatively higher VFA concentrations, *Methanosarcina* can convert both acetate and H_2/CO_2 to methane, and can thus simultaneously play a role in the aceticlastic and hydrogenotrophic methanogenesis under inhibition by acids and ammonium. It was reported in the literature that *Methanosarcina* was dominant in full-scale digesters under high levels of ammonia and acids [132]. Otherwise, OTU A1 related *Methanothrix* is less tolerant to

high VFA concentrations than *Methanosarcina*. Therefore, the accumulation of VFAs on day 581 may have be influenced the growth of *Methanosarcina*, which potentially may have enhanced and resilient the performance of CB. When the reactors were stabilized, except for CR, on day 920, the genus *Methanothrix* was dominant in all samples. Interestingly, OTU A1 was dominant in CR and FR, while the same genus *Methanothrix* but belonging to OTU A2 was dominant in CB and FB. Especially, the transient OTU from A1 to A2 was observed in FB from day 581. The microbial community shift may have influenced the stability of FB as opposed to that of FR. In addition, the presence of *Methanospirillum* related to OTU A7, which can act to syntrophically degrade propionate as a hydrogen scavenger, was stronger in CR and FR than in CB and FB. This indicated that the accumulated VFAs with longer-chain propionate and i-valerate in CR and FR were likely consumed by the microbial community that shifted from hydrogenotrophic methanogens of *Methanobacterium* and *Methanospirillum*.

In P4, when CR and CB were fed with FW alone, the methanogenesis pathway shifted from aceticlastic methanogens to hydrogenotrophic methanogens compared to that in P1 in which FW alone was treated. This is because the low C/N ratio (12.8) in P4 compared to that in P1 (21.3) could have had a stronger effect on the high levels of FA (374 and 195 mg NH_3-N/L in CR and CB, respectively), which contributed to the shift in the methanogenesis pathway. In particular, the presence of OTU A4 in CB declined, and then, the hydrogenotrophic methanogens belonging to OTUs A3 and A7 became dominant. This implies that stable reactor performance without VFA accumulation at relatively high FA concentrations contributed to the major hydrogenotrophic methanogenesis and reduced the growth of *Methanosarcina* at low VFA concentrations. By contrast, *Methanothrix* related to OTU A1 (48.5%) in CR continued to be present dominantly, and *Methanobacterium* related to OTU A3 (41.9%) was abundant as well. The differences in the microbial community were attributable to the process efficiency of CR and CB, in that a higher level of methane production was sustained in CB compared to that in CR. Moreover, the significant community shift in both reactors indicated that the systems adapted successfully to the inhibition of FA concentrations owing to fluctuations in substrate composition over long-term reactor operation.

Again, CR and CB were operated with 20% co-digestion in P5, and the differences in their archaeal community were distinct. In CR, OTU A1 was the most abundant, with a presence of more than 83%; OTUs A1 and A2 in CB, represented by the aceticlastic genus *Methanothrix*, accounted for 52%, and the hydrogenotrophic OTUs A3 and A7 occupied more than 42%. This distinct shift in dominance suggests that the co-digestion of KW contributed to an environmental change that changed the microbial community structures significantly. In addition, these structural and metabolic changes in the methanogen community pointed to differences in process performance. In this phase, CB produced more methane than CR, possibly because methane production in CB was enhanced through diverse methanogenesis pathways associated with aceticlastic and hydrogenotrophic methanogens. Therefore, CB with bioaugmentation could perform stably and resiliently against fluctuations in FW compositions owing to favorable changes in the underlying microbial community structure.

Table 4-6. Relative abundance and taxonomic affiliation of major bacterial OTUs (>3% relative abundance in at least one library)[a].

				CR								CB								FR						FB							
OTUs	Classification[b]	AS	RC	8	331	497	581	681	920	1000	1145	8	331	497	581	681	920	1000	1145	8	331	581	681	920	1145	8	331	581	681	920	1145	Closest species[c]	Sim (%)[d]	Accession no.
B1	*Candidatus* Cloacamonas	0.0	14.3	5.7	17.2	36.6	26.8	17.2	12.1	9.7	13.4	3.0	17.6	31.9	23.7	3.6	14.0	14.7	16.3	0.1	27.5	28.1	3.8	15.9	0.4	2.7	2.4	20.9	19.0	9.2	2.9	*Candidatus* Cloacamonas acidaminovorans	91.7	CU466930
B2	*Coriobacteriaceae*	0.4	19.6	0.9	4.8	0.8	0.1	9.2	0.8	1.9	0.6	1.1	12.6	0.9	0.4	0.9	0.0	0.0	3.2	0.6	6.8	0.0	29.2	0.6	0.7	1.9	2.9	0.1	0.7	0.8	1.1	*Atopobium parvulum*	95.5	NR102936
B3	*Porphyromonadaceae*	0.2	0.5	8.6	0.7	0.5	9.5	1.0	6.1	8.2	15.8	0.7	1.1	1.1	2.0	9.1	5.6	11.5	12.1	0.5	0.4	4.9	7.0	5.5	23.9	0.8	6.6	19.9	1.7	4.5	11.6	*Petrimonas sulfuriphila*	97.6	LT558828
B4	*Bacteroidetes*	0.0	0.0	0.0	0.6	0.0	0.2	0.2	7.9	15.6	12.4	0.0	0.3	0.0	0.1	0.1	9.5	5.5	4.1	0.0	0.0	0.2	1.5	21.2	5.7	0.0	0.0	0.2	0.4	15.4	11.1	*Ralstonia solanacearum*	90.7	CP011998
B5	*Bacteroidetes*	0.5	0.0	0.7	3.1	11.8	13.9	1.1	0.3	0.2	12.2	0.5	2.9	18.7	15.0	4.0	0.5	1.8	7.9	0.4	4.4	16.5	5.7	0.5	15.8	0.3	0.0	1.9	4.9	0.3	15.6	*Mariniphaga sediminis*	92.4	NR137221
B6	*Sedimentibacter*	17.1	0.5	7.1	0.0	0.1	0.2	0.1	0.1	0.1	0.2	7.7	0.0	0.1	0.1	0.1	0.1	0.1	0.0	7.3	0.2	0.3	0.0	0.1	0.1	6.7	0.0	0.2	0.1	0.1	0.1	*Sedimentibacter sp*	96.9	EF059533
B7	*Ruminococcaceae*	0.2	0.1	0.4	0.4	0.9	9.8	4.2	16.9	11.1	4.6	0.2	0.1	0.2	5.5	7.6	6.4	7.3	4.0	0.2	2.0	10.3	5.9	12.1	5.6	0.2	0.1	6.5	0.7	15.4	6.5	*Flintibacter butyricus*	94.4	NR144611
B8	*Olsenella*	0.1	0.0	0.4	5.7	1.6	0.5	9.9	1.0	4.5	9.3	0.0	2.9	0.9	0.9	0.5	0.2	2.5	6.4	0.1	3.1	0.2	7.2	0.9	6.6	0.0	14.8	0.9	2.8	1.2	11.1	*Olsenella uli*	99.3	NR074414
B9	*Candidatus* Endomicrobium	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.4	0.0	0.0	14.6	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.1	0.0	10.2	0.0	1.0	*Endomicrobium sp*	97.2	KY665292
B10	*Enterococcus*	0.1	12.9	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.2	0.2	0.0	0.0	0.6	0.1	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.3	0.1	0.0	0.2	*Enterococcus lemanii*	100.0	NR114648
B11	*Mesotoga*	8.3	0.8	5.2	0.7	0.9	0.1	0.0	0.0	0.0	0.0	11.6	1.1	1.7	0.1	0.0	0.0	0.0	0.1	12.3	1.1	0.3	0.0	0.0	0.0	11.2	1.4	0.2	0.0	0.0	0.0	*Mesotoga prima*	99.7	NR102952
B12	*Porphyromonadaceae*	0.9	0.3	1.3	12.0	0.2	1.1	1.9	1.5	1.7	0.2	1.3	10.7	1.2	1.0	1.6	0.7	1.4	0.4	1.4	5.3	1.0	0.5	0.6	0.6	1.3	2.8	1.5	0.9	0.7	0.4	*Petrimonas mucosa*	98.6	NR148808
B13	*Firmicutes*	4.8	2.6	8.1	7.8	5.9	6.0	3.8	3.3	4.9	0.9	3.6	7.0	5.8	7.9	5.1	5.0	4.6	2.7	3.3	7.5	9.1	1.3	3.8	0.6	3.0	11.1	7.9	6.2	4.0	2.2	*Salinithrix halophila*	88.9	NR134171
B14	*Bacteroidetes*	0.3	0.1	0.1	0.2	0.2	0.8	5.1	1.5	0.6	0.9	0.2	0.4	0.2	0.3	10.3	1.0	5.7	2.0	0.2	0.1	2.7	2.7	6.2	2.0	0.3	0.2	0.7	6.2	0.7	2.1	*Porphyromonas pogonae*	86.9	NR136443
B15	*Bacteroidetes*	0.0	10.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	*Solitalea canadensis*	89.7	NR074099
B16	*Firmicutes*	0.4	0.1	0.2	1.0	0.2	1.6	10.0	9.9	4.7	1.5	0.4	1.2	0.3	4.3	5.5	3.9	2.6	2.3	0.6	0.8	0.4	4.8	2.7	3.2	0.4	2.5	0.5	1.1	6.0	4.2	*Ruminiclostridium cellobioparu*	88.9	NR113360

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B17	*Cloacimonetes*	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	9.1	0.0	0.0	0.9	0.1	0.0	0.7	*Agromyces ramosus*	80.5	NR026165
B18	*Actinomyces*	0.0	1.8	1.5	2.6	8.7	0.7	0.0	0.0	0.0	0.5	0.1	4.5	2.2	2.2	0.1	0.0	0.0	0.1	0.0	1.4	0.2	0.0	0.0	0.6	0.2	2.0	0.1	0.0	0.0	0.5	*Actinomyces polynesiensis*	97.9	NR144691
B19	*Petrimonas*	0.1	0.4	0.0	0.2	0.1	0.1	3.6	1.3	2.2	0.1	0.1	0.3	0.0	0.0	1.1	1.6	0.6	0.1	0.1	0.1	0.2	0.9	2.0	0.1	0.1	3.5	1.0	3.5	7.9	0.1	*Fermentimonas caenicola*	100.0	NR148809
B20	*Bacteroidetes*	2.1	5.5	0.6	4.4	2.7	1.0	0.7	0.6	1.3	3.0	3.4	5.8	2.7	2.0	0.7	5.0	2.7	3.7	1.5	4.8	0.9	0.4	2.4	2.8	5.1	7.6	6.6	5.0	2.8	1.8	*Lentimicrobium saccharophilum*	89.2	NR149795
B21	*Clostridiales*	2.8	0.8	1.6	0.1	0.0	0.5	0.7	2.0	2.0	3.5	4.0	0.2	0.0	0.2	0.4	7.4	4.0	1.8	6.7	0.1	0.1	0.2	3.0	1.4	4.5	0.3	0.6	0.3	2.6	2.2	*Sporanaerobacter acetigenes*	87.6	NR117381
B22	*Candidatus* Saccharibacteria	0.0	0.0	0.1	2.2	1.3	0.3	0.0	0.0	0.1	2.1	0.0	1.0	2.1	0.2	0.1	0.0	0.0	1.7	0.0	2.9	0.0	7.1	0.0	1.5	0.0	7.3	0.0	0.0	0.1	0.2	*Geobacter metallireducens*	78.0	NR075011
B23	*Bacteria*	2.1	0.0	4.0	2.1	6.6	0.4	0.0	0.0	0.0	0.0	2.4	1.5	6.8	0.6	0.0	0.0	0.0	0.0	1.5	4.8	1.2	0.1	0.0	0.0	1.5	0.0	1.2	0.0	0.0	0.0	*Rubrobacter spartanus*	85.8	NR158052
B24	*Bacteroides*	0.0	0.8	0.1	0.6	0.0	0.0	0.1	0.1	0.0	0.3	0.1	1.3	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.2	0.4	0.2	0.0	1.2	0.0	5.7	0.6	0.0	0.0	0.8	*Bacteroides pyogenes*	100.0	NR113048
B25	*Desulfomicrobium*	0.1	0.0	0.2	0.3	0.1	0.4	0.3	5.1	2.0	1.6	0.5	0.4	0.1	1.1	1.9	3.7	0.3	0.8	0.7	0.1	0.5	0.5	1.8	1.1	0.3	0.5	1.0	1.4	1.9	1.0	*Desulfomicrobium orale*	97.9	NR113205
B26	*Thermovirga*	2.0	2.7	1.6	0.2	0.1	0.1	0.0	0.0	0.0	0.0	3.4	0.4	0.2	0.1	0.0	0.0	0.0	0.0	4.6	0.5	0.1	0.0	0.2	0.1	4.3	0.8	0.2	0.0	0.0	0.0	*Thermovirga lienii*	94.1	NR074606
B27	*Bacteroidetes*	3.7	0.0	4.4	0.6	0.2	0.5	0.1	0.0	0.0	0.0	3.5	0.6	0.3	0.6	0.1	0.0	0.0	0.0	3.3	0.2	0.2	0.1	0.0	0.1	3.0	0.3	0.2	0.2	0.1	0.1	*Prolixibacter denitrificans*	90.7	NR137212
B28	*Actinomyces*	0.0	2.8	0.0	0.0	0.0	0.0	0.3	1.4	0.4	0.5	0.0	0.0	0.0	0.0	0.2	0.8	0.6	0.5	0.0	0.0	0.0	2.0	0.7	0.2	0.2	0.0	0.0	4.3	3.0	0.3	*Actinomyces europaeus*	99.7	NR114971
B29	*Porphyromonadaceae*	0.4	0.0	0.4	0.2	0.2	1.4	0.1	2.9	2.9	0.8	0.3	0.3	0.1	1.0	0.2	4.0	4.1	0.7	0.4	0.2	0.9	0.1	1.6	0.4	0.3	0.4	0.7	0.0	2.2	2.2	*Proteiniphilum acetatigenes*	89.2	NR043154
B30	*Pseudomonas*	0.0	0.0	0.2	0.1	0.7	0.1	0.5	0.0	0.0	0.0	0.1	0.0	0.6	0.4	1.6	0.0	0.0	0.0	0.0	0.0	0.5	3.9	0.0	0.0	0.0	0.0	1.2	1.6	0.0	0.0	*Pseudomonas caeni*	99.7	NR116388
B31	*Clostridiales_Incertae Sedis XI*	0.0	0.0	0.0	0.2	0.0	0.2	0.5	0.2	1.1	0.1	0.0	0.2	0.0	0.7	3.6	0.5	1.1	2.4	0.0	0.2	0.5	2.3	1.9	0.3	0.0	0.3	0.3	1.0	2.5	0.4	*Parvimonas micra*	93.4	NR114338
B32	*Syntrophomonadaceae*	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.3	0.2	0.0	0.0	0.0	*Syntrophomonas zehnderi*	96.2	NR044008
B33	*Bacteria*	0.0	0.0	0.2	0.2	0.0	1.5	0.5	1.6	1.4	0.2	0.1	0.0	0.0	1.5	1.2	3.3	0.9	0.4	0.0	0.1	1.5	0.2	2.7	0.1	0.1	0.2	1.3	0.3	1.9	0.0	*Planifilum composti*	87.7	NR135724
B34	*Firmicutes*	0.0	0.0	0.0	0.2	0.1	0.3	2.9	3.1	0.9	0.2	0.0	0.4	0.1	0.2	1.5	0.5	1.5	1.4	0.0	0.1	0.1	0.3	0.2	0.2	0.0	0.1	0.1	2.7	0.1	0.4	*Irregularibacter muris*	87.2	NR144613

B35	*Porphyromonadaceae*	0.6	0.1	0.4	0.5	0.4	0.8	3.1	0.7	0.3	0.4	0.4	0.3	0.7	0.9	1.9	0.6	0.6	0.9	0.5	0.2	0.4	0.3	0.6	0.1	0.5	0.1	0.9	1.3	0.6	0.1	*Dysgonomonas capnocytophagoides*	92.7	NR113133
B36	*Ruminococcaceae*	0.0	3.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.2	0.1	0.0	0.3	0.0	0.3	0.2	0.3	0.0	0.0	0.0	0.0	0.2	0.2	0.3	0.1	0.2	0.5	0.4	0.2	*Desulfobulbus mediterraneus*	87.5	NR025150
B37	*Proteobacteria*	0.6	0.9	0.2	3.0	0.4	0.2	0.0	0.0	0.0	0.0	0.5	0.6	1.0	0.4	0.0	0.0	0.0	0.1	0.2	1.8	0.2	0.0	0.1	0.2	0.2	0.4	0.7	0.2	0.3	0.3	*Thiohalocapsa marina*	92.6	NR115047

[a] Cells with relative abundance values are colored in a heatmap-like fashion: Samples are labeled with the corresponding reactor name and sampling time in days.

[b] The lowest rank classified against the NCBI 16S rRNA sequence database down to the genus level.

[c] Closest cultivated sequences were determined by BLAST search against the NCBI 16S rRNA sequence database.

[d] Sequence similarity.

Table 4-7. Relative abundance and taxonomic affiliation of major archaeal OTUs (>3% relative abundance in at least one library)[a].

				CR								CB								FR						FB							
OTUs	Classification[b]	AS	RC	8	331	497	581	681	920	1000	1145	8	331	497	581	681	920	1000	1145	8	331	581	681	920	1145	8	331	581	681	920	1145	Closest species[c]	Sim (%)[d]	Accession no.
A1	*Methanothrix*	95.9	30.7	51.8	69.9	32.1	43.0	26.2	61.7	48.5	83.3	93.3	85.8	64.1	54.6	7.8	1.4	3.1	27.9	95.3	67.2	35.5	23.7	32.9	48.9	91.6	82.0	22.2	2.2	0.1	0.3	*Methanothrix harundinacea*	97.8	NR043203
A2	*Methanothrix*	0.0	0.0	5.5	0.3	0.5	0.4	0.2	3.4	1.1	0.1	0.0	0.1	0.1	4.0	2.2	45.4	27.2	23.1	0.0	0.0	2.1	0.5	21.3	2.7	0.0	1.3	23.2	23.3	69.2	30.9	*Methanothrix soehngenii*	98.2	NR102903
A3	*Methanobacterium*	0.4	2.6	0.4	7.1	36.0	38.2	56.9	21.3	41.9	9.6	0.4	10.0	26.8	30.0	34.4	14.9	37.2	13.6	0.5	8.5	19.8	47.3	31.5	36.8	0.6	10.5	41.5	42.6	22.4	55.0	*Methanobacterium beijingense*	99.6	NR028202
A4	*Methanosarcina*	0.0	0.5	0.0	0.0	0.0	0.0	1.4	0.1	0.1	0.0	0.0	0.0	0.0	0.0	49.3	29.7	6.2	0.7	0.0	0.0	0.4	2.0	0.6	0.1	0.0	0.2	0.0	0.0	0.0	0.0	*Methanosarcina flavescens*	98.9	NR148758
A5	*Methanobacterium*	0.1	41.9	2.0	0.1	0.4	0.1	0.1	0.1	0.0	0.0	1.5	0.1	0.4	0.2	0.0	0.3	0.4	0.2	0.1	0.1	0.0	0.0	0.0	0.1	2.0	0.1	0.2	0.2	0.1	0.3	*Methanobacterium ferruginis*	99.6	NR113045
A6	*Methanoregulaceae*	0.5	0.0	9.6	9.6	2.0	3.0	8.3	0.2	0.1	0.0	1.4	1.0	0.5	0.8	0.8	0.0	0.0	0.0	0.8	17.0	36.2	23.4	0.2	0.1	1.1	0.1	0.1	0.1	0.0	0.0	*Methanolinea tarda*	97.8	NR028163
A7	*Methanospirillum*	0.3	0.0	0.9	6.7	4.3	1.6	2.4	11.4	7.8	5.7	0.3	1.8	3.5	1.6	1.9	4.8	24.2	28.5	0.3	4.5	1.2	1.5	10.5	5.6	0.3	3.6	5.1	7.2	2.4	6.9	*Methanospirillum hungatei*	98.2	NR074177
A8	*Methanothrix*	0.1	0.5	22.3	3.3	21.7	8.6	2.0	0.3	0.1	0.0	0.2	0.1	1.0	1.1	0.2	1.0	0.6	0.5	0.1	0.0	0.7	0.0	0.6	0.1	0.4	0.4	1.6	0.6	1.4	0.7	*Methanothrix soehngenii*	98.9	NR104707
A9	*Methanosarcina*	0.0	20.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	*Methanosarcina soligelidi*	98.5	NR109423
A10	Archaea	0.7	0.0	3.6	2.4	0.6	1.8	0.0	0.0	0.0	0.3	1.2	0.8	1.9	2.4	0.1	0.0	0.0	4.3	0.7	1.7	0.2	0.0	1.0	5.1	0.9	1.1	2.5	19.4	2.4	4.8	*Methanothermus fervidus*	85.7	NR102926

[a] Cells with relative abundance values are colored in a heatmap-like fashion: Samples are labeled with the corresponding reactor name and sampling time in days.

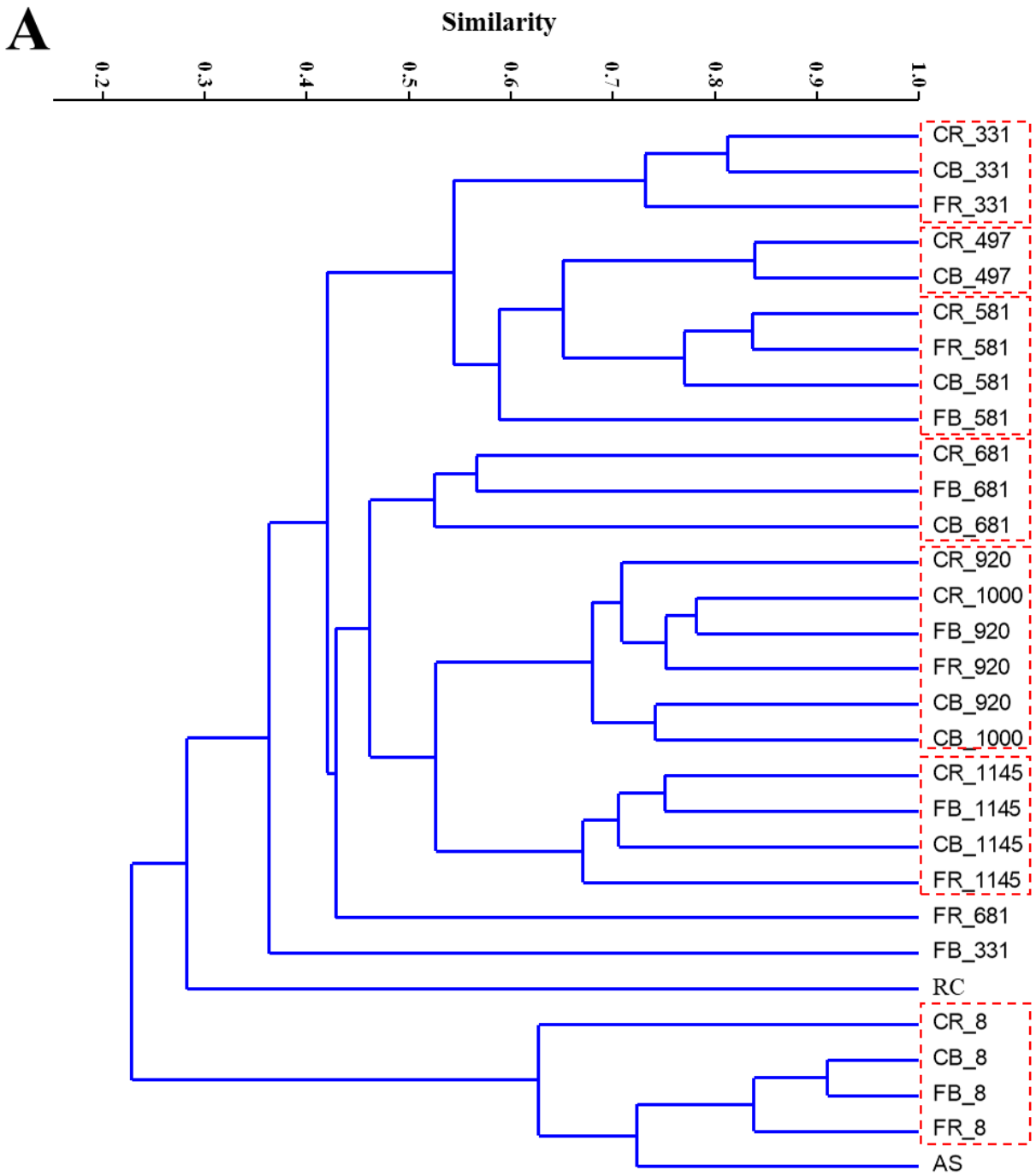
[b] The lowest rank classified against the NCBI 16S rRNA sequence database down to the genus level.

[c] Closest cultivated sequences were determined by BLAST search against the NCBI 16S rRNA sequence database.

[d] Sequence similarity.

The cluster dendrograms and NMS plots show that both bacterial and archaeal community structures changed during the experiment (Figs. 4-12 and 4-13). NMS is an ordination method that can indicate microbial community structures by means of points in an ordination space so that communities with similar structures are closely located in the space. Therefore, the NMS plot can be considered representative of the degree and direction of community structure shifts during the experimental periods. The cumulative r^2 of the ordination axes was 0.79 and 0.94 in the bacterial and archaeal NMS plots, respectively, indicating that each two-dimensional plot explained 79% and 94% of the total variability in the analyzed community data. The final stress (<14.3) and instability levels ($<10^{-4}$) were adequately low from the viewpoint of reliable ordination of the final solutions [109]. Interestingly, both bacterial and archaeal community structures changed significantly over the experimental phases in response to fluctuations in substrate composition. In the cluster dendrograms (Fig. 4-12), significant community shifts in CR and CB were observed after the co-digestion on day 497 (Sorensen similarity index (Ss) <0.55), which was associated with increases in bacterial *Candidatus* Cloacamonas and *Bacteroidetes* and archaeal *Methanobacterium* with the disappearance of *Porphyromonadaceae* (Tables 4-6 and 4-7). In addition, the archaeal community profiles between CR and CB were more distantly related to the bacterial community profiles, which were associated with the dominant *Methanothrix* divided into OTUs A1 and A8 in CR. These significant changes were likely associated with the adaptation of the microbial community to fluctuations in substrate composition from FW to FKW. In the presence of process imbalance in CR, CB, and FR on day 581, remarkably different structures in FB with stable performance were observed compared to those in other reactors (Ss, <0.6 and <0.32 in bacterial and archaeal community, respectively). The microbial profiles in FB were closely related to the changes in environmental factors, that is, FA, CH_4 production, and CODr in the NMS plots. These findings suggest that the microbial community shift in FB possibly played a role in maintaining stable reactor performance against the inhibitory effect of high FA concentration. During recovery periods on day 681 with substrate changes, with dramatic variations in the bacterial and archaeal communities in CB, the bacterial and archaeal communities in CR and CB diverged greatly from each other to remarkably different structures; the same was true for the bacterial and archaeal communities in FR and FB as well. These differences can be attributed to different resilience levels resulting from the different residual acids and ammonia concentrations. In this period, CB exhibited the highest methane yield without accumulation of VFAs compared to the other reactors. This result suggests that the microbial community structure in CB was affected to a greater extent by the diverse microbial groups in RC for treating FKW owing to the fluctuation in substrate composition. Especially, the shift in dominance to *Methanosarcina* (Table 4-7) and syntrophic bacteria such as *Petrimonas sulfuriphila* and *Candidatus* Endomicrobium (Table 4-6) with hydrogen-utilizing methanogens may have been significantly related to process performance and microbial community. After the feed to CR and CB was changed to 100% FW on day 1000, changes to the archaeal community in CB (Ss, 0.5) were more dynamic than those to the bacterial community in CB (Ss, 0.74) and the archaeal

community in CR (Ss, 0.8). With the reapplication of co-digestion in CR and CB between days 1000 and 1145, the microbial community structures in the two reactors differed significantly from each other with the similarity (Ss 0.53–0.65). These results indicate that the significant changes could be associated with fluctuations in substrate characteristics. In particular, the structural changes to the archaeal community (Ss, 0.32) between CR and CB were more pronounced than those to the bacterial community (Ss, 0.71) on day 1145. This could possibly be associated with the less diverse nature of archaeal communities than that of bacterial communities in AD environments [127]. The dramatic dominant shifts between hydrogenotrophic methanogens and aceticlastic methanogens owing to changes in substrate compositions are noteworthy. The changes in CB were more pronounced because of its more stable and efficient performance with bioaugmentation than those in CR. The cluster dendrograms and NMS plots, therefore, indicate that the microbial community structures changed significantly according to fluctuations in substrate composition and bioaugmentation with RC over long-term operation. Moreover, the changes in the microbial community structures significantly influenced reactor performance in terms of stability and resilience with fluctuations in substrate composition.



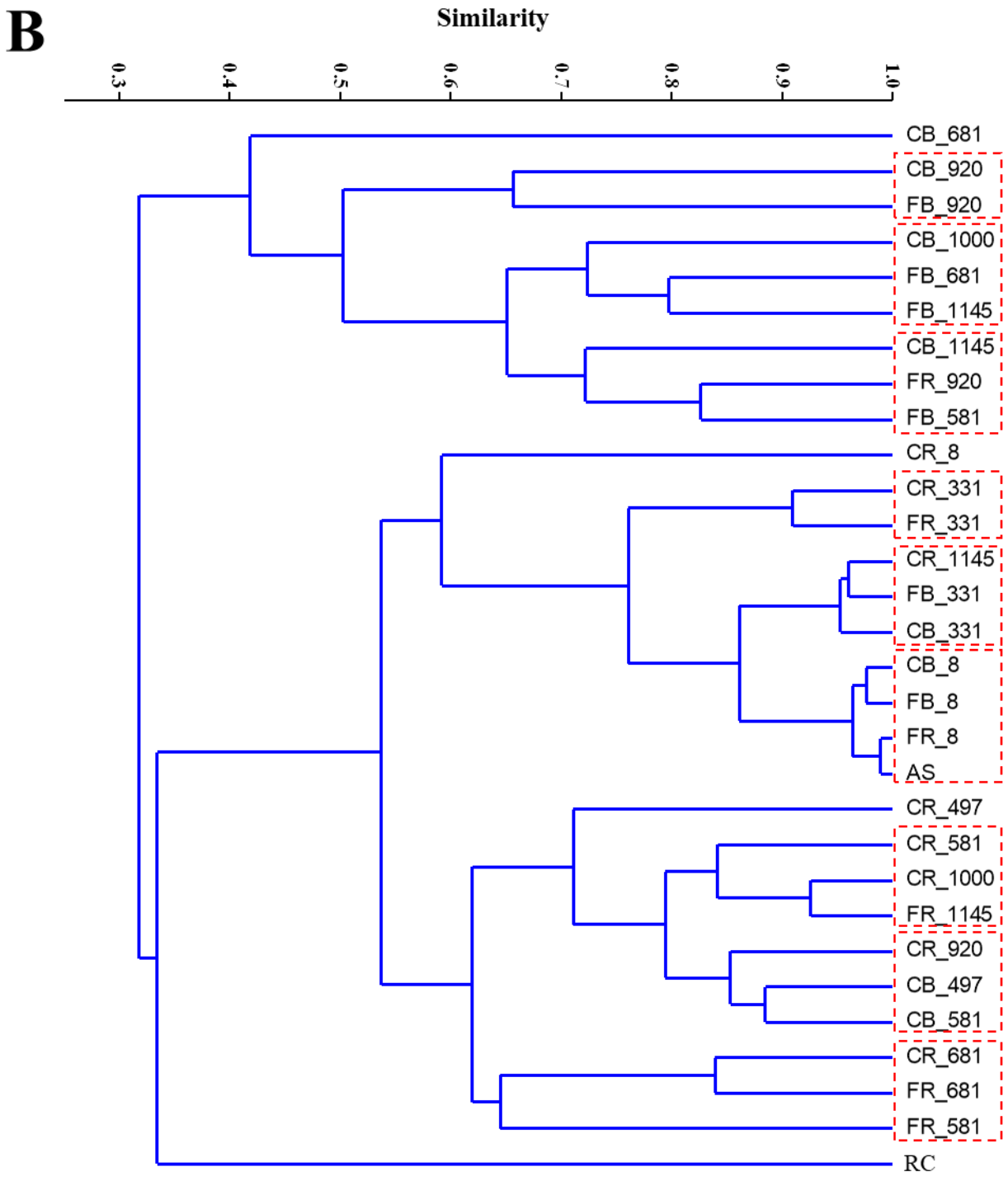
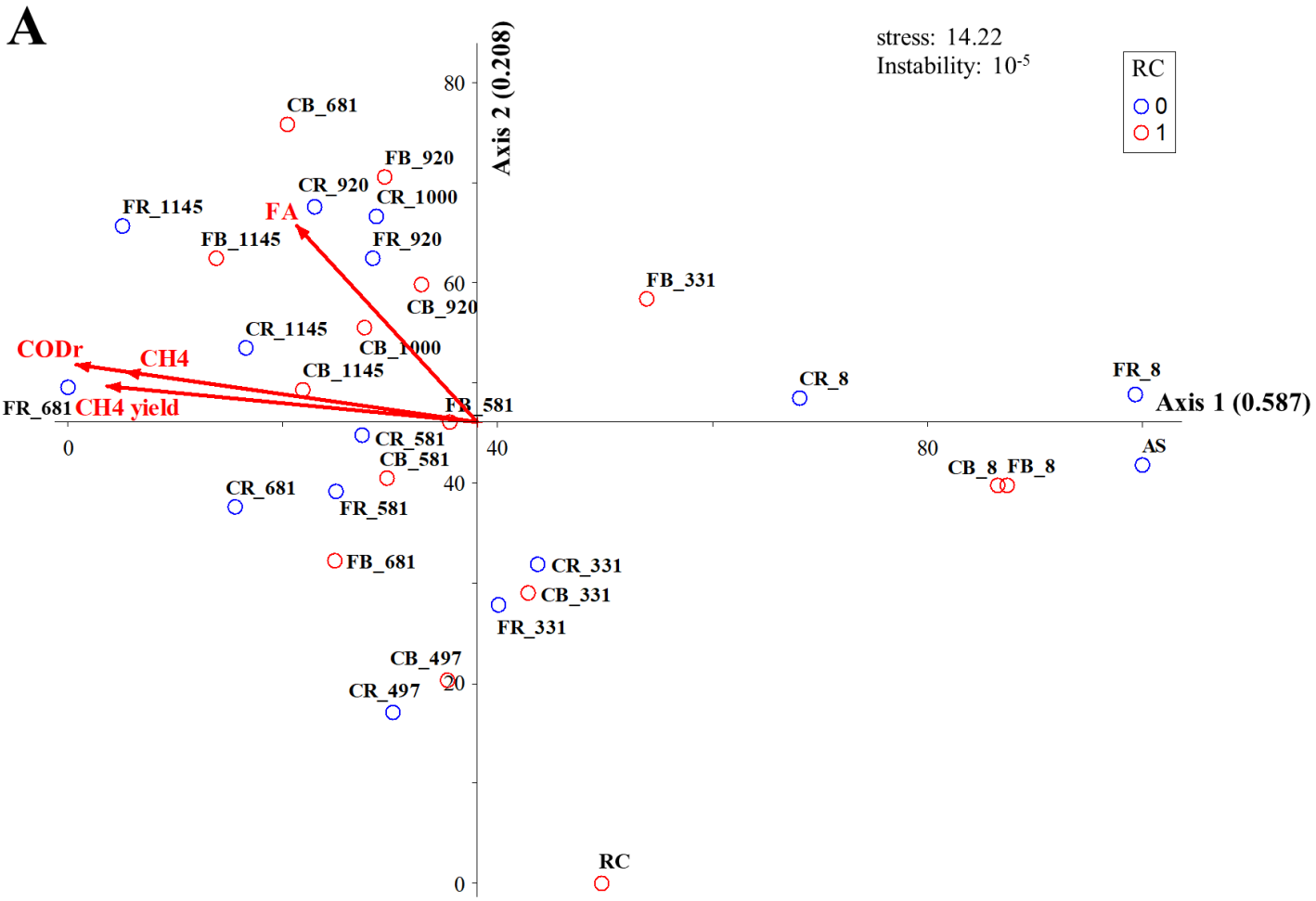


Figure 4-12. Cluster dendrograms constructed based on the OTU distribution in the bacterial (A) and archaeal (B) 16S rRNA gene libraries.



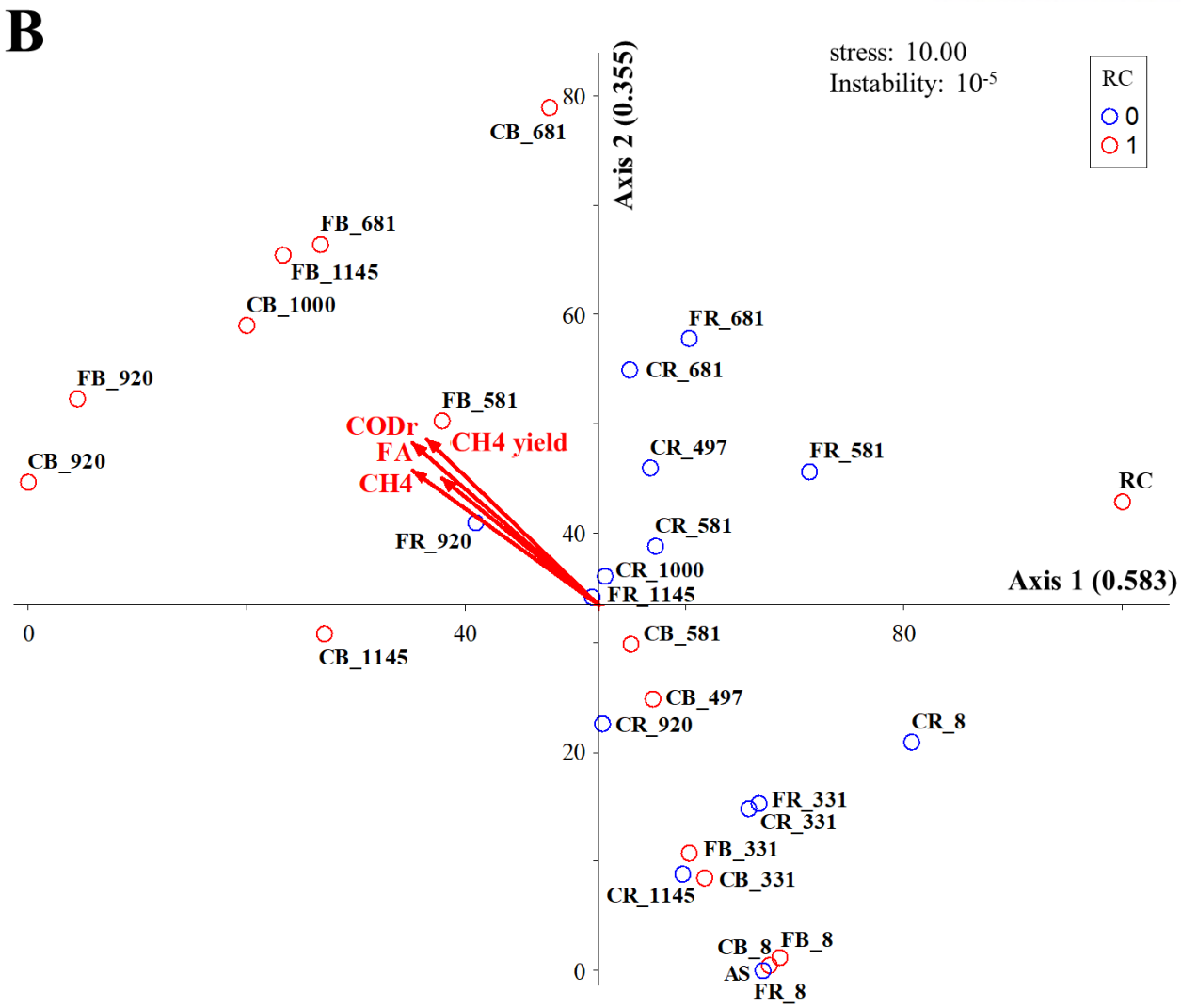


Figure 4-13. NMS plots of CH_4 production, CH_4 yield, COD removal (CODr), free ammonia (FA) constructed based on OTU distribution in the bacterial (A) and archaeal (B) 16S rRNA gene libraries. Arrows indicate the direction (angle) and magnitude (length) of the correlation between the environmental variable and the ordination axes. The blue circles denote the absence of RC (CR and FR), and the red circles denote bioaugmentation with RC (CB and FB).

4.4. Summary

Four CSTRs were operated to examine the stability and efficiency of reactor performance under bioaugmentation with RC against fluctuations in the composition of Korean FW with co-digestion of KW as the seasonal variation. Before co-digestion, the reactors performed similarly in terms of methane production because of their similar microbial community structures. After co-digestion, the performance of CB improved (by 12.3% increases in methane yields) and became more stable compared to the performance of

the other reactors in the steady state during P2−P5. In addition, the methane yields of 0.500–0.556 L CH_4/g VS_{fed} in CB were higher than the values of 0.356–0.478 L CH_4/g VS_{fed} reported in previous studies on the biomethanation of Korean FW [1, 84, 129, 130]. Even when ammonia inhibition on low C/N ratio substrates affected process performance, leading to decreased methane production and VFA accumulation, CB with RC bioaugmentation exhibited superior performance and rapid recovery compared to CR. Moreover, FB with RC bioaugmentation exhibited more stable performance without VFA accumulation compared to FR. Furthermore, the microbial community structure in CB changed distinctly to a structure favorable from the viewpoint of process performance, in particular, the dominance shift in the archaeal community with *Methanosarcina* or other hydrogenotrophic methanogens with diverse acidogenic bacteria probably from RC. The process in CB performed in a stable manner over a long period (over 3 years) even as the substrate composition changed seasonally. This indicates that the co-digestion of KW under RC bioaugmentation could be enhanced and operated stably with significant structural changes to the underlying microbial community despite changes in substrate composition. Therefore, bioaugmentation with RC has the potential to enhance the sustainability and resilience of AD for the long-term treatment of Korean FW with seasonal fluctuations.

5. [Further study] Partial nitritation anammox process for treating anaerobic digestate

5.1. Introduction

AD has an advantage to biologically degradation of organic waste such as FW, however, anaerobic digester effluent still should be treated. Because the effluent remains to high ammonium nitrogen concentration which cause environmental problems including dissolved oxygen depletion, eutrophication, odor, ammonia toxicity [141]. Conventional biological nitrogen removal (BNR) technologies rely on sequential nitrification and denitrification (Fig. 5-1). Nitrification involves two consecutive oxidation steps; the oxidation of ammonia to nitrite by ammonia oxidizing bacteria (AOB) and the oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB). These reactions are regarded as a rate limiting step because of slow growth rate of AOB. In particular, AOB are sensitive to environmental conditions such as a high ammonia concentration, dissolved oxygen (DO) concentration, temperature. Moreover, these reactions need to much more oxygen for oxidation of ammonia which highly increase the operational cost for aeration. It accounts for about 20% in total operational cost of sewage management plant. Denitrification in anaerobic condition performs to conversion of nitrate to N_2. But, this reaction requires to organic carbon source for the reaction which contain less in anaerobic digester effluent. For the denitrification, thus, external carbon source i.e., methanol has been added into BNR process. It also causes the increase of operational cost for BNR process. Therefore, to reduce the operational cost and improve the process efficiency, it requires alternative

method.

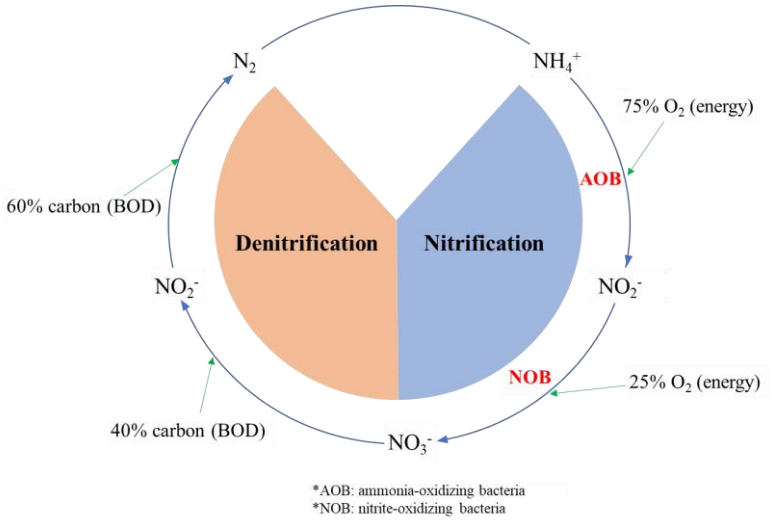


Figure 5-1. BNR process of nitrification-denitrification.

Recently, anammox-based BNR processes, particularly those combining partial nitritation and anammox (PNA), have been extensively studied and applied as a sustainable alternative to conventional BNR processes at different scales [142, 143]. PNA is a completely autotrophic process where ammonium is partially oxidized to nitrite under aerobic conditions by ammonium-oxidizing bacteria (AOBs) (Eq. 5-1), followed by the anoxic oxidation of remaining ammonium to nitrogen gas using nitrite as electron acceptor by anammox bacteria (AMBs) (Eq. 5-2).

$$NH_4^+ + 1.38O_2 + 0.09HCO_3^- \rightarrow 0.98NO_2^- + 0.02C_5H_7O_2N + 1.04H_2O + 1.89H^+ \quad (5\text{-}1)$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (5\text{-}2)$$

Therefore, PNA reduces the requirements for aeration and organic carbon source by 100% and more than 50% compared to conventional nitrification/denitrification [144]. PNA has been successfully implemented in the treatment of high-strength wastewater such as anaerobic digestion reject water [143]. While early PNA implementations employed two-stage configuration (i.e., separate reactors for partial nitritation and anammox connected in series) for better control of nitritation, recent focus has turned mainly to single-stage systems (i.e., PNA completed in one reactor due to the lower capital and operating costs [143, 145, 146]. However, maintaining a balanced activity of AOBs and AMBs while suppressing the growth of nitrite-oxidizing bacteria (NOBs) in one reactor is a difficult challenge in single-stage PNA (S-PNA) processes

[147]. Both AOBs and AMBs grow very slowly (doubling time, 0.3–2.1 days for AOBs [148, 149] and 7–20 days for AMBs [150, 151]) and their high retention in the reactor is critical for stable S-PNA performance. AMBs are sensitive to environmental conditions, such as dissolved oxygen (DO), temperature, pH, and organic matter, and applying unfavorable or suboptimal operating conditions can result in a significant deterioration of anammox activity [152].

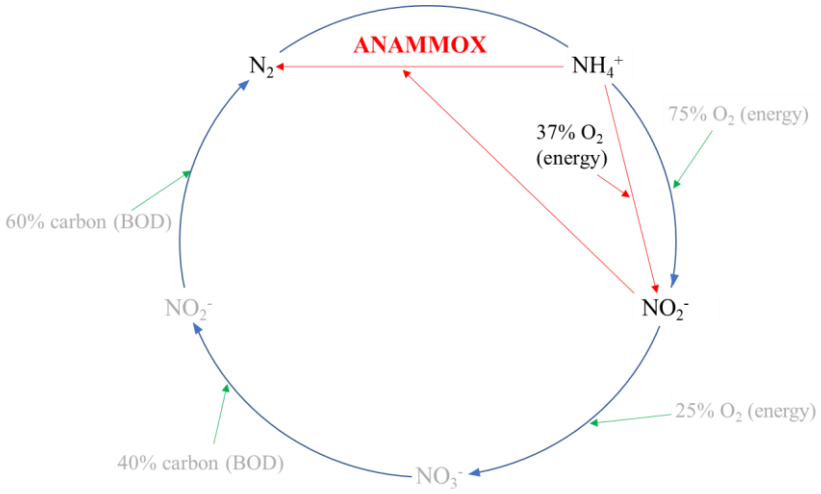


Figure 5-2. Anammox process with partial nitrification.

Cell immobilization is a promising method for enriching slow growth microorganisms. Immobilization technology has been used to provide higher cell density, biomass retention, easier solid-liquid separation, and resistance to toxic matters of adverse environmental conditions [153]. Among the various immobilization techniques, cell entrapment using polyvinyl alcohol (PVA) as an immobilization matrix has been widely used for biological wastewater treatment because these polymers are non-toxicity to microorganisms, low cost, higher mechanical strength, and chemical stability [154]. The different making method of PVA gel has been introduced in previous studies i.e., PVA-alginate, PVA-boric acid, and PVA-cryogel formation. Among these methods, PVA-cryogel formation is to fabricate hydrophobic hydrogel method using freezing and thawing, while other methods using chemical crosslinking (Fig. 5-3). Previous studies have reported to the enrichment of anammox bacteria using PVA-alginate and PVA-cryogel [153, 155-157]. Therefore, anammox bacteria immobilization using PVA cryogel is considered as a successful start-up method for solving the problem of low growth rate.

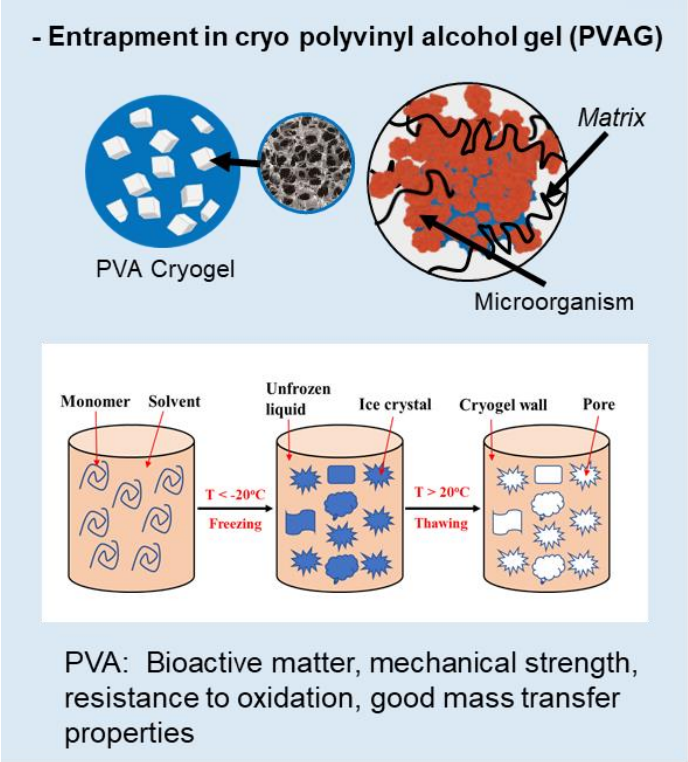


Figure 5-3. Entrapment on PVA-cryogel.

In this study, to examine the potential of anammox process for anaerobic digester liquor treatment, anammox bacteria was firstly cultivated from the activated sludge treating anaerobic digester digestate. For the enrichment of anammox bacteria, PVA-cryogel was applied to entrapment method due to higher stability of biomass retention in bioreactor. After the enriching anammox bacteria, the bacteria has been being utilized to operate partial nitritation and anammox process. Preliminarily, to examine the novel upflow dual-bed gel-carrier reactor (UDGR) system in PNA process, the system was operated using synthetic wastewater under low nitrogen load and then increased nitrogen load. From the preliminary test, modified UDGR system will be operated to treat high nitrogen wastewater using synthetic wastewater. After stabilizing this process, digester liquor will be supplemented to synthetic wastewater with gradually increasing ratio of 33, 66, and 100%.

5.2. Materials & methods

5.2.1. Experimental design for preliminary tests

Return activated sludge taken from a conventional activated sludge in Yongyeon plant treating municipal wastewater (CAS-Y) was sieved through an 860-μm mesh and concentrated by gravity settling to a VSS concentration of 6.5 g/L. A 20% (w/v) solution of polyvinyl alcohol (PVA; 100% saponification, 2000

degrees of polymerization, SHOWA) was autoclaved at 121°C for 30 min and then cooled to 37°C. The prepared seed sludge and PVA solution were mixed at a 1:1 volume ratio to produce a 10% PVA (w/v) mixture [158] and then poured into rectangular trays (18 × 24 cm) to a depth of 4 mm. The mixture was gelled by freezing (–20°C for 24 h) and thawing (room temperature for 1 h) twice. The prepared gel plates were cut into cubes of approximately 4 mm and rinsed with sterile water.

The gel-immobilized activated sludge was cultivated in two identical UDGRs, namely Ra1 and Ra2, for the enrichment of anammox bacteria. Each reactor had a working volume of 1.6 L with a gel packing ratio of 19% (v/v). One half volume of the anammox gel carriers (0.15 L) was added to each of the upper and lower compartments of a UDGR, which were separated by a 1-mm stainless steel mesh. The reactors were operated continuously at a HRT of 8 h, corresponding to a nitrogen loading rate (NLR) of 0.33 kg $N/m^3 \cdot d$, with a synthetic medium containing per liter: $(NH_4)_2SO_4$, 55 mg-N; $NaNO_2$, 60 mg-N; $NaHCO_3$, 80 mg-C; KH_2PO_4, 6 mg-P; $MgSO_4 \cdot 7H_2O$, 12 mg-Mg; $CaCl_2 \cdot 2H_2O$, 48 mg-Ca, and 1 mL each of trace element solutions I and II [157]. The reactor temperature was maintained between 34 and 35°C, and the pH was not controlled. The UDGR configuration and experimental setup are shown in Fig. 5-4.

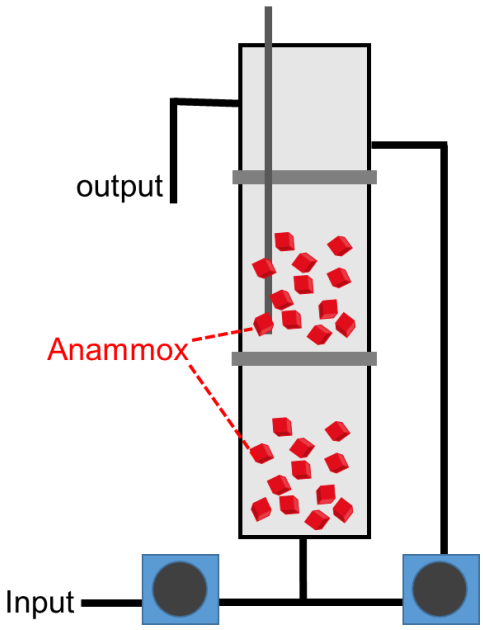


Figure 5-4. UDGR system configuration.

5.2.2. High-throughput 16S rRNA gene sequencing

Community DNA of CAS-Y was extracted using an automated nucleic acid extractor (Exiprogen, Bioneer) as described previously [105]. A 1-mL aliquot of CAS-Y was centrifuged at 13,000 *g* for 3 min, and the

supernatant was decanted. The pelleted biomass was then washed by repeated resuspending (up to 1 mL), decanting, and pelleting (13,000 *g* for 3 min) in distilled water to remove debris and impurities. A 200-μL portion of the final resuspension was loaded onto the extractor. The extracted DNA was eluted in 100 μL of elution buffer. Cryogel carriers for microbial community analysis were cut into fine pieces (<1 mm) with sterile scissors [153] and then subjected to total DNA extraction using the MoBio PowerSoil DNA Kit (MoBio Laboratories) following the manufacturer's protocol. The extracted DNA was stored at –20°C until use.

The 16S rRNA gene libraries for HTS were prepared from the purified DNA samples by PCR with universal prokaryotic primers 515F and 806R [108]. An Illumina adapter sequence was attached to the 5′ end of each primer. PCR was conducted using the following thermal cycling program: an initial denaturation at 94°C for 10 min, 30 cycles of amplification (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C), and a final elongation at 72°C for 7 min. The resulting PCR products were cleaned and sequenced on the Illumina MiSeq platform at Macrogen, Inc. Reads with low quality scores, ambiguous bases, or potential chimeric sequences were excluded from subsequent analyses. The trimmed sequences were aligned and clustered using CD-HIT-OTU (http://weizhongli-lab.org/cd-hit-otu/) with an operational taxonomic unit (OTU) definition of <3% divergence. The taxonomic classification of OTUs was performed by comparing a representative sequence against the RDP database using UCLUST [159] in the QIIME suite (ver. 1.8.0) [160].

5.2.3. Analytical methods

Cations (including NH_4^+) and anions (including NO_2^- and NO_3^-) were measured using two ion chromatographs (Dionex ICS-1100, Thermo Scientific) equipped with an IonPac CS12A column and an IonPac AS14 column, respectively. Samples for ion analysis were prepared by filtration through a 0.22-μm pore-size syringe filter. Solids were measured according to the protocols in Standard Methods for the Examination of Water and Wastewater [52]. All analyses were replicated at least twice.

5.3. Results & discussion

5.3.1. ANAMMOX enrichment reactors

As the first step, ANAMMOX biomass was enriched from CAS-Y using the UDGR system. Residual nitrogen concentrations and nitrogen removal efficiency (NRE) of anammox process (Ra) were shown in Fig. 5-5. During the initial 90 days, total NRE was gradually decreased with increasing residual NO_2^-–N and no significant NH_4^+–N removal. The residual NO_2^-–N and NH_4^+–N were rapidly decreased over 106 days. It

indicated that Ra showed a lag period of 106 days. The result of lag phase was similar with a previous study which represented to anammox activity related to PVA-cryogel thickness [156]. According to the study, the lag period was significantly higher with increasing thickness from 1 to 3mm (the lag phase 94 days at thickness 3mm). After 132 days, the average NRR and NRE were maintained at 0.30 ± 0.03 kg $N/m^3 \cdot d$ and 90.0 ± 4.3% on NLR of 0.34 ± 0.02 kg $N/m^3 \cdot d$, respectively. During this period, the average residual NO_2^-–N and NH_4^+–N were stably maintained below 10 and 1 mg/L, respectively. The results indicate that the ANAMMOX reaction was successfully achieved in the UDGR systems even though CAS was used as an inoculum source.

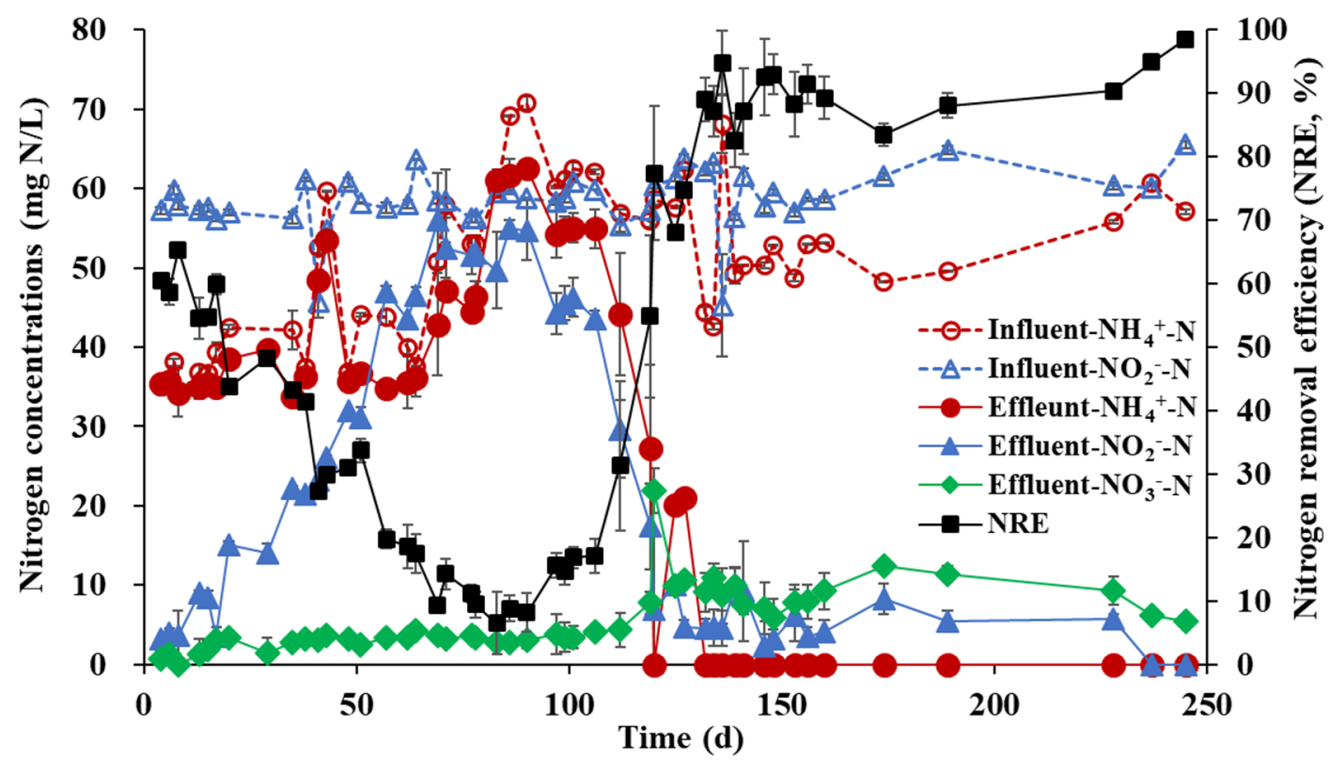


Figure 5-5. The average values of the enrichment process in both Ra reactors.

In steady anammox reaction, the consumed ratio of NO_2^-–N and NH_4^+–N were 1.06 ± 0.17 and the ratio of the produced NO_3^-–N to consumed NH_4^+–N were 0.13 ± 0.05. These results were generally lower than the theoretical stoichiometric ratio of 1.32 and 0.26, respectively [161]. Low DO in the substrate storage may affect to the conversion from ammonium to nitrite by AOB. However, it would be low portion from the calculation of stoichiometric ratio due to the low DO in the anaerobic system [157]. The oxidants released under the biomass stress conditions from mixed culture using conventional activated sludge may cause the overconsumption of ammonium with low ratio of NO_2^-–N to NH_4^+–N. Because the free radicals or reactive oxygen species derived from the dead cells, which could not adapt to new environmental condition for anammox friendly, could be used for ammonium oxidation as electron acceptors [162]. In addition to, the

degradation of the biomass produces the organic carbon and ammonium which could have an effect on the heterotrophic denitrification by providing organic source for denitrifier in anaerobic condition. Thus, low NO_3^-–N/NH_4^+–N ratio suggests that co-existence and cell lysis of diverse microbial cells from activated sludge could contribute to the TN removal efficiency. Previous studies also represented to the observation of an extremely low NO_3^-–N/NH_4^+–N ratio using conventional sludge [157, 163].

5.3.2. Microbial community characterization

The three samples for microbial community analysis were collected on CAS-Y and days 256 in Ra. A total of 69,011 bacterial reads without archaeal reads were obtained from the samples by NGS analysis. A total of 49 bacterial order were identified from the retrieved 16S rRNA gene sequences. *Rhodocyclales*, *Sphingobacteriales*, and *Methylophilales* were dominant order in CAS-Y, while *Candidatus* Brocadiales, *Anaerolineales*, *Xanthomonadales*, *Burkholderiales*, and *Ignavibacteriales* were dominant order in Ra1 and 2 (Fig. 5-6). In particular, *Candidatus* Brocadiales involved to anammox bacteria was dominated in Ra1 and 2. It indicates to the enrichment of anammox bacteria from activated sludge in UDGRs. The dominant order *Rhodocyclales* in CAS-Y is related to nitrogen fixing bacteria in aerobic condition. This order may play a role for the treatment of sewage wastewater in wastewater treatment plant.

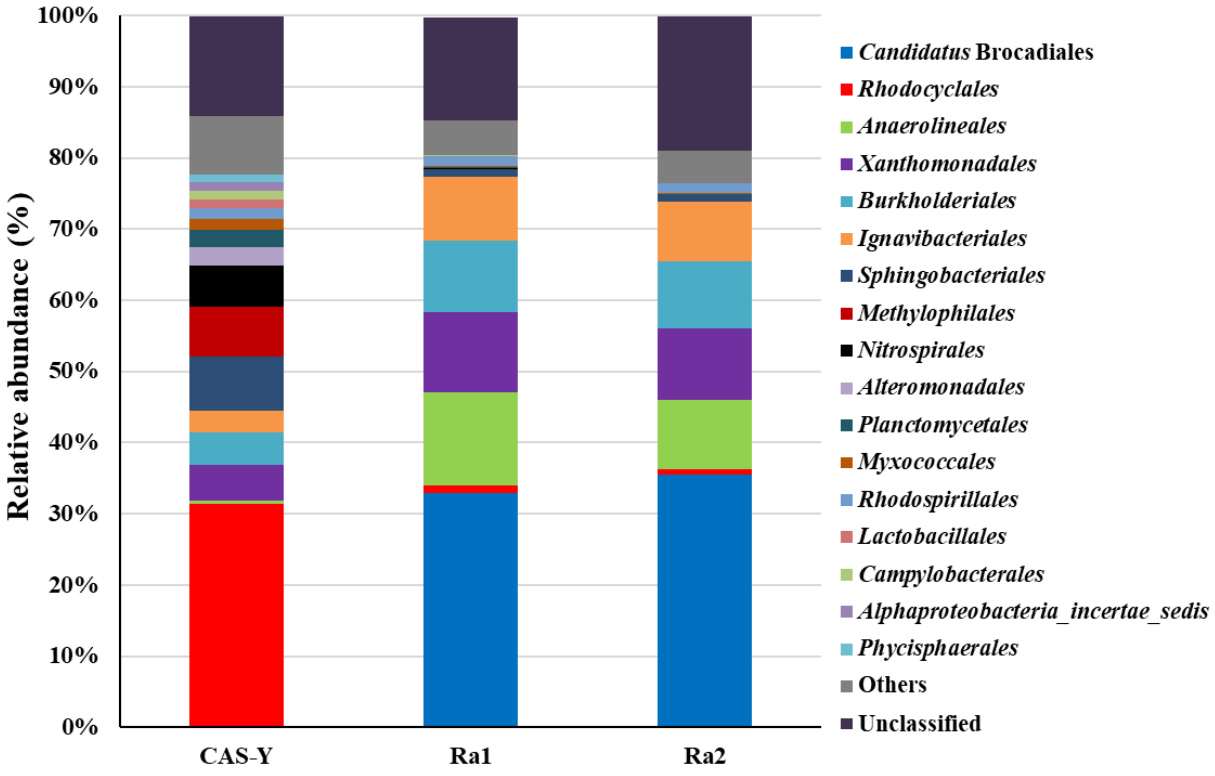


Figure 5-6. Bacterial community structures characterized at the order level.

The 468 bacterial OTUs were clustered from the recovered sequences and the major OTUs (>1% relative abundance in at least one library) are listed in Table 5-1. OTU B1 was dominant in Ra1 and 2, which was assigned to genus *Candidatus* Jettenia showed a considerable similarity (98.3%) to an anammox bacteria utilizing NH_4^+ and NO_2^- [164]. The greater prevalence of OTU B1 in anammox process reflect to successful enrichment and immobilization of anammox bacteria from activated sludge using PVA-cryogel. Therefore, OTU B1 could mainly carry out the anammox reaction in the study. Most of the major OTUs (B2, 6, 7, 9, 10, 13) above 3% relative abundance related to nitrogen utilization in CAS-Y nearly disappeared to anammox process. It might be caused by changing operational condition i.e., substrate, anaerobic, and HRT. Because conventional BNR process is different from anammox process, especially, aerobic bacteria would not be tolerated under anaerobic condition. On the other hands, various OTUs (B1, B5, B12, B14, and B16) related to denitrification and anammox reaction were appeared in anammox process. In addition, B3 assigned to *Anaerolineaceae* family can provide a carbon source for denitrification by fermenting macromolecules. The symbiotic relationship between these bacteria were likely to help the adaptation on the anammox process condition and play a role for nitrogen removal in this study. Interestingly, OTU B4 was affiliated with the genus *Povalibacter*, which is capable of utilizing polyvinyl alcohol [165]. This OTU was only detected in Ra1 and 2. It potentially indicated that PVA-cryogel may be broken in the early part of formation.

Table 5-1. Relative abundance and taxonomic affiliation of major bacterial OTUs (>1% relative abundance in at least one library)[a].

OTU no.	Classification[b]	CAS-Y	Ra1	Ra2	Nearest species[c]	Similarity (%)[d]	Accession no.
B1	*Candidatus* Brocadiaceae	0.0	32.9	35.5	*Candidatus* Jettenia asiatica	98.3	AB973443
B2	*Rhodocyclaceae*	23.5	0.0	0.0	*Azoarcus olearius*	96.6	NR108183
B3	*Anaerolineaceae*	0.0	12.7	9.5	*Thermanaerothrix daxensis*	86.7	NR117865
B4	*Povalibacter*	0.0	10.6	9.3	*Povalibacter uvarum*	98.0	NR126172
B5	*Bacteria*	0.1	5.9	9.6	*Ammoniphilus oxalaticus*	86.2	NR026432
B6	*Methylophilaceae*	6.8	0.0	0.0	*Methylotenera versatilis*	97.9	NR074693
B7	*Thauera*	6.5	0.0	0.0	*Thauera phenylacetica*	98.6	NR027224
B8	*Ignavibacterium*	0.0	5.4	4.8	*Ignavibacterium album*	98.3	NR074698
B9	*Dokdonella*	4.9	0.0	0.0	*Dokdonella immobilis*	97.6	NR108377
B10	*Nitrospira*	3.7	0.1	0.1	*Nitrospira lenta*	92.5	NR148573
B11	*Bacteria*	0.0	3.6	3.6	*Ignavibacterium album*	85.7	NR074698
B12	*Janthinobacterium*	0.0	3.5	3.2	*Janthinobacterium svalbardensis*	97.9	NR132608
B13	*Gammaproteobacteria*	3.3	0.0	0.0	*Alkalimarinus sediminis*	93.8	NR137384
B14	*Betaproteobacteria*	0.0	2.5	2.6	*Methyloversatilis universalis*	93.5	NR043813
B15	*Burkholderiales*	2.5	0.0	0.0	*Chromatocurvus halotolerans*	97.3	NR115058
B16	*Betaproteobacteria*	0.1	2.5	2.2	*Zoogloea ramigera*	94.2	NR113749
B17	*Ignavibacterium*	2.5	0.0	0.0	*Ignavibacterium album*	96.2	KF528150
B18	*Nitrosomonas*	2.4	0.0	0.0	*Nitrosomonas sp.* Nm59	98.3	AY123811
B19	*Simplicispira*	0.6	2.3	1.8	*Simplicispira piscis*	98.3	NR145892
B20	*Chitinophagaceae*	2.3	0.0	0.0	*Pseudobacter ginsenosidimutans*	91.0	CP042431
B21	*Nitrospira*	2.0	0.0	0.0	*Nitrospira sp. Ecomares* 2.1	98.3	HQ686082
B22	*Bacteroidetes*	1.8	0.0	0.0	*Bacteroidetes bacterium*	90.3	HQ675539
B23	*Bacteria*	0.0	1.6	1.7	*Phycisphaera mikurensis*	80.6	NR074491
B24	*Acidobacteria*	0.0	1.3	1.7	*Limibacillus halophilus*	88.4	NR137248
B25	*Saprospiraceae*	1.3	0.0	0.0	*Phaeodactylibacter xiamenensis*	92.1	NR134132
B26	*Alphaproteobacteria*	1.2	0.0	0.0	*Candidatus* Sphaeronema italicum	98.3	AY428765
B27	*Trichococcus*	1.2	0.0	0.0	*Trichococcus pasteurii*	98.6	MK138630
B28	*Cytophagales*	1.2	0.0	0.0	*Chryseolinea serpens*	89.7	NR108511
B29	*Saprospiraceae*	1.2	0.0	0.0	*Lewinella cohaerens*	94.2	KF228160
B30	*Alphaproteobacteria*	1.1	0.0	0.0	*Candidatus* Combothrix italica	98.3	AY590698
B31	*Rhodospirillales*	0.0	0.9	1.1	*Dongia soli*	91.8	NR146690

[a] Cells with relative abundance values are colored in a heatmap-like fashion: Samples are labeled with the corresponding reactor name and sampling time in days.

[b] The lowest rank classified against the NCBI 16S rRNA sequence database down to the genus level.

[c] Closest cultivated sequences were determined by BLAST search against the NCBI 16S rRNA sequence database.

[d] Sequence similarity.

5.4. Plan for further study

Further study aims to i) enrich the anammox bacteria and AOB at high NLR condition in order to ii) apply for single PNA process of the treatment of AD effluent as high ammonium wastewater. Each anammox sludge and AOB sludge collected from the enrichment process will be utilized to the partial nitritation and anammox process with PVA-cryogel using synthetic wastewater. The novel reactor configuration of UDMR with separated nitrifying gel and anammox gel on top and bottom, respectively under intermittent aeration on top part will be applied like two-phase system; aerobic AOB part in top and anaerobic anammox part in bottom. After stabilizing the process, the process will be applied to treat the AD effluent instead of synthetic wastewater.

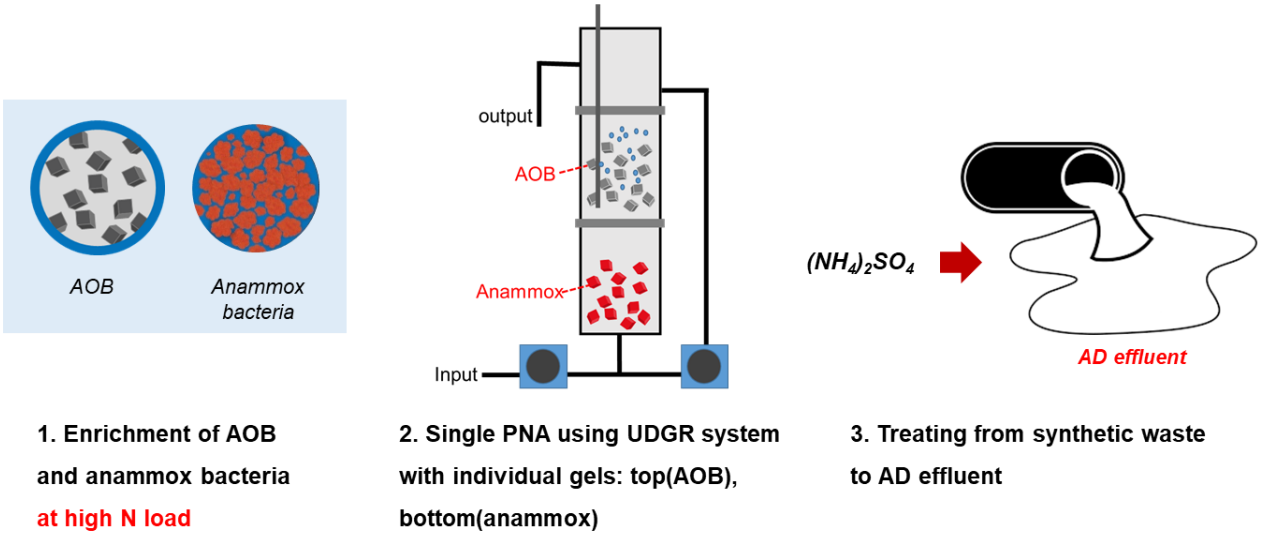


Figure 5-7. Further study scheme.

6. CONCLUSIONS

Bioaugmentation in AD can be a biological management strategy for boosting (enhancing the performance) and remediation (recovering deteriorated performance). RC consisting of versatile hydrolytic bacteria and methanogens was customized to degrade refractory organic matter related to plants in an anaerobic digester. RC has potential to be used as a bioaugmentation source for enhancing the AD of complex organics found in FW. Korean FW consists of approximately 55% vegetable and fruit waste, which increases to about 20% of the total FW during Kimjang season. Wastes consisting of vegetables and fruits contain high levels of dietary fiber, which is not easily biodegraded. Thus, hydrolysis of FW may be important for enhancing AD performance in Korea. In this study, the feasibility of bioaugmentation with RC for enhancing AD was confirmed using real Korean FW.

In study 1, the potential of bioaugmentation with RF in AD was demonstrated to improve the biomethanation of FW in batch and continuous systems. The results of study 1 implied that bioaugmentation with RF was beneficial for realizing enhanced (more than 8.5% increase in biogas yield) and resilient AD performance after encountering accidental process problems in the continuous AD of FW. Significant changes in the microbial community, especially bacterial communities, with bioaugmentation were related to syntrophic relationships between syntrophic bacteria and hydrogen-utilizing methanogens. These changes possibly contributed to the beneficial effect on AD performance.

In study 2, to derive an optimal system for AD with RF bioaugmentation, single- and two-phase systems were compared at various OLR conditions under uncontrolled pH. The results demonstrated that the single-phase system was superior to the two-phase system in terms of process efficiency and stability under conditions of higher OLR. In addition, the single-phase system was found to be easy to operate and less expensive to install and operate, and it was selected as the optimal process in study 2.

In study 3, the feasibility of bioaugmentation with RC as a strategy to enhance the biomethanation of FW in both batch and long-term continuous experiments was examined. The batch test, conducted using three inocula and two substrates, indicated that the mixed-culture inoculum was suitable for enhancing the biomethanation of KW and cellulose. Based on the results of the batch test, RC was applied as a bioaugmentation source in the long-term continuous experiment with various amounts of KW (0–20% of the total substrate VS) added to FW. The results of the continuous test demonstrated that bioaugmentation with RC in CB sustainably improved AD (an increase of up to 12.3% in methane yield compared to the control without bioaugmentation) for treating Korean FW with co-digestion of KW over the long-term (more than 38 months). Moreover, the methane yields of 0.500–0.556 L CH_4/g VS_{fed} in CB were higher than those in the previous studies (0.356–0.478 L CH_4/g VS_{fed}) in which Korean FW was treated in batch tests. The significant shifts in microbial community structures corresponding to bioaugmentation and the adaptation to fluctuations in substrate composition, such as the dominant shift to hydrogenotrophic methanogenesis and hydrolytic/acidogenic bacteria originating from RC, were observed.

In conclusion, the present study verified that bioaugmentation with RC enhanced the AD of Korean FW in a stable and sustainable performance even as the substrate composition fluctuated owing to the addition of KW. The findings of this study contain useful information from the viewpoint of managing AD plants treating FW. In addition, they provide information for understanding bioaugmentation with RC.

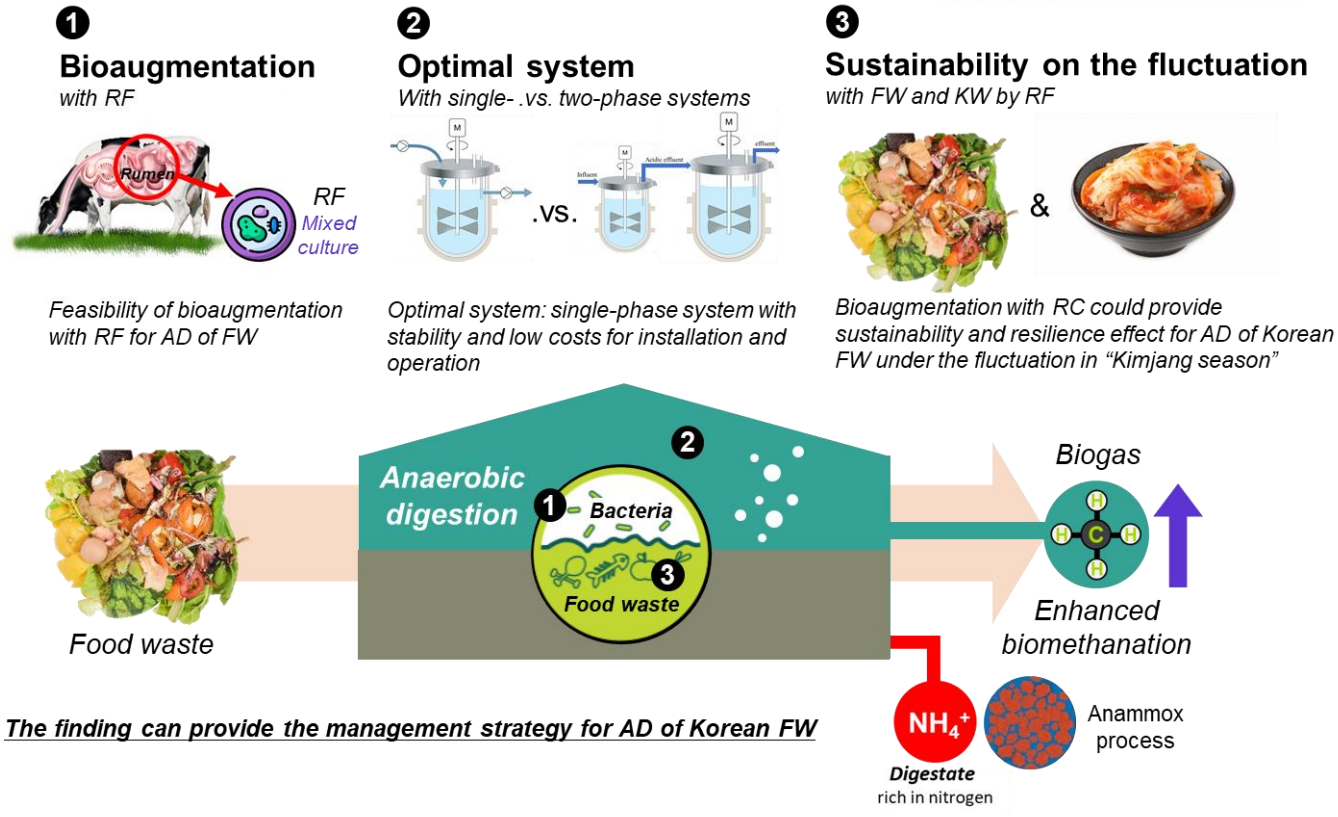


Figure 6-1. Summary of dissertation.

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감사의 말

박사학위 논문의 마지막 글을 작성하기까지 부딪혔던 어려움을 모두 지혜롭게 해결해 갈 수 있도록 인도해주신 하나님께 먼저 감사드립니다.

학위논문을 완성하기 까지의 시간을 되돌아보면 혼자 모든 것을 하고자 했지만 너무 부족한 것 투성이라 부끄럽고, 부족했던 저를 도와준 좋은 사람들 덕분에 지금까지 포기하지 않고 달려왔다는 생각이 듭니다. 지면을 빌어 그 분들께 감사를 전하고 싶습니다.

가장 먼저, 지도교수님이신 이창수 교수님께 감사 인사를 드리고 싶습니다. 때로는 선생님으로서, 인생 선배로서 학부생 시절부터 지금까지 아무것도 모르던 저를 물심양면으로 가르쳐 주시고 연구자로 성장할 수 있도록 도와주셔서 감사합니다. 부족한 제자이지만 긍정적으로 봐주시고 언제나 열심히 연구하시는 모습을 통해서 많은 것을 배울 수 있었습니다. 좋은 스승을 만난 덕분에 재밌는 연구를 하고 무사히 학위를 마칠 수 있게 되었습니다. 더불어, 바쁘신 와중에 시간을 할애하여 학위 심사를 해주시고 연구에 대한 조언을 통해 제 연구의 완성도를 높여주신 학위 심사위원 분들께도 감사의 인사를 드립니다. CRC과제 (science walden)에 참여하여 실험실에서 경험해보지 못했던 파일럿 규모의 공정 운전과 똥본위화폐라는 환경공학, 철학, 순환경제, 인문학이 결합된 개념을 통해 새로운 연구를 경험할 수 있게 해주신 조재원 교수님, 해외에 계셨지만 흔쾌히 학위 심사를 수락해주시고 조언을 해주신 조경화 교수님, 아나목스 공정에 대해 잘 모를 때 친절한 설명과 여러 조언을 아끼지 않으시고 처음 쓰는 아나목스 공정 논문에 도움을 주신 KIST 조경진 박사님, 실험실에서 처음으로 도전한 연구에 당황하고 고민하던 시기에 언제나 친절하게 조언해주시고, 얼마 없던 아나목스 종균도 흔쾌히 내주신 부산대학교 배효관 교수님께도 깊은 감사를 드립니다. 석박사통합 1, 2년차 시절 과제를 담당하여 매우

부족했던 저에게 언제나 친절하게 대해 주시고 많은 조언을 해주신 GS건설 황광현 박사님께도 감사를 드립니다. 그때 담당했던 과제 덕분에 학위 주제에 대한 아이디어를 얻고 연구에 매진할 수 있었습니다. 실험에 대해 전혀 모르던 아기 대학원생 시절부터 어머니처럼 하나하나 가르쳐 주시고, 챙겨주셨던 김자애 박사님, 박사님의 그늘 덕분에 아기 대학원 생활에 적응하는데 큰 힘이 되었고, 늘 감사하게 생각합니다.

그리고 학위 과정 중에 가장 많은 시간을 함께 한 우리 ABLE 랩 구성원들에게도 감사를 전합니다. 좋은 교수님과 박사님 밑에 좋은 랩 구성원들이 함께하여 학위 과정 동안 연구와 생활하는데 큰 힘이 되었습니다. 먼저 같은 시기에 입학하여 가장 오랫동안 함께 하고 고생한 1기 랩장 가현이, 미국가서도 일은 여전히 잘할 거라 생각하고 사고 없이 무사히 포닥을 마치길 바랄게. 또 함께 졸업하는 동기 희정이, 똑부러지는 성격만큼 미국에서도 잘 적응할 거라 생각하고 무사히 포닥을 마치길 바랄게. 첫 석사 졸업생으로 모든게 낯설었을 학찬이형, 처음 후배로 들어와서 많이 도와주진 못했지만 언제나 아이디어가 샘솟고 질문을 많이 해줬던 규철이, 과제를 도와주다가 칼에 손이 베어서 미안했던 현정이, 독특한 말과 행동으로 언제나 웃음을 주었던 진수, 헬스보이였지만 허리가 아파서 걱정되는 한웅이, 원칙주의/청결주의의 만화 캐릭터 같은 단비, 고등학교/대학교/대학원도 같아 신기하고 실험도 도와주면서 많은 도움이 된 형민이, 그리고 냄새나고 더러울 수도 있는 일들을 항상 도와줬던 이제는 대학원생이 된 지훈이를 비롯한 인턴친구들, 다들 너무 고마워. 주변에 우리 랩실을 소개할 때 항상 즐거운 마음으로 얘기하고 추천할 수 있었던 건 다들 너무 좋은 사람들이기 때문에 가능한 일이었어. 다들 너무 고맙고, 덕분에 지루할 수 있는 일상에 재밌는 에피소드와 추억을 쌓을 수 있었어.

수험생 시절 함께 동고동락했던 멘토 친구들과 선생님, 재밌게 놀았던 만큼 더 열심히 공부하게 만든 원동력이었습니다. 특히 공부뿐만 아니라 인생에 대해 지금까지도 조언을 아끼지 않

으시는 배우엽 선생님께 언제나 감사하다는 말씀드리고 싶습니다. 고등학교 시절부터 나랑 비슷해서 더 친해졌고 절친인 홍조, 오랜 시간 우정을 나눌 친구가 있어서 언제나 든든해! 농구로 맺어진 인연으로 항상 열정적으로 같이 운동한 동아리 '아낙수나문' 친구들도 고마워. 대학에 들어와 진로에 대해 고민할 때 도시환경공학부를 추천해주었던 선배 승후형, 그 조언 덕분에 생각해보지 않았던 전공에 관심을 가지고 공부를 하게 되었습니다. 세미나에서 혐기소화에 대해 소개 해주셨던 강사님, 재미있는 설명과 함께 '미생물을 이용한 폐기물 처리 및 에너지 생성'이라는 연구에 대해 알게 되었고, 세미나를 들으면서 처음으로 연구를 해보고 싶다는 생각이 들게 했습니다. 그 분은 다음해에 학교로 부임하셨고, 저를 첫 제자로 받아 주신 지도교수님인 이창수 교수님이십니다. 그때 세미나를 가지 않았다면 교수님을 뵙지 못했다면 대학원에 가지 않았을 것이라 생각합니다. 그만큼 우연한 만남을 통해 진학하게 된 대학원이었지만 이 선택이 제 인생에서 가장 잘한 선택 중 하나라는 생각이 듭니다. 또 때로는 친구로서, 대학원생으로서 나와 가장 가까운 거리에서 지켜봐 주고 내가 부족한 부분을 많이 채워준 지현이, 스트레스 받고 지치고 고민할 때 언제나 웃게 해주고, 에너지를 불어넣어줘서, 그리고 학위 마무리할 때까지 도와줘서 너무너무 고마워! 덕분에 박사 학위를 무사히 마칠 수 있게 됐어.

마지막으로 내가 제일 아끼고 사랑하는 우리 가족에게도 감사를 전합니다. 언제나 힘들어도 내색하지 않으시고 그저 가족이 우선이셨던 어머니의 헌신적인 사랑과 굳건한 신앙과 기도 덕분에 성공적으로 학위를 마칠 수 있었습니다. 언제나 성실하셨던 모습 덕분에 저도 그 성실함을 본받아 연구하는데 큰 힘이 되었습니다. 그리고 어머니께서 기도할 때 함께 기도해주신 이종홍 목사님 및 교회분들에게도 감사를 드립니다. 일찍 돌아가신 아버지의 빈자리로 어머니 홀로 두 아들들을 키우시느라 많이 힘드셨을 텐데 공부한다는 핑계로 많이 도와드리지 못해 늘 죄송하고, 이 글을 빌어 항상 사랑하고 감사한다고 말씀드리고 싶습니다. 그리고 칭찬에 내색하셨던 아버지셨지만 누구보다 하늘에서 기뻐하고 계시리라 생각합니다. 항상 내 기억 속에는

개구쟁이 동생이지만, 어느새 어머니 곁을 지키는 듬직한 동생 규진이 항상 고마워. 태어날 때부터 지금까지 아들처럼 사랑해주신 셋째이모와 이모부, 언제나 그 사랑에 감사를 드리고 항상 건강하세요. 그리고 새로운 가족이 되어준 우리 고양이들 쪼리, 오쯔, 바나. 지쳐서 집에 갈때마다 냥냥 반겨주고 큰 활력소가 되어줘서 고마워.

박사 학위를 받는 순간 더 이상 학생이 아닌 연구자로서 첫발을 딪는 순간이 떨리기도 하고 두렵지만, 제 인생의 멘토이신 배우엽 선생님과 지도교수님이신 이창수 교수님의 가르침 아래 새로운 도약을 잘할 수 있으리라 생각합니다. '전문가가 되려면 자기 확신이 있어야 한다'고 하셨던 말씀처럼 여전히 너무 부족하지만 제가 하고 있는 일에서 만큼은 확신을 가지고 일하는 전문가가 되도록 하겠습니다. 또한, 인생에서 만난 소중한 인연들과 내가 어떤 일을 하든 항상 응원해주는 든든한 가족들 덕분에 성공적으로 박사 학위를 마칠 수 있었다고 생각합니다. 박사 학위가 끝이 아닌 시작이지만, 언제나 감사한 마음을 가지고 새로운 인생의 전환점을 맞이하겠습니다. 감사합니다.

2020 년 1월 ABLE lab에서

조 예 담