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Acclimation of Microbial Consortia to Ammonia and Salt in Methane Fermentation

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Abstract: As methane fermentation is inhibited by ammonia derived from organic waste, anaerobic microbial communities tolerant to enriched wastewater with high concentrations of ammonia and salt must be obtained for methane fermentation. Therefore, acclimation cultures were prepared in bottles for 60–80 weeks with artificial wastewater medium added every 2 weeks, using three types of sludge from wastewater treatment plants in food factories. These cultures were maintained without substantially decreasing methanogenesis and gradually increasing NH_4-N and salt concentrations to 5 and 34 g/L, respectively, via the accumulation of ammonia and salt through anaerobic digestion and direct addition. The culture did not show the severe inhibition of methanogenesis or the accumulation of volatile fatty acids (VFAs) such as acetic and propionic acids. The analysis of bacterial consortia in the acclimated sludge based on the 16S rRNA sequence showed that hydrogenotrophic methanogenic bacteria of the genus *Methanoculleus* were dominant among archaea, whereas bacteria from the orders *Clostridiales* and *Bacteroidales* were dominant among eubacteria. Further, VFA-assimilating bacteria, including synthetic acetate-oxidizing bacteria coupled with hydrogenotrophic *Methanoculleus* to convert methane from acetate, were present to prevent the excessive accumulation of VFAs in the acclimation culture. The proposed acclimation process can enhance the anaerobic digestion of wastewater for methane production.

Keywords: acclimation; ammonia; methane fermentation; microbial consortia; repetitive batch culture; salt

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1. Introduction

Ammonia is a major reactive nitrogen compound, which is used as a raw material in fertilizers, polymers, and other products. The industrial production of ammonia from gaseous nitrogen and hydrogen using the Haber–Bosch process has overcome the limitations of biological nitrogen fixation by some microorganisms, in which nitrogenase catalyzes the reduction of N_2 to NH_3, leading to increased food production and population growth. However, the increase in industrial reactive nitrogen species, such as nitrate esters, amines, amides, nitriles, nitro, azo, and heterocyclic compounds, which are used to produce drugs, fibers, reagents, plastics, rubbers, and dyes, is of increasing concern. Some of these compounds are highly toxic and their flow into the environment can exert various adverse effects [1]. The current condition of the biogeochemical flow through nitrogen and phosphorus recycling is serious throughout the planet, as is the extinction rate of organisms [2]. There are concerns that anthropogenically fixed reactive nitrogen will diffuse and accumulate in the atmosphere and water systems without being recycled, thereby polluting the environment and adversely affecting humans and ecosystems. In the updated report, however, the situation is getting worse. The planetary

boundary has been assessed and found to be significantly transgressed: anthropogenically fixed nitrogen use of 190 ton g N $year^{-1}$ compared to the agricultural use limit of nitrogen resources of 62 ton g N $year^{-1}$ [3]. Therefore, it is necessary to consider the cycle of nitrogen resources as well as carbon resources. Chemical and biological technologies are thus required to recycle ammonia and other reactive nitrogen species that accumulate in the environment. Ammonia recovery from wastewater involves membrane separation processes [4] and the biological digestion of organic materials by anaerobic methane fermentation. Industrial and domestic wastewater contains dilute amounts of nitrogen compounds such as ammonia that could be concentrated by membrane separation and recovered to recycle nitrogen resources. Thus, industrial wastewater can be concentrated using membrane separation technology, allowing the concentrations of NH_4-N and salt in industrial wastewater to reach as high as 5 and 35 g/L, respectively [4,5]. Subsequently, biological anaerobic digestion is expected to decompose organic compounds in concentrated wastewater to methane, providing an energy resource [6].

Methane fermentation is widely used to decompose and purify organic matter from various wastewaters, including municipal wastewater, industrial wastewater, and livestock waste, allowing the recovery and utilization of the methane in the produced biogas as an energy resource [7]. Methane fermentation is performed by a complex microbial community comprising several microbial species (eubacteria and archaea) involved in the decomposition of organic matter (carbohydrates, proteins, and lipids) to low-molecular-weight volatile fatty acids (VFAs) such as propionic and acetic acids; it also results in the production of biogases such as hydrogen, carbon dioxide, and methane. However, ammonia derived from food and animal waste as well as protein degradation inhibits methane production [8–12]. To overcome ammonia-induced inhibition, acclimation cultures have been prepared using livestock waste such as chicken manure as a seed slurry, and acclimation to a maximum of 7 g NH_4-N/L has been achieved [13]. Ammonia-induced inhibition occurs with the accumulation of VFAs owing to the varying tolerance of methanogens to ammonia. The pathways for methanogenesis from acetic acid include the direct conversion of acetate to methane by acetoclastic methanogens, as well as the conversion of VFAs to hydrogen and CO_2 by syntrophic acetate-oxidizing bacteria (SAOB) and their conversion to methane by hydrogenotrophic methanogens. The latter pathway is considered important at high ammonia concentrations because hydrogenotrophic methanogens are more tolerant to ammonia than acetoclastic methanogens [14]. However, this may be a rate-limiting step in methanogenesis, because owing to the limited energy exchange between bacteria and methanogens, SAOB grow slowly [15]. As marine microorganisms have been reported to show tolerance to 4% or higher salinity [16–18], bioaugmentation with marine sediment-derived microbial consortia can alleviate the inhibition of CH_4 production under NH_4^+ or salt stress [19]. However, wastewater that has been concentrated by membrane separation processes for ammonia recovery is likely to be enriched not only with ammonia but also with salt and other constituents. The anaerobic digestion of organic matter in such concentrated wastewater requires the establishment of a microbial community capable of anaerobic digestion under multiple stress conditions. The acclimation of microbial communities tolerant to both ammonia and salt has not been reported, except a few reports in marine sediments and waste [19,20]. To acclimate to multiple stresses, stress loading while gradually increasing their concentrations would be desirable.

Therefore, in this study, we aimed to construct methane-fermenting microbial communities tolerant to both ammonia and salt, which slowly increased in concentration in anaerobically digested sludge from three food factory wastewater treatment plants. We then analyzed the structure of the microbial community to compare variations over time, determine differences between samples, and identify the characteristic bacteria present in the communities that are tolerant to both ammonia and salt. Reactors have been used to acclimate stress-tolerant microbial communities for fed-batch and continuous cultures [21]; however, multiple systems are required when large numbers of samples are evaluated simultaneously for acclimation. In contrast, small bottles (up to approximately 1 L), can easily be used for the simultaneous culture of multiple samples under different culture conditions. Therefore, in this study, we used bottles for repeated batch cultures with three

types of samples under two different conditions so as to acclimate to high concentrations of ammonia and salt.

2. Materials and Methods

2.1. Anaerobic Digestion and Acclimation Culture

Sludges A, B, and C were derived from the wastewater treatment facilities of three different food factories. To evaluate methane production using anaerobic digestion, synthetic wastewater medium (glucose 2.3 g/L, peptone 0.9 g/L, yeast extract 1.1 g/L) containing sodium bicarbonate (10 g/L) was added as a substrate to a 50 mL serum bottle. The bottles were sealed with butyl rubber stoppers (NN-1GS, NGS Ltd., Higashi-Hiroshima, Japan), and the headspace was flushed with nitrogen gas. They were then incubated at 37 °C with shaking at 150 rpm (NR-30; Taitec Co., Koshigaya, Japan). For acclimation to ammonia and salt, 200 mL of synthetic wastewater medium, including sludge A, B, or C, was prepared in 800 mL serum bottles (NNM-1GL, NGS Ltd., Higashi-Hiroshima, Japan) with butyl rubber stoppers (NN-2GS, NGS Ltd., Higashi-Hiroshima, Japan). Ammonium chloride or sodium chloride was added to the bottles depending on the acclimation conditions. Unless otherwise stated, the reagents were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The volume of biogas in the headspace was measured weekly in a sodium chloride-saturated solution using a graduated cylinder. The biogas composition was analyzed using gas chromatography. After sampling 10 mL of the culture medium every 2 weeks, an equal volume of fresh medium was added to the bottles. The amount and composition of biogas and the concentrations of VFAs, salt, and NH_4-N were measured weekly. Data about the acclimation cultures were obtained by averaging the values from three bottles containing each sludge and evaluating the standard errors.

2.2. Preparation of DNA Samples and 16S rRNA Gene Amplicon-Based Metagenomic Analysis

Sludge was collected from one of three bottles containing each sludge via centrifugation at 20,400× *g* for 10 min at 4 °C. Genomic DNA was extracted using a NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The bacterial consortia were then analyzed based on the sequence of the V4 region of the 16S rRNA gene. To construct the library for sequencing, the 16S rRNA gene sequences were amplified using Ex Taq HS (Takara Bio Inc., Kusatsu, Japan) with the relevant primers (515F: ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-GTGCCAGCMGCCGCGGTAA and 806R: GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCT-NNNNN–GGACTACHVGGG TWTCTAAT). The polymerase chain reaction (PCR) mixtures (10 µL) contained 0.5 mM of each primer, 200 mM Ex buffer, 0.5 U ExTaq HS (Takara Bio Inc., Kusatsu, Japan), and 1 ng DNA. The thermocycling conditions comprised a denaturation step performed at 94 °C for 2 min; 30 amplification cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and a final polymerization step at 72 °C for 5 min. The PCR products were purified using AMPure XP (Beckman Coulter Inc., Brea, CA, USA) according to the manufacturer's instructions. The second PCR was performed with 10 ng of the PCR product and primers (F: AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC and R: CAAGCAGAAGACGGCATACGAGAT-Index1-GTG ACTGGAGTTCAGACGTGTG), using the same amplification conditions (except 10 amplification cycles were used) and reaction mixture (except for the primers and DNA template) as those in the first PCR. After confirming the quantity and quality of the PCR products, library samples were prepared using Synergy H1 (BioTek Instruments, Inc., Winooski, VT, USA), the QuantiFluor dsDNA System (Promega Co., Madison, WI, USA), a Fragment Analyzer, and a dsDNA 915 Reagent Kit (Agilent Technologies Inc., Santa Clara, CA, USA). DNA sequencing was performed using MiSeq and the MiSeq Reagent Kit v3 (Illumina Inc., San Diego, CA, USA).

Only read sequences whose beginning matched perfectly with the primer sequences were extracted using the fastx_barcode_splitter tool in the FASTX-Toolkit (ver. 0.0.14). If the primer sequences contained N-mixes, this procedure was repeated considering the number of Ns (six

forward × six reverse = 36). Primer sequences were removed from the extracted reads using Fastx Trimmer from the FASTX-toolkit. Sequences with quality values less than 20 were then removed using Sickle (ver. 1.33), and sequences less than 130 bases in length and paired sequences were discarded. The paired-end read-joining script, FLASH (ver. 1.2.11), was used to join reads under the following conditions: joined sequence length, 250 bp; read length, 230 bp; and overlap length, 10 bp. After removing the chimera and noise sequences using the dada2 plugin in Qiime2 (ver. 2022.8), representative sequences and amplicon sequence variant (ASV) tables were generated. The feature classifier plugin was used to compare the obtained representative sequences with 97% of the operational taxonomic units (OTUs) in the Greengene database (ver. 13.8) for the obtainment of phylogenetic inferences. Alpha and beta diversities were analyzed using the diversity plugin Qiime 2 (ver. 2023.5). Library construction and amplicon sequence analysis were performed by the Bioengineering Lab. Co., Ltd. (Sagamihara, Japan). Raw reads were deposited in the DNA Data Bank of Japan under accession numbers DRR503689-DRR503712.

2.3. Analysis

Gas content in the bottle headspace was analyzed using gas chromatography linebreak (Nexis GC-3020; SHIMADZU Co., Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a Micropacked-ST column (3.0 m × 1.0 mm I.D., Shinwa Chemical Industries Ltd., Kyoto, Japan). The injection port and column were maintained at 300 °C and 80 °C, respectively, during the analysis. Argon was used as the carrier gas. The gas sample was injected (600 µL) using a gastight syringe (MS-GAN100; Ito corporation, Fuji, Japan). Data were collected and further analyzed using a Chromatopac GC-8RA (SHIMADZU Co., Kyoto, Japan). VFAs such as acetate, propionate, and formate were quantified using high-performance liquid chromatography (HPLC; JASCO Co., Tokyo, Japan) equipped with a refractive index (RI) detector, an ion exclusion chromatography column (RSpak KC-811; 7.8 mm internal diameter (ID) × 300 mm L; Resonac Co., Tokyo, Japan), and a guard column (RSpak KC-G 6B; 6.0 mm ID × 50 mm L; Resonac Co., Tokyo, Japan). The products were eluted at 40 °C using Milli-Q water as the mobile phase at a flow rate of 1.0 mL/min. Supernatants of each sample were obtained via centrifugation at 7300× *g* for 5 min at 4 °C and used for the subsequent analysis. Ammonia concentrations were quantified using an ammonia assay kit (TNT832; DKK-TOA Co., Tokyo, Japan), according to the manufacturer's instructions. Salinity was measured based on NaCl concentrations using a conductivity meter (EC-33B; HORIBA Ltd., Tokyo, Japan). Volatile solids (VSs) were measured using sludge samples dried in an oven (DX302; Yamato Scientific Co., Ltd., Tokyo, Japan) and muffle furnace (300 Plus, DENKEN-HIGHDENTAL Co., Kyoto, Japan). The content of total solids (TSs) was measured after drying 3 g of sludge samples at 105 °C for 4 h. The content of ignition loss (IS) was measured after the TS samples were incubated at 600 °C for 3 h. The VS content was calculated by subtracting IS from TSs.

3. Results and Discussion

3.1. Anaerobic Digestion of Sludge from Wastewater Treatment Plants in Food Factories with Ammonia or Salt

Three different types of sludge, A, B, and C, were obtained from the wastewater treatment plants of food factories where stable anaerobic digestion was used. Their characteristics were measured as follows: salinity (g/L; A: 0.73, B: 1.56, C: 24.5), NH_4-N (mg/L; A: 0.704, B: 25.8, C: 6.72), VS (g/L; A: 55.7, B: 8.94, C: 36.8), and chemical oxygen demand (g/L; A: 18.0, B: 26.0, C: 37.6). Sludges B and C contained high concentrations of ammonia and salt. The sludge samples may have contained microorganisms tolerant to ammonia or salt, which were expected to become dominant after the acclimation culture.

To evaluate the inhibition of methane production by ammonia or salt in the original source sludge for the acclimation culture, we compared the cumulative methane production between samples containing ammonium chloride or sodium chloride, using one bottle per each sample. Ammonium chloride was added to bottles containing sludge at different concentrations (NH_4-N; 0 g/L, 1 g/L, 2 g/L, 3 g/L, 4 g/L, and 5 g/L) prior to incubation,

and the methanogenic potential was evaluated (Supplementary Figure S1). In sludges A and B, methane production did not decrease with NH_4-N addition up to 5 g/L, but methanogenesis was delayed at 5 g/L. In sludge C, a decrease in methanogenic potential was observed in the system with an NH_4-N concentration of 5 g/L compared to that in the sample without ammonia addition. Based on these results, the optimal ammonium chloride concentration to be added was determined to be within 3 g/L at the beginning of the acclimation culture, which was gradually increased by digestion to reach an NH_4-N concentration of 5 g/L or higher.

We preliminarily evaluated the salt tolerance of each sludge with the addition of sodium chloride (0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, and 60 g/L; Supplementary Figure S2). The amount of methane produced in sludges A and C with the addition of up to 10 g/L of sodium chloride did not decrease compared to that in the sludge without sodium chloride addition. The methane production of all sludges decreased significantly when 30 g/L or more of sodium chloride was added. In the sample containing 20 g/L of NaCl added to sludges A and B, methane production was delayed and its amount decreased, indicating the need for the acclimation culture for tolerance acquisition in the consortia. Based on these results, the initial concentration of sodium chloride added was set at 10 g of NH_4-N/L at the beginning of the acclimation culture, which was gradually increased to a final salt concentration of 20 g/L or higher by repeatedly adding medium containing inorganic salts. Inhibition by the simultaneous addition of salt and ammonia was not evaluated; however, inhibition by salt was assumed to be more pronounced than that by ammonia.

3.2. Acclimation Culture for High Concentrations of Ammonia and Salt

When ammonium chloride (1.0 g/L) was added to bottles containing sludge with 10 g of VS/L in a 1-week repetitive batch culture for 8 weeks, methanogenesis decreased over time. In contrast, VSs increased to 12 g/L after decreasing to 2 g/L in the first week, and the VFAs, i.e., acetic and propionic acids, accumulated to over 50 and 10 mM, respectively (data not shown). The accumulation of VFAs indicated that the decomposition of organic matter progressed relatively quickly compared to the formation of long-chain and volatile fatty acids, but subsequent methane production took time because of the high substrate load. In this case, methane production decreased over time despite the increase in VSs. However, the low methane production suggested that the dominant species in the bottles may have been microorganisms other than methanogens. Therefore, the initial VS concentration was changed from 10 to 20 g/L to increase the initial number of microorganisms for methane fermentation, and the interval between successive substrate additions was extended from one week to two weeks. To prevent the pH from decreasing owing to acid production from substrate digestion, sodium bicarbonate was added at the beginning of the incubation period, and the pH was adjusted between 7.8 and 8.6 by adding sodium bicarbonate only upon a significant change in pH.

Acclimation cultures were performed with sludge A, B, and C using sodium chloride at an initial concentration of 10 g/L. The bottles were filled with nitrogen gas, 2.0 g of sodium bicarbonate, and VSs at 20 g/L of sludge in synthetic wastewater medium. Three bottles were prepared per sludge. Batch cultures were repeated by adding a volume of 20 times concentrated synthetic wastewater medium equal to the volume of the liquid (10 mL) sampled after 2 weeks.

Both sludges stably produced methane and maintained their VS levels. The VSs increased once after the start of incubation and then settled to a constant value. Salinity increased over time; however, when NaCl was added at the 8-week mark to bring the salt concentration to 26 g/L, methane production decreased considerably in sludges A and C. However, methanogenesis was only delayed, and after 2–4 weeks, methanogenesis was recovered to the level before addition. Ammonium chloride was not added to the bottles, but the NH_4-N concentration was gradually increased as ammonia was accumulated in the bottles because of the consumption of repeatedly added synthetic wastewater containing

peptone, a proteolytic degradation product (Figure 1). No significant differences in the amount of methane production; VSs; and concentrations of ammonia, salt, and volatile organic acid were observed among the three bottles in the acclimation culture using the same sludge (A, B, or C) except for several time points. It was difficult to gradually increase the ammonia concentration by adding reagents; however, this could be achieved through increases in ammonia concentration associated with substrate digestion. The accumulation of VFAs, such as acetic and propionic acids, was within a few millimoles. However, acetate was increased after 18 weeks in sludge C, indicating that the rate of acetate consumption and methane production was slow. In addition, the physical form of sludge A and B was granular, while that of sludge C was muddy. Thus, the physical forms were obviously different between them. These characteristics might affect the stability of microorganisms and their enzymatic activities in methane fermentation. Granulated sludge could be acclimated to methane fermentation with this repetitive batch culture, since granulated sludge is still susceptible to stresses such as ammonia, even though methane fermentation with granules is known to facilitate high volume loading, long biomass retention, and effective bioenergy recovery [22,23]. Despite concerns regarding a drop in pH due to the lack of sodium bicarbonate, all methane production remained at approximately pH 8, indicating steady methanogenesis. After acclimation to salt addition, ammonium chloride was added twice between 30 and 40 weeks to bring the concentration to 4 g/L and added two times between 50 and 56 weeks to bring the concentration to 5 g/L; however, both bottles showed stable methanogenesis.

(A)

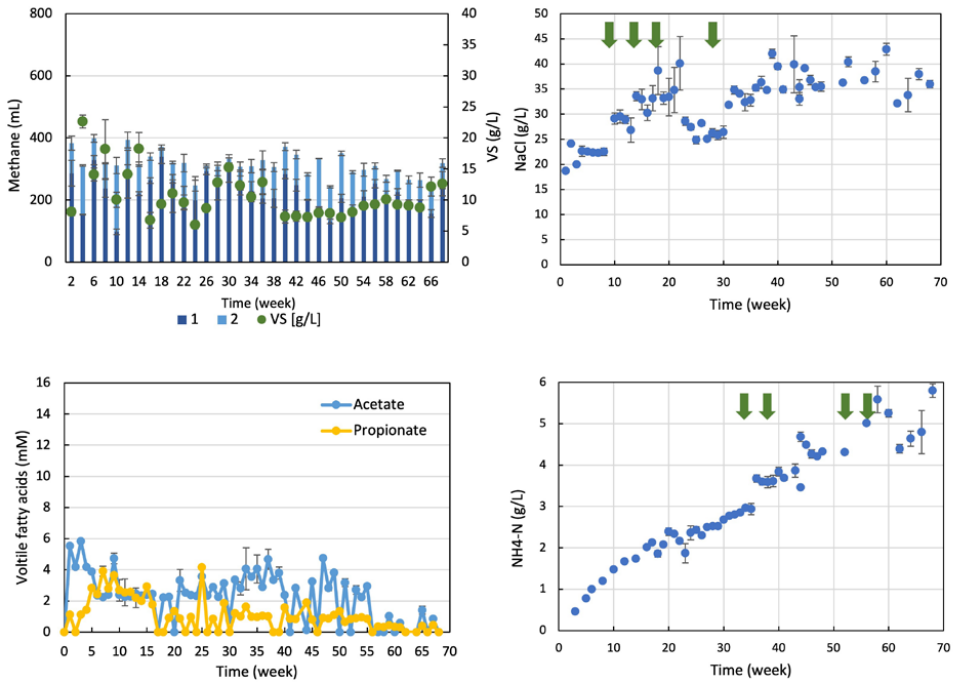


Figure 1. *Cont.*

(**B**)

(**C**)

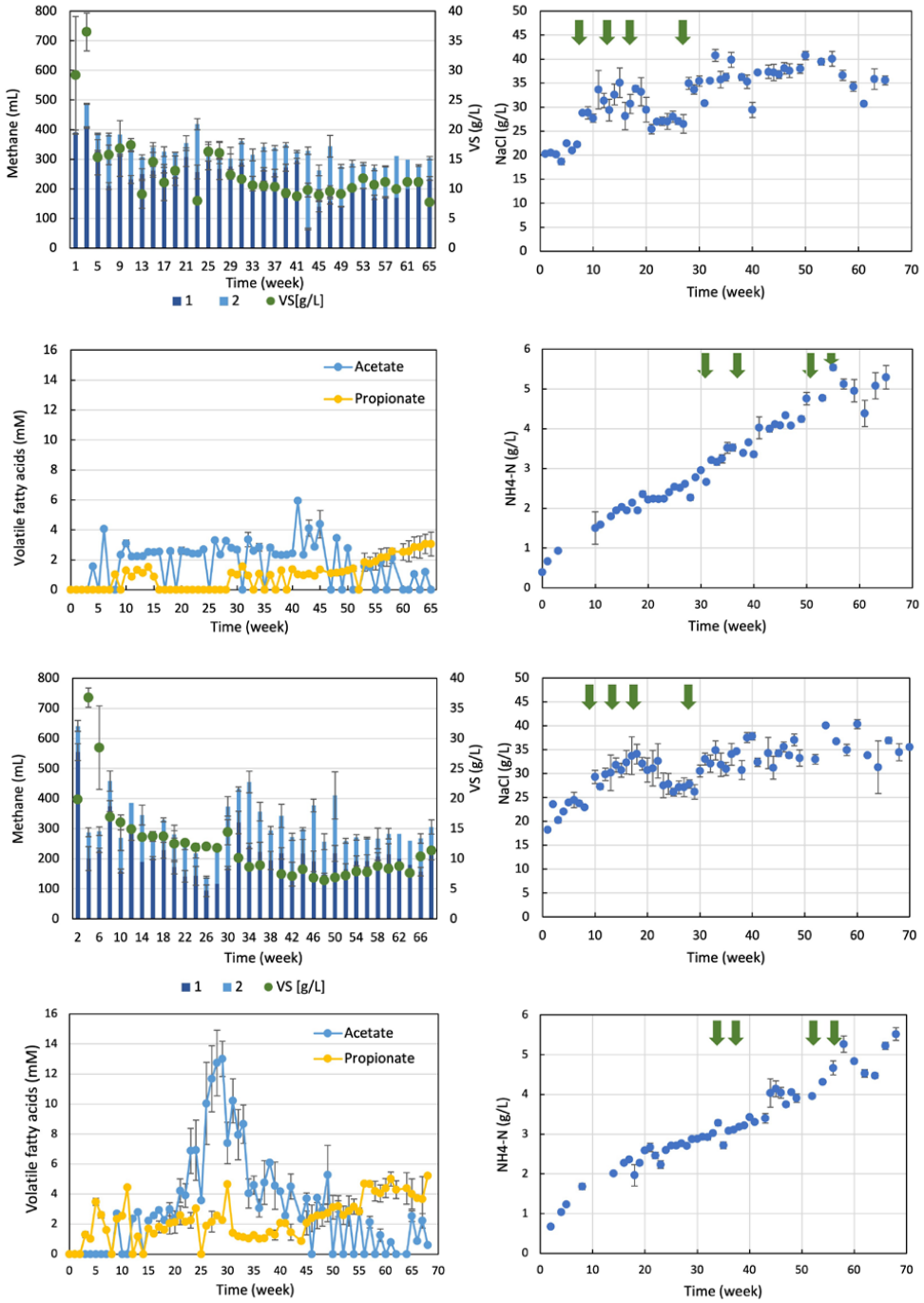


Figure 1. Methane production, volatile solids (VSs), salinity, and concentration of volatile fatty acids (VFAs) and NH_4-N during the acclimation culture started with the addition of 10 g/L of sodium chloride in (**A**) sludge A, (**B**) sludge B, and (**C**) sludge C. The amount of methane produced every two weeks is shown as a single integrated bar (first week: blue bar; second week: light blue bar). Arrows indicate the addition of sodium chloride or ammonium chloride. Data were obtained by averaging the values from three bottles in each sludge, with error bars indicating standard error.

Other acclimation cultures were performed with the initial addition of ammonium chloride to achieve an NH_4-N concentration of 3 g/L. One bottle was prepared per sludge. These cultures also exhibited stable digestion and methane production when ammonium chloride was added several times to reach an NH_4-N concentration of 5 g/L (Figure 2). The accumulation of VFAs (acetic and propionic acids) was maintained within a few millimoles, which is relatively low compared to our first trial of the acclimation experiment described above in this section (a

1-week repetitive batch culture; over 50 and 10 mM, acetic and propionic acids, respectively). For sludge C, methane production peaked at the third sampling (weeks 5 and 6), and then decreased until week 14. Simultaneously, acetic and propionic acids accumulated to 9.5 and 7.3 mM, respectively. After recovery from unstable digestion, sludge C was acclimated to ammonia without inhibiting methane production. Sodium chloride was added to the bottles to achieve a salt concentration of 34 g/L after acclimation to ammonia for 54 weeks. An increase in salinity was also observed owing to the addition of ammonium chloride.

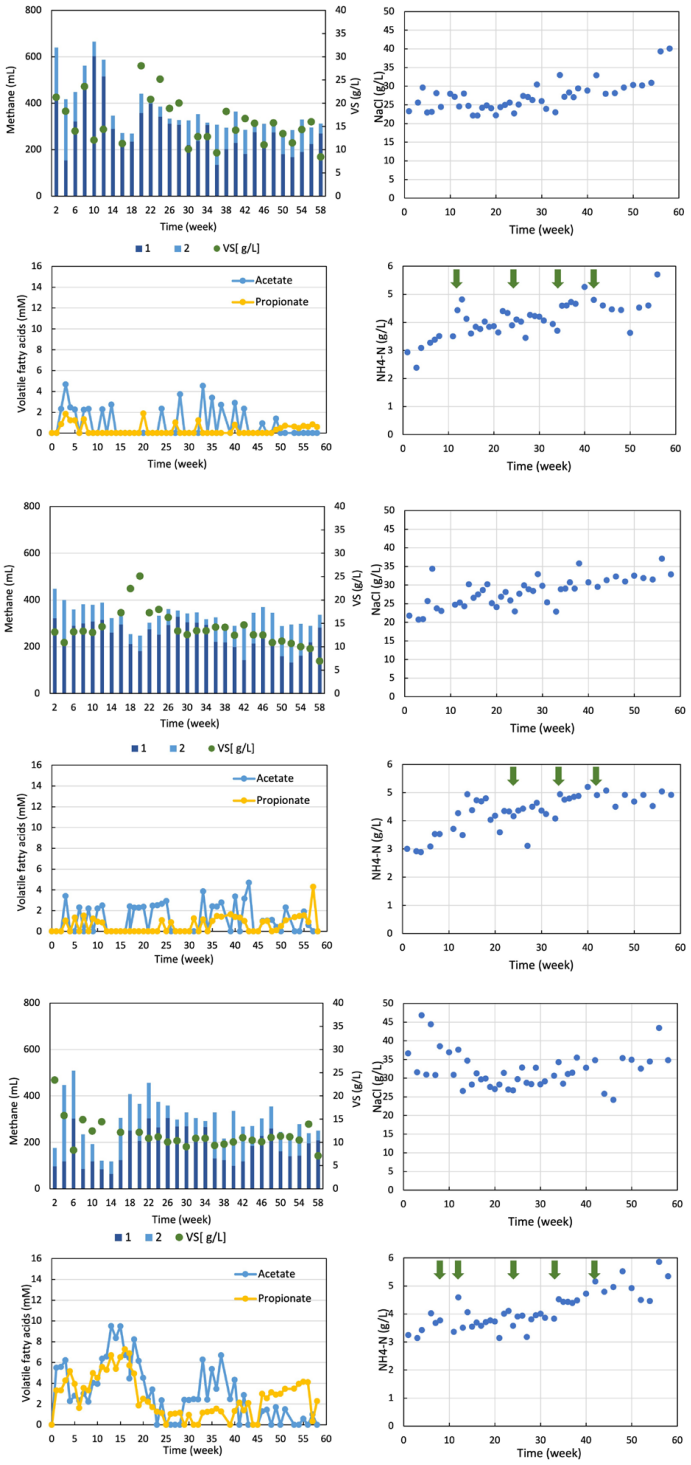


Figure 2. Methane production, volatile solids (VSs), salinity, and concentration of volatile fatty acids (VFAs) and NH_4-N during the acclimation culture initiated with 3 g/L NH_4-N addition in (**A**) sludge

A, (**B**) sludge B, and (**C**) sludge C. The amount of methane produced every two weeks is shown as a single integrated bar (first week: blue bar, second week: light blue bar). Arrows indicate the addition of sodium chloride or ammonium chloride.

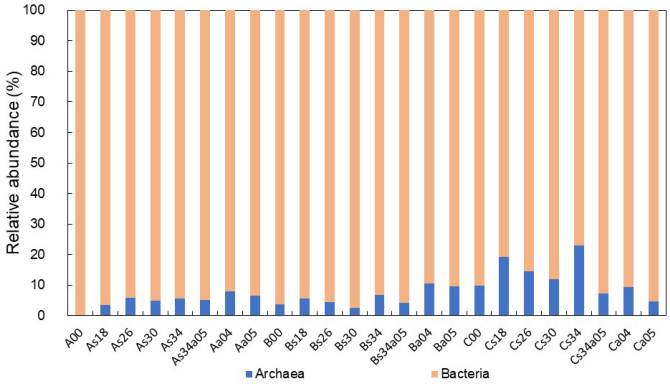
Acclimation to salt and ammonium chloride resulted in the formation of methanogenic microbial consortia capable of producing methane under the high concentrations of salt and ammonia. This indicates that the gradual increase in ammonia and salt concentrations allowed acclimation without the excessive addition of ammonia and salt. Sludge from different types of food factory wastewater treatment plants could also be acclimated using the same method. Therefore, this acclimation culture showed that the potential microorganisms in the anaerobically digested sludge can acclimate to high concentrations of ammonia and salt.

3.3. Structure of Bacterial Communities Tolerant to High Concentrations of Ammonia and Salt

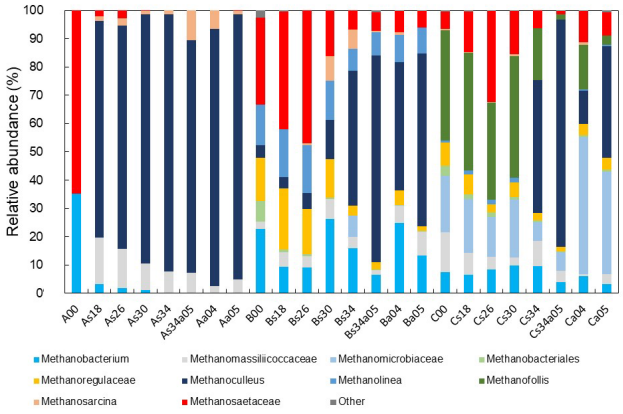
In this study, the acclimation culture was performed and NH_4-N and salt were added until the NH_4-N and salt concentrations reached 5 and 34 g/L, respectively, since granulated sludge is still susceptible to stresses such as ammonia, although even though methane fermentation with granules is known to be advantageous in the facilitation of high volume loading, long biomass retention, and effective bioenergy recovery via accumulation in each bottle. To investigate whether the microbial community was changed during the acclimation culture and to determine the dominant microbial species, we compared the bacterial community between sludge samples at the respective concentrations of NH_4-N and salt during the process of the acclimation culture using 16S rRNA V4 region amplicon sequencing. Samples in salt acclimation cultures were collected at the time of acclimation to different concentrations of salt or NH_4-N as follows: A00: original source sludge A for the acclimation culture; As18, As26, As30, and As34: acclimated to 18 g/L, 26 g/L, 30 g/L, and 34 g/L of salt in sludge A, respectively; As34a05: acclimated to 34 g/L of salt and 5 g/L of NH_4-N in sludge A; and Aa04 or As05: acclimated to 4 g/L or 5 g/L of NH_4-N in sludge A, respectively. The cultures of sludges B and C were named similarly.

Fluctuation in the abundance of bacterial consortia was compared in each acclimation culture based on 16S rDNA sequence analysis. The abundance of archaea, including methanogenic bacteria, in the original source sludges A (A00) and B (B00) was relatively low at 0.137% and 3.66%, respectively. These increased to approximately 5–10% at the end of the acclimation culture (As34a05, Aa05, Bs34a05, and Ba05). However, their abundance in sludge C was 9.89% in the original source sludge, increased by over 20% in the acclimation culture for salt (Cs34), and finally decreased to 7.34% (Cs34a05) and 4.70% (Ca05) in the acclimation culture for salt and ammonia (Figure 3A). Regarding the archaeal composition (Figure 3B) in sludge A, the families *Methanosaetaceae* (64.8%) and *Methanobacterium* (35.2%) were dominant before the acclimation culture, whereas after acclimation at a salt concentration of 18 g/L, the order *Methanoculleus* (76.6%) became dominant and then progressively increased in As26 (78.9%), As30 (88.4%), and As34 (90.8%). In sludges B and C, the abundance of *Methanosaetaceae* was lower than that in sludge A (B00: 31.6%, C00: 5.94%); however, it increased in the acclimation culture samples with up to 26 g/L of salinity (Bs18: 41.8%, Bs26: 46.8%, Cs18: 14.5%, Cs26: 32.2%). A temporal increase in the order *Methanosarcina* was also detected in sludge B at salinities of 30 g/L and 40 g/L (Bs30 and Bs34). *Methanosaricina* has been reported to be the dominant acetate-degrading methanogen in culture under ammonia-induced stress conditions [24]. Thus, a small but consistent number of acetate-utilizing methanogens were present in the consortia. The abundance of hydrogenotrophic methanogens such as *Methanobacterium*, *Methanoculleus*, *Methanolinea*, and other species in the family of Methanoregulaceae except for *Methanolinea* was increased. The family Methanomassiliicoccaceae was also slightly detected at a high concentration of NH_4-N and salt; these organisms cannot use acetate but are reported to use methanol as well as mono-, di-, and trimethylamine with hydrogen to produce methane and are reported to be highly abundant among ammonia-acclimated methanogenic consortia [25].

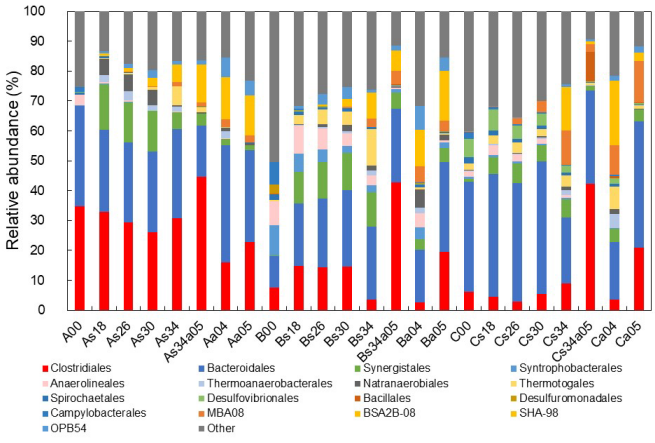
In sludge C, a similar fluctuation in abundance was observed with an increase in acetate-forming methanogens up to 26 g/L of salinity followed by a decrease, which remained until the end of the acclimation culture. The abundance of hydrogenotrophic methanogens such as those belonging to the genus *Methanofollis* (C00: 39.2%, Cs18: 41.7%, Cs26: 34.3%, Cs30: 42.9%) and the family Methanomicrobiaceae (Ca04: 48.5%, Ca05: 36.4%) was dominant, which was different from that in sludge A and B, whereas the genus *Methanoculleus* was generally dominant at the end of culture in all sludges (As34a05: 90.8%, Aa05: 93.9%, Bs34a05: 73.5%, Ba05: 61.3%, Cs34a05: 80.7%, Ca05: 39.5%). Therefore, methane was considered to be produced mainly from hydrogen and carbon dioxide, while part of it was produced from acetate under high concentrations of ammonia and salt.



(**A**)



(**B**)



(**C**)

Figure 3. Abundance of microbial organisms in samples during the acclimation culture. (**A**) Total abundance of archaea and eubacteria together, (**B**) archaea, and (**C**) eubacteria.

Among the eubacterial consortia, including organic material decomposers and acid-producing bacteria (Figure 3C), orders of *Bacteroidales* and *Clostridiales* initially accounted for 70% of the total in sludge A, less than 20% in sludge B, and approximately 40% in sludge C. The abundance of the orders *Synergistales*, SHA-98, and OBP54 decreased slightly in sludge A, whereas those of SHA-98 and OPB54 increased in sludges B and C. *Synergistales* included those of the genus *Aminobacterium* and the family Aminiphilaceae in the major group, which have peptides and amino acids as preferred substrates. *Aminobacterium* is considered an SAOB candidate [26], SHA-98 is known to be involved in formate oxidation [27], and OPB54 is involved in synthetic acetate oxidation [28]. Besides *Bacteroidales* and *Clostridiales*, *Syntrophobacterales*, mainly the genus *Syntrophobacter*, are known to be responsible for propionate oxidation [29–31]. MBA08 is thermophilic, similar to *Thermotogales,* and may be involved in stress tolerance [32]. Despite differences in origin among A, B, and C, SHA-98, OPB54, and *Syntrophobacter* were associated with VFA consumption. This was consistent with the fact that VFA accumulation was not excessive during the acclimation process and did not inhibit methanogenesis. As described in previous reports [13,33], hydrogenotrophic *Methanoculleus* methanogenesis was dominant in the acclimation culture, and acetotrophic *Methanosaeta* methanogenesis was reduced. Further, acetic acid is converted to hydrogen and CO_2 by SAOB at high ammonia concentrations [33,34], and the OPB54 identified in this study is thought to play a role in this process. Bioaugmentation with the hydrogenotrophic methanogens and syntrophic VFA-oxidizing bacteria found in this study was thus expected to alleviate the ammonia and VFA stress in the anaerobic digestion, as reported at high concentrations of ammonia or salt [19,35–37].

Regarding alpha diversity, the Shannon index reached a plateau above the 5000 sequencing depth for all samples and decreased after acclimation and incubation compared to that in the original source sludge (A00: 5.72 ± 0.02, B00: 7.02 ± 0.02, C00: 6.04 ± 0.03) for each sludge series (As34a05: 4.93 ± 0.02, Bs34a05: 4.90 ± 0.02, Cs34a05: 4.97 ± 0.02, Aa05: 4.79 ± 0.02, Ba05: 5.45 ± 0.01, Ca05: 4.87 ± 0.02). This indicated that the number of microorganisms in consortia A, B, and C decreased after acclimation. The differences in β-diversity among the three groups of sludge A, B, and C were presented as phylogenetically weighted distances between bacterial communities using two-dimensional principal coordinate analysis plots of weighted UniFrac distances (Figure 4). The β-diversity indices of the three original source and initial phase of the acclimation culture sludge groups A, B, and C (Cluster A: A00; Cluster B: B00, Bs18, Bs26, and Bs30; Cluster C: C00, Cs18, Cs26, Cs30, and Cs34) were scattered in each group, and they clustered close to each other as the acclimation culture progressed (Cluster A': As18, As26, As30, As34, As34a05, Aa04, and Aa05; Cluster B': Bs34, Bs34a05, Ba04, and Ba05; Cluster C': Cs34a05, Ca04, and Ca05). As expected given the similar acclimation process, the microbial communities showed a similar community structure as the acclimation culture progressed between three types of sludge, and also between two different acclimation cultures. The results indicate that the ammonia- or salt-tolerant microorganisms were present in each consortium and they could be selected and become dominant during the acclimation culture. Therefore, the microbial communities identified in this study contained key populations that contribute to salt and ammonia tolerance. In future, these characteristic microorganisms could be used as candidates for constructing microbial communities to mitigate salt and ammonia stresses. Sludge acclimated to both ammonia and salt can also be used as a source for bioaugmentation [19,35–37] in anaerobic digestion under high concentrations of ammonia and salt. The supplementation of hydrogenotrophic *Methanoculleus* enhanced the anaerobic digestion with the increase in methane yield and decrease in VFA accumulation [38]. Bacterial consortia containing stress-tolerant bacteria such as *Methanoculleus*, SAOB, and VFA-oxidizing bacteria constructed in this study contribute to the understanding of the mechanism of stress tolerance and the improvement in anaerobic digestion under both ammonia and salt stress.

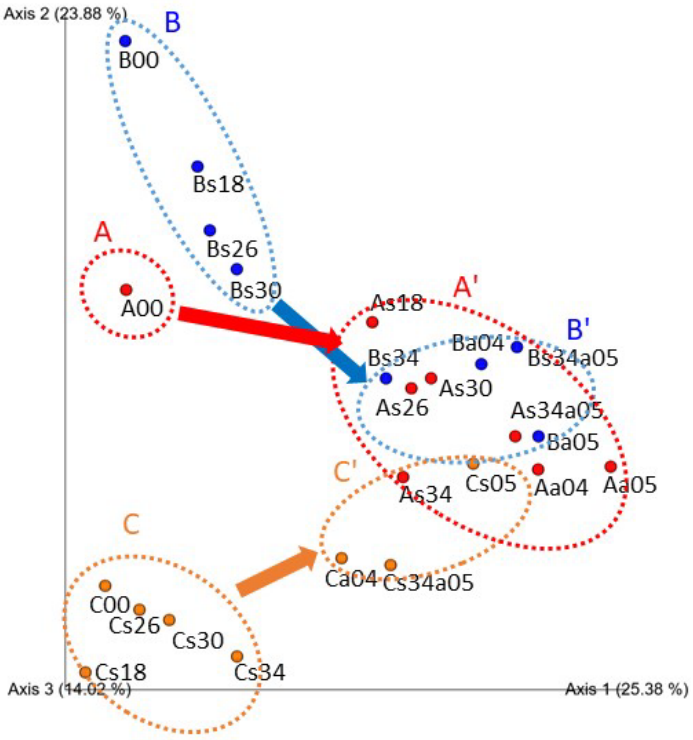


Figure 4. Principal coordinate analysis plots of weighted UniFrac distance in the beta diversity of samples during the acclimation culture.

4. Conclusions

In this study, we constructed microbial consortia tolerant to both ammonia and salt stress using an acclimation culture without the extreme accumulation of VFAs in the presence of gradually increasing ammonium chloride and sodium chloride during repeated batch cultures. The results showed that both acclimated cultures contained hydrogenotrophic methanogens such as the genus *Methanoculleus* and the family Methanomicrobiaceae as major dominant archaeal bacteria, and SAOB (*Aminobacterium* and OPB54) and other VFA-oxidizing bacteria (SHA-98 and *Syntrophobacter*) in addition to organic matter degraders (*Clostridiales* and *Bacteroidales*). They formed similar bacterial communities after acclimation to both ammonia and salt stress, even with different types of source sludge from food companies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10020098/s1, Figure S1: Methane production in anaerobic digestion with NH_4-N (0, 1, 2, 3, and 5 g/L) by sludge A, B, and C used for acclimation; Figure S2: Methane production in anaerobic digestion with sodium chloride (0, 10, 20, 30, 40, 50, and 60 g/L) by sludge A, B, and C used for acclimation.

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