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Comparison of Different Carbon Sources on Biomethane Production with *Clostridium cellulovorans* and Methanogens

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Abstract: Methane (CH_4) has attracted attention as not only one of the hydrogen carriers in terms of energy density, but also synthetic natural gas. In nature, the decomposition of organic compounds is performed with bacterial ecosystems that can produce CH_4. *Clostridium cellulovorans* as a decomposer was cultivated with pig manure (PM) as an unused biomass in this study. As a result of high-performance liquid chromatography (HPLC) analysis, while formate and lactate were decreased in the *C. cellulovorans* medium containing 0.5% PM, acetate and butyrate were increased in it. Accordingly, in order to compare with the effect of carbon sources for methane production, the cocultivation of *C. cellulovorans* and the methanogenesis of *Methanosarcina mazei* or microbial flora of methane production (MFMP) was carried out in the *C. cellulovorans* medium. As a result, only the cocultivation with *C. cellulovorans* and MFMP showed methane production in 0.5% acetate medium. Moreover, in comparison with a carbon source in either 1% acetate or 1% methanol medium, MFMP was only cultivated after being precultivated with 0.5% glucose medium for 12 h. The results revealed that MFMP with a 1% methanol medium produced methane approximately eight times higher than with 1% acetate medium. After cultivation with 1% acetate or 1% methanol, next-generation sequencing (NGS) analysis of MFMP was carried out. Interestingly, *Methanofollis* (0.211%), belonging to methanogens through the CO_2 reduction pathway, was dominant in the 1% acetate medium for 72 h cultivation, while *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in 1% methanol medium for 72 h cultivation. Since *Methanosarcina* spp. belong to acetoclasts (acetoclastic pathway), methanol could promote the growth of *Methanosarcina* spp., rather than acetate. Therefore, it seems that *Methanosarcina* spp. may play a key methanogenesis role in MFMP. Thus, these results will provide important information for low-cost biomethane production.

Keywords: methanogenesis; pig manure; carbon sources; *C. cellulovorans*; methanogens

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1. Introduction

Anaerobic digestion (AD) consists of a series of biochemical processes such as hydrolysis, fermentation (acidogenesis), acetogenesis and methanogenesis performed by various interacting microorganisms, including bacteria such as acidogens and acetogens, and archaea (methanogens). It is also clear that the cumulative CH_4 production from the three different substrates varied significantly and was not in agreement with the expected, according to the theoretical value calculated (Table 1) (formate 82.35 N mLCH_4/gVS, acetate 273.17 N mLCH_4/gVS, H_2/CO_2 414.81 N mLCH_4/gVS) [1]. Since methanogenesis is the final step in anaerobic carbon transformation and is of critical concern in thawing permafrost peatland systems where CH_4 release is increasing rapidly, predicting the magnitude of carbon loss as CO_2 or CH_4 is hampered by our limited knowledge of the microbial metabolism of organic matter in these environments [2].

Genome-centric metagenomic analysis of microbial communities provides the necessary information to examine how specific lineages transform organic matter during permafrost thaw [3]. The biomethanation process in nature relies on the microbial interactions between three main metabolic groups of anaerobes: fermentative, acetogenic, and methanogenic microorganisms [4–6]. Whereas the first two groups decompose complex organic matters to acetate—H_2 and CO_2, which are the key precursors for methanogenesis, methanogens further convert these metabolites to CH_4 by two major routes: the acetoclastic pathway and the CO_2 reduction pathway [7]. On the other hand, although the growth behavior of a donor bacterium, *Sulfurospirillum multivorans*, in the modified *Methanococcus voltae* (acceptor) medium with pyruvate alone as substrate was similar to that in the medium originally used for the cultivation of *S. multivorans*, the morphology of *S. multivorans* cells was unaltered in the *M. voltae* medium and independent from the type of cultivation—fermentatively or respiratory [8]. In this case, the new medium with lactate as the sole growth substrate instead of formate and acetate could not promote growth for pure *S. multivorans* cultures. Furthermore, 15 mM lactate was consumed in approximately 2 weeks, while methane was produced in the corresponding coculture, indicating lactate fermentation by *S. multivorans* and H_2 transfer to *M. voltae* as a syntrophic partner. Therefore, the coculture system seems to include system unique advantages, composition, products, and interaction mechanisms.

Table 1. Methanogenic reactions from typical substrates.

Reactions	DG^0 (kJ/mol CH_4)	Microorganisms
I. Hydrogen $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−135	Most methanogens
II. Formate $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	−130	Many hydrogenotrophic methanogens
III. Acetate $CH_3COOH \rightarrow CH_4 + CO_2$	−33	*Methanosarcina* and *Methanosaeta*

The elaboration of the underlying mechanism in microbial communities, such as the exchange of intermediate metabolites, cell-to-cell electrical connections, communications, etc., would guide the design of artificial microbial consortia and further improve the robustness and stability of the cocultivation systems [9–12]. Therefore, these artificial microbial consortia interact mutually through the interaction of synergism, commensalism, competition, mutualism, and so on [12]. Diverse microbial communities within the same or different species have been set up to realize more complicated tasks [8,13,14]. In particular, the greatest advantage of coculture systems consists of the combination of the metabolic capacity of two or more microorganisms that allows for the utilization of more complex substrates and the production of specific products [14]. In addition, the treatment of wastewater, biodegradation of textile azo dye and disposal of contaminated soil have also recently been applied to produce biofuels, bulk chemicals, and natural products by cocultivation systems [15–26].

Cellulose is most abundant on the Earth and is not easily degraded and utilized. In addition to cellulosic sources, various other carbohydrates, carbon monoxide and syngas can also be processed using these systems [27]. The cellulolytic system of *Clostridium cellulovorans* mainly consists of a cellulosome that synergistically collaborates with non-complexed enzymes [28,29]. IBE (isopropanol-butanol-ethanol) fermentation by the cocultivation of *C. cellulovorans* and *C. beijerinckii* was performed using mandarin orange wastes [30], and methane was produced from sugar beet pulp [31] and mandarin orange peel under cocultivation with *C. cellulovorans* and methanogens [32]. Furthermore, two coculture models combining *C. cellulovorans* with *Methanosarcina barkeri* Fusaro or *M. mazei* Gö1 were established for the direct conversion of cellulose to CH_4 [33]. Coculturing *C. cellulovorans* with *M. barkeri* or *M. mazei* not only enabled the direct conversion of cellulose to CH_4,

but also stabilized pH for *C. cellulovorans*, resulting in a metabolic shift and enhanced cellulose degradation. The other approach involved implementing nanotechnology in combination with *C. cellulovorans* through a consolidated bioprocessing (CBP) method to produce hydrogen from raw corn cob [34].

In this study, we observed the cocultivation of *C. cellulovorans* and *M. mazei* or microbial flora of methane production (MFMP) for the different carbon sources between sugars such as glucose and cellobiose, which are the products from cellulose degraded by *C. cellulovorans,* and acetate metabolized from glucose through the TCA cycle. Furthermore, pig manure (PM) was used for the *C. cellulovorans* cultivation and was analyzed with organic acids. In addition, we investigated the cultivation manner of MFMP in comparison with acetate and methanol as the sole carbon source. Finally, 16S rRNA analysis in MFMP was performed by next-generation sequencing (NGS) after cultivations with acetate or methanol as a carbon source.

2. Materials and Methods

2.1. Microorganism

C. cellulovorans 743B (ATCC35296) was anaerobically grown as described previously [28], with pig manure (PM) (Mie University, Tsu, Japan) as a carbon source. *M. mazei* (DSM# 3647) was purchased from the German Collections of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and was cultivated with the JCM230 medium [35].

2.2. Culture Conditions

Moreover, 0.5% (*w*/*v*) PM, 0.5% (*w*/*v*) glucose, 0.5% acetic acid (FUJIFILM Wako Chemicals, Osaka, Japan), and 0.5% (*w*/*v*) cellobiose (Sigma, St. Louis, MO, USA) were used as the sole carbon source in 10 mL or 50 mL of *C. cellulovorans* media and was anaerobically cultivated. The microbial flora of methane production (MFMP) was obtained from methane fermentation digested liquid in January 2017 in Gifu, Japan [32]. *C. cellulovorans* was precultured with 0.5% cellobiose for 12 h at 37 °C and *M. mazei* and MFMP were precultured with 0.5% glucose for 12 h at 37 °C, respectively. Cocultivation was performed as approximately 1000 RLU of *C.c* cells and approximately 20,000 RLU of MFMP cells (*C. cellulovorans*:MFMP = 1:20) and approximately 1000 RLU of *C. cellulovorans* cells and approximately 3000 RLU of *M. mazei* cells (*C. cellulovorans*:*M. mazei* = 1:3), respectively. In comparison with cocultivations with 1% acetate and 1% methanol media, *C. cellulovorans* was precultured with 0.5% cellobiose for 12 h at 37 °C and *M. mazei* and MFMP were precultured with 0.5% glucose for 12 h at 37 °C, respectively. The precultured cells were inoculated into each medium with 1% acetate or 1% methanol. After the cells were collected by centrifugation, the supernatants and the cells were used for each experiment.

2.3. 16S rRNA Sequencing

Samples for bacterial cells cultivated in the culture medium were crashed by Shake Master Neo (bms, Tokyo, Japan) and DNA was extracted by Fast DNA spin kit (MP Bio, Irvine, CA, USA). iSeq 100 (Illumina, San Diego, CA, USA) was used for sequencing under the condition of 2 × 150 bp. The 16S Metagenomics App performed the taxonomic classification of 16S rRNA targeted amplicon reads using a version of the GreenGenes taxonomic database curated by Illumina. The primer sequences used in the protocol were: PCR1_Forward (50 bp): 5′–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGG GNGGCWGCAG–3′ and PCR1_Reverse (55 bp): 5′–GTCTCGTGG GCTCGGAGATGTGTAT AAGAGACAGGACTACHVGGGTATCTAATCC–3′, respectively. The 16S rRNA sequences of MFMP previously reported [31] were deposited in the DDBJ database (accession no. DRR160954).

2.4. Gas and Organic Acid Concentrations

The total gas amount and the concentration of organic acids were measured as previously described [31]. The produced gas after the cultivation was recovered by downward

displacement of water by a syringe (Terumo, Tokyo, Japan) and measured by gas chromatography (Shimadzu, Kyoto, Japan). The concentration of organic acids was measured by high-performance liquid chromatography with a UV detector (Shimadzu, Kyoto, Japan). The data represent at least three independent experiments.

3. Results

3.1. Cultivation of C. cellulovorans with Pig Manure

In order to promote the utilization of pig manure (PM) as an unused biomass, the cultivation of *C. cellulovorans* was carried out. PM was pretreated with 0.45 µm filter to remove the inhibitor for bacterial cell growth and 0.5% (*w*/*v*) pretreated PM was used as the sole carbon source in the *C. cellulovorans* medium. *C. cellulovorans* was inoculated into the PM medium and then organic acids were measured by HPLC. The results suggested that *C. cellulovorans* was able to grow in the 0.5% PM medium and that acetate and butyrate were increased, while formate and lactate decreased after increasing once at 1-day post-cultivation (Figure 1). Total concentrations of acetate and butyrate at 14 days were approximately 2300 mg/L and 820 mg/L, respectively, showing that PM would be an excellent biomass for methanogenesis.

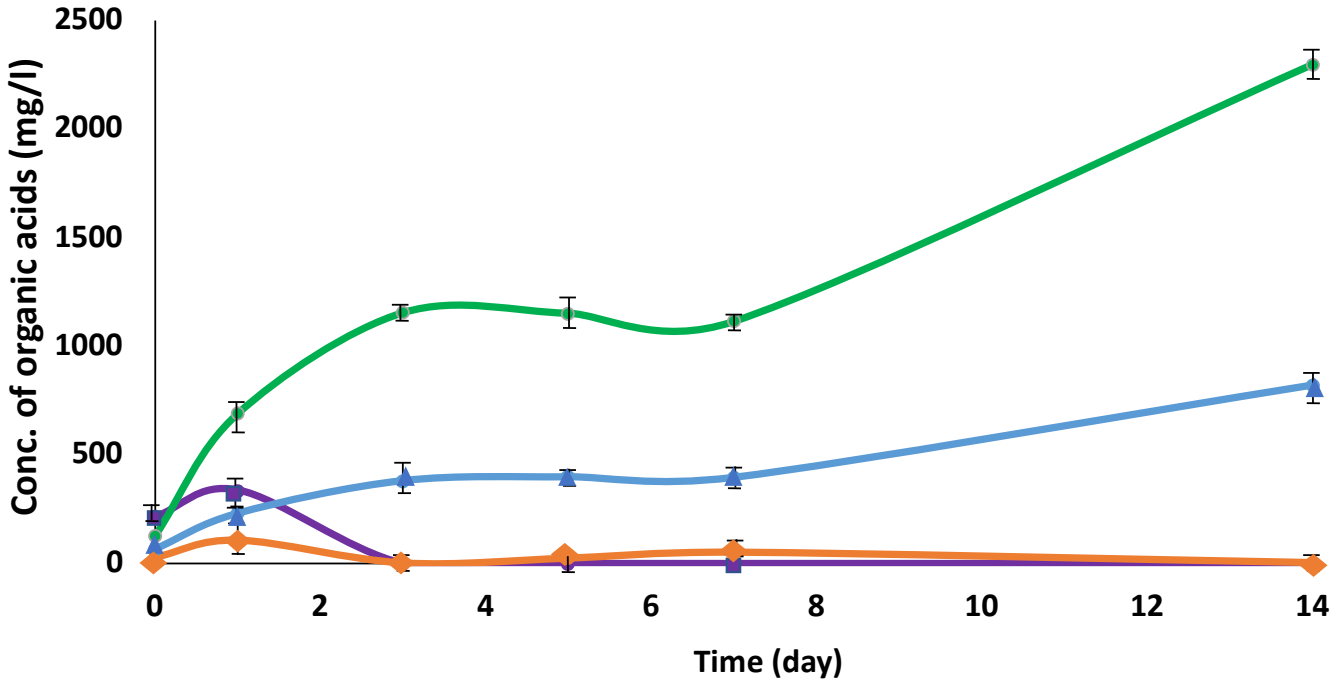

Figure 1. Measurement of organic acids from 0.5% pig manure (PM) cultivated by *C. cellulovorans*. Symbols: rhombus, formate; square, lactate, circle, acetate; triangle, butyrate. The data represent at least three independent experiments.

3.2. Cocultivation of C. cellulovorans with Methanogens or M. mazei

CH_4 production by coculturing *C. cellulovorans*–methanogens (MFMP) was examined with 0.5% (*w*/*v*) glucose, 0.5% (*w*/*v*) cellobiose, and 0.5% (*v*/*v*) acetate, respectively, while the cocultivation of *C. cellulovrorans–M. mazei* was conducted with 0.5% cellobiose as the sole substrate. As shown in Figure 2A, the cell growth in each coculture was observed and showed different patterns. On the other hand, the cocultivation of *C. cellulovorans*-MFMP showed CH_4 production only with 0.5% acetate, whereas the cocultivation of *C. cellulovorans-M. mazei* with the 0.5% cellobiose medium led to no methanogenesis during the cultivation period, showing that *M. mazei* could never use cellobiose for its growth (Figure 2B). These results suggested that methanogenesis promotes not sugars such as glucose or cellobiose but acetate as the carbon source.

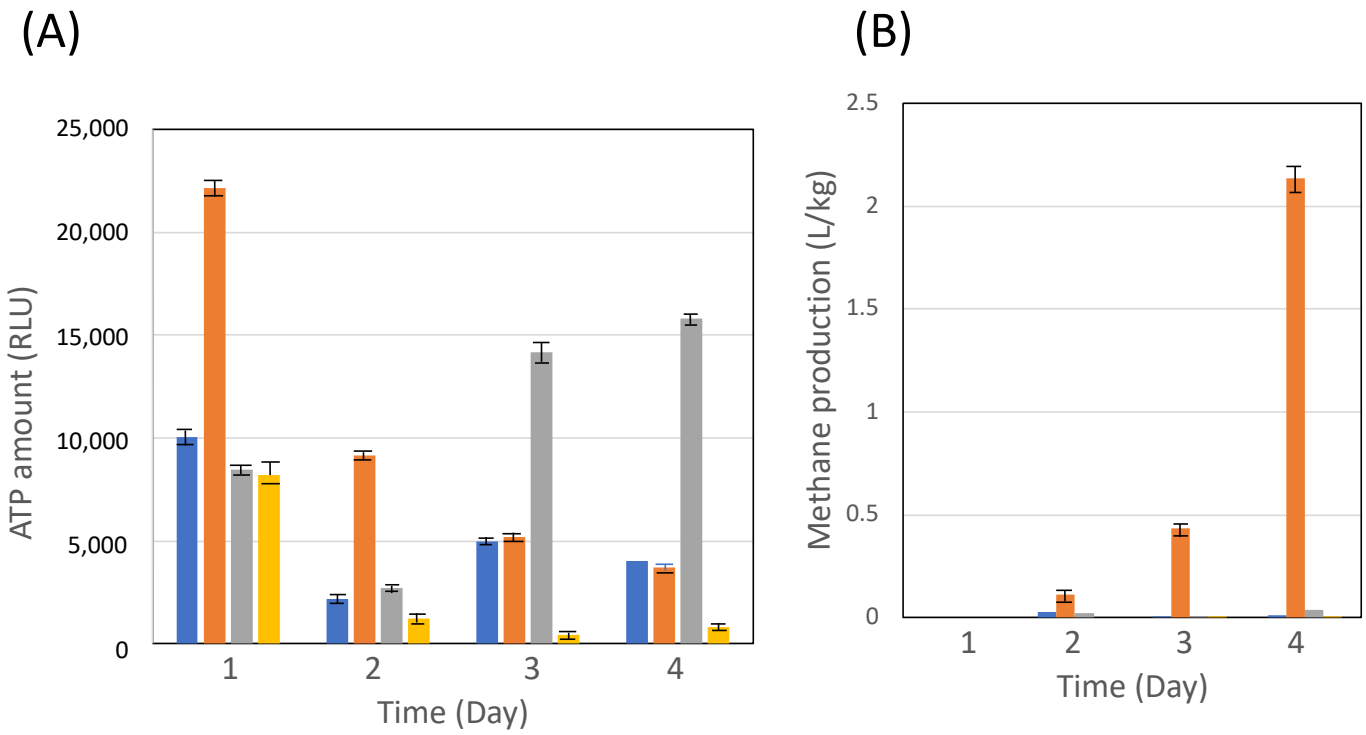

Figure 2. Measurement of ATP amount (RLU) (**A**) and methane production (**B**) with cocultivation of *C. cellulo vorans* and MFMP or *M. mazei*. Bars: blue, 0.5% cellobiose cultivated with *C. cellulovorans* and MFMP; orange, 0.5% acetate cultivated with *C. cellulovorans* and MFMP; gray, 0.5% glucose cultivated with *C. cellulovorans* and MFMP; yellow, 0.5% cellobiose cultivated with *C. cellulovorans* and *M. mazei*. The data represent at least three independent experiments.

3.3. Effect of Carbon Sources with Methanogens

In order to produce CH_4 efficiently, MFMP was examined with the culture media of 1.0% (*v*/*v*) acetate and 1.0% (*v*/*v*) methanol, respectively (Figure 3). The cell growth in the medium of 1.0% acetic acid was at peak at 1 day, while that in the medium of 1.0% methanol was at peak at 16 days (Figure 3A). On the other hand, CH_4 production on the methanol medium was increased from 8 days, and then the maximum production of methane was at peak at 16 days (Figure 3B). In case of the acetic acid medium, CH_4 production was lower than that of the methane medium, resulting in the difference in the metabolic pathway of methanogenesis in MFMP. These results indicated that methanogenesis easily occurs for not acetate but methanol, and the production of methane by 1.0% methanol was 8 times higher than that by 1.0% acetate.

3.4. Identification of Methanogens Cultivated with the Different Carbon Sources

MFMP was precultivated with 0.5% glucose medium for 12 h at 37 °C and then 1000 RLU of MFMP cells was inoculated into the *C. cellulovorans* medium containing 1% acetate or 1% methanol at 37 °C for 72 h. After DNA extraction from the growth cells of each medium, 16S rRNA analyses were carried out by a next-generation sequencer. As shown in Figure 4, *Methanofollis* was the majority of the archaea and was 0.211% in 1% acetate medium for 72 h cultivation. On the other hand, *Methanofollis* in 1% methanol medium was found, i.e., 0.007% for 24 h cultivation and 0.490% for 72 h cultivation, respectively. On the other hand, *Methanosarcina barkeri* was a typical methanogen and was 0.011% for 24 h cultivation and 0.015% for 72 h cultivation, respectively, in 1% acetate medium. Interestingly, for 72 h cultivation, 0.004% of *M. mazei* was found in 1% methanol medium, while 0.571% of *M. barkeri* was detected in the same medium. These results indicated that the growth of methanogens was dependent on the carbon sources,

and the growth trends of individual methanogens seemed remarkably different under the sole carbon sources.

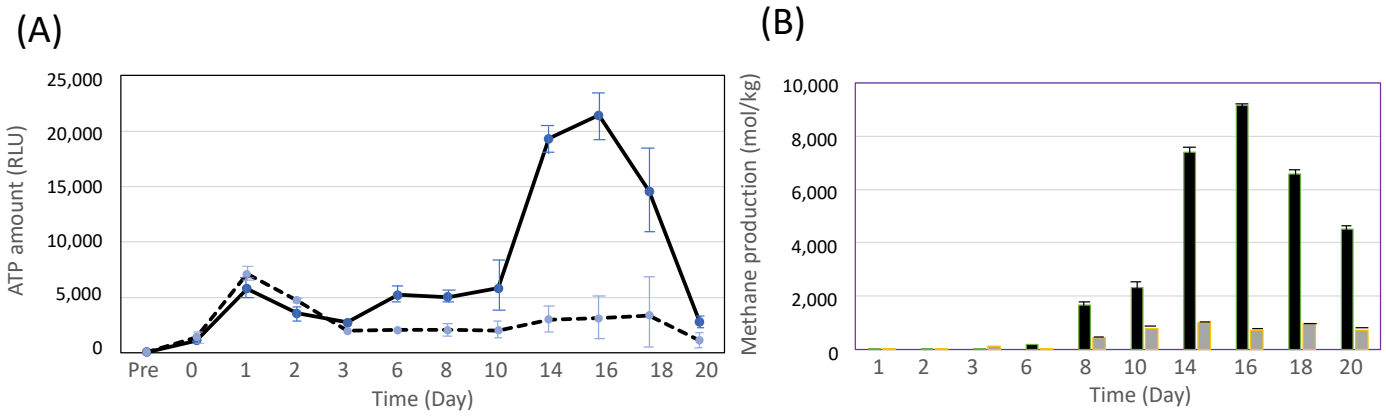

Figure 3. Measurement of ATP amount (RLU) (**A**) and methane production (**B**) in MFMP cultivation. (**A**) black line, 1% methanol; wavey line, 1% acetic acid. (**B**) black bar, 1% methanol; gray bar, 1% acetic acid. The data represent at least three independent experiments.

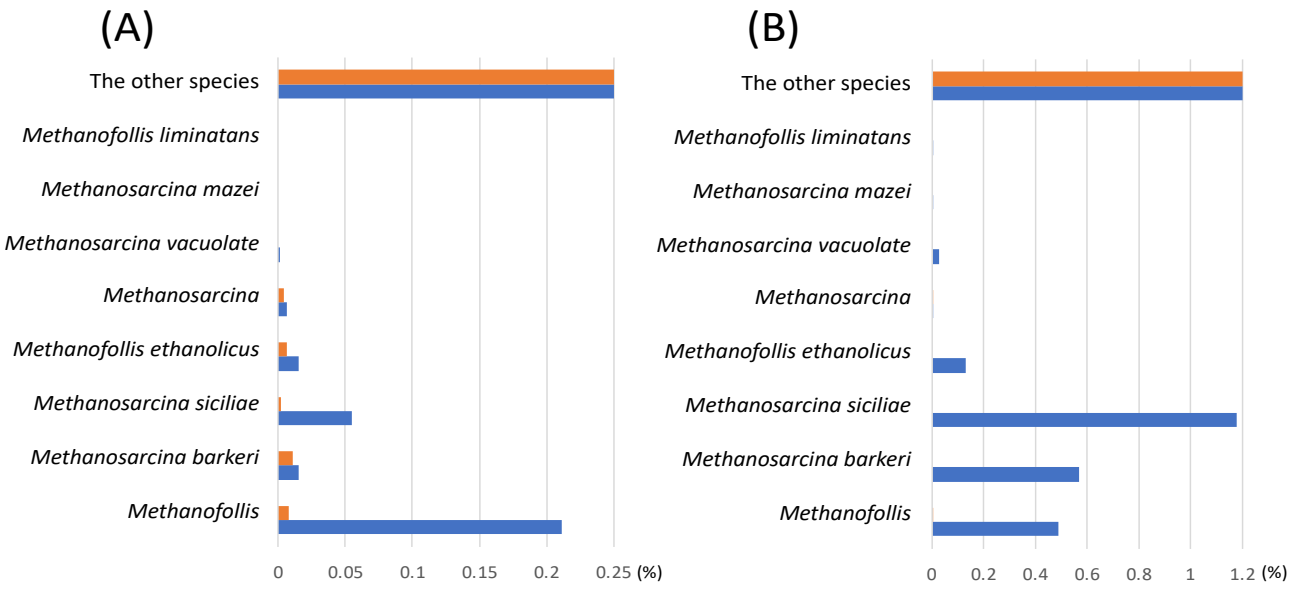

Figure 4. Relative abundance ratio of archaea in MFMP after cultivated with the different carbon sources. After 16S rRNA analysis, the identified archaea were compared. (**A**) 1% acetate cultivation; (**B**) 1% methanol cultivation. Bars indicate orange (after 24 h cultivation) and blue (after 72 h cultivation), respectively. The others show the rest of total percentage.

4. Discussion

With simultaneous renewable energy production, anaerobic digestion (AD) has been widely applied for organic solid waste or wastewater treatment. AD consists of a series of biochemical processes (i.e., hydrolysis, fermentation (acidogenesis), acetogenesis and methanogenesis) performed by various interacting microorganisms, containing bacteria (i.e., acidogens, acetogens) and archaea (methanogens). Through these processes, polymers such as carbohydrates, lipids and proteins are ultimately converted into CH4 and CO_2. In some cases, hydrolysis as the first step is considered as the rate-limiting step by affecting the overall process kinetics, especially when treating recalcitrant fibrous materials. In other cases, especially when the feedstock consists of easily biodegradable organic matter, methanogenesis is considered as the rate-limiting pathway. About 25.31 million tons of food wastes from consumer households, food manufacturing and retail in Japan was generated

in 2018 [36]. In addition to maximizing social and economic benefits, such a management process for appropriate food waste should be implemented to minimize the environmental impacts. Although recycling food waste is preferred to composting and converting to animal feed in Japan, the composting of food waste is still in high demand from farmers, due to the relatively low price and a shortage of cropland for application [37–39]. Therefore, since the most successful application at the commercial scale has so far been AD, which has been widely adopted for waste treatment, a plentiful source of organic compounds such as pig manure (PM) can be used as feedstock in AD. Namely, since the fermented liquid feed (FLF) for pigs contains several nutrients required for bacterial growth, recycling food waste has been considered a possible alternative for many years. Additionally, due to the accumulation of volatile fatty acids (VFAs), PM with a high buffering capacity possibly protects AD against failures [40–42]. The food-waste mixing ratio for methane yield reported that the feedstock composition of 60:40 (volatile solid basis) significantly enhanced methane yield [43]. On the other hand, another group reported that the co-substrate using vegetable processing wastes could improve methane yield up to three-fold with a feedstock ratio of 50:50 (dry weight basis) [44]. Thus, since several potential co-substrates have been examined to assess the effect of varying feedstock compositions on improving AD process performance and increasing methane yield, the VFAs of the *C. cellulovorans* medium containing PM were measured in this study. As a result, acetic acid (approx. 2300 mg/mL) and butyric acid (approx. 820 mg/mL) were accumulated for 14 days, respectively (Figure 1). In this study, since the high concentration of ammonia might inhibit bacterial activity in AD [45–49], in order to enhance methane production, the filtration (0.45 μm filter) was used for the pretreatment of PM before the inoculation of *C. cellulovorans*. By adjusting the ratio of carbon-to-nitrogen (C/N), the co-digestion of PM with organic wastes including high carbon dilute seemed to enhance the macro- and micro-nutrient balance in the feedstocks and improve the inhibitory effect of ammonia [50,51]. On the other hand, cow manure (CM) is rich in nutrients and can provide strong buffer capacity, and thus CM seems more robust than other manures in AD [52]. Therefore, the alleviation of ammonia inhibition when CM is used in AD seems not that urgent and should not be the priority of co-digestion. Additionally, CM is categorized as lignocellulosic waste due to its high amount of lignocellulose (50% in dry matter), which is relatively low in other types of manure [53]. Hence, to make full use of CM to produce more methane via co-digestion, attention should be paid to how to improve the degradation of recalcitrant lignocellulose in CM. In addition, the current study determined biogas production in single-stage and two-stage AD using sheep manure (SP) as substrate and yak rumen fluid as the inoculum. Yak rumen fluid is rich in hydrolytic bacteria [54] and, consequently, its inclusion should improve the degradation of lignocellosic biomass, leading to high biogas production.

All archaeal metagenome-assembled genomes (MAGs) could reveal the reconstruction of pathways related to methanogenesis and relevant energy conservation systems [55]. Methane production from H_2/CO_2 or acetate as sole substrates has also been widely reported. On the contrary, only scarce knowledge on formate as a methanogenic substrate is available despite its reported important role in interspecies electron transfer. Furthermore, it could be evaluated by the average RPKM of genes in each KEGG module among the holistic microbial community activity [56]. However, all methanogenic environments in natural conditions are mixed cultures, such as anaerobic granules, municipal waste digesters, soil and anaerobic aquatic systems. Additionally, almost all of the kinetic studies have not been combined with an analysis of the microbial composition, which has a direct impact on CH4 production kinetics parameters. Thus, by maintaining the methanogenic activity of the microbial community, such a syntrophic behavior is required to synthesize numerous metabolites. An overall shift of the microbial activity was observed in the majority of the KEGG modules after H_2 addition. Moreover, H_2 also enhanced the activity of the glyoxylate cycle and the biosynthesis of lipids and specific amino acids. In addition to H_2, formate, as a similarly formed product during fermentative metabolism, is an important electron carrier in the syntrophic fatty acid-degrading methanogenic consortia [13]. In

fact, formate was in low concentration and immediately consumed in the PM medium (Figure 1). Therefore, other anaerobes might utilize both formate and H_2 as an electron donor for methanogenesis or sulfate respiration.

Clostridium coculture systems are typically used to produce biofuels such as H_2 and CH_4, solvents, and organic acids [57]. Because cellulosic materials are commonly found in nature [18], the specific metabolic capacities of cellulolytic strains and producers in co-culture systems have attracted significant attention and offered many long-term prospects for development. Furthermore, since the combination of genome-centric metagenomics and metatranscriptomics has successfully revealed individual functional roles of microbial members in methanogenic microcosms, these results assigned a multi-trophic role to *Methanosarcina* ssp., suggesting its ability to perform simultaneous methanogenesis from acetate, CO_2 and methanol/methylamine [55]. The MFMP used in this study originally consisted of *C. butyricum* (0.005%), identified as the same genus of *C. celulovorans* and *M. mazei* (1.34%) found among methanogens [32]. Furthermore, other methanogens such as *Methanosaetaceae*, *Methanosaeta*, and *Methanospirillaceae* were also identified in MFMP. The genus *Methanosaeta*, which utilizes only acetate, was a large portion of the ratio, next to *Methanosarcina*. On the other hand, 1% acetate or 1% methanol was used as the sole carbon source for MFMP cultivation in this study. As a result, while *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in the 1% methanol medium for 72 h cultivation, *Methanofollis* (0.211%) was dominant in the 1% acetate medium for 72 h cultivation (Figure 4). It is thought that all methanogens are physiologically specialized and able to scavenge the electrons from H_2, formate, acetate, and methanol, having CH_4 as the final product [49]. The *Clostridium* coculture system can also produce CH_4 in addition to producing H_2 and solvents, in particular the coculture of cellulolytic *Clostridia* and methanogens, including *M. barkeri* Fusaro, *M. mazei*, and *Methanothermobacter thermautotrophicus*. The methanogens utilized H_2 and CO_2, acetate, and even formate that was generated by the cellulolytic *Clostridia* from cellulose to produce CH_4 [33,58]. In this study, CH_4 production by cellobiose was not found in the cocultivation of *C. cellulovorans*-*M. mazei* (*C.c*:*M.m* = 1:3), while only acetate led to methanogenesis in the cocultivation of *C. cellulovorans*-MFMP (Figure 2). In addition, since *M. barkeri* was more dominant than *M. mazei* in MFMP cultivation according to the 16S rRNA analysis (Figure 4), it seemed that *Methanosarcina* spp. may play a key role on the methanogenesis of MFMP. So far, it has been reported that CH_4 production was investigated with sugar beet pulp [16] and mandarin orange peel [17] in the cocultivation of *C. cellulovorans*-MFMP (*C.c*:MFMP = 1:20). Therefore, carbon sources such as acetic acid and methanol were compared by the production of CH_4 in this study. As expected, CH_4 production from methanol was approximately eight times higher than that from acetic acid, related to the cell growth of MFMP (Figure 3). Thus, methanogens seemed to be altered in their flora, dependent on the sole carbon source.

5. Conclusions

In this study, *C. cellulovorans* was cultivated with PM and the cocultivation of *C. cellulovoroans*-*M. mazei* or *C. cellulovorans*-MFMP was performed with different carbon sources. Since the cultivation of *C. cellulovorans* with PM had much acetic acid, it was thought to be one an excellent biomass for methane production. On the other hand, methanol was the best carbon source for CH_4 production with MFMP. Regarding the next-generation sequence analysis of MFMP for 72 h cultivation, while *Methanofollis* (0.211%) was dominant in the 1% acetic acid medium, *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in 1% methanol medium. Therefore, it seemed that *Methanosarcina* spp. may play a key role in the methanogenesis of MFMP.

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