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Article

Effects 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 synthetic natural gas, but also one of the hydrogen carriers in terms of energy density. On the other hand, there exist bacterial ecosystems in nature that can decompose organic compounds to produce CH_4 and CO_2. In this study, *Clostridium cellulovorans* was first cultivated with pig manure (PM) as an unused biomass. Regarding the measurement of organic acids by high-performance liquid chromatography (HPLC), acetate and butyrate were increased in the *C. cellulovorans* medium containing 0.5% PM, while formate and lactate were decreased in it. Next, in comparison with carbon sources such as glucose, cellobiose, and acetate, cocultivation of *C. cellulovorans* and *Methanosarcina mazei* or microbial flora of methane production (MFMP) was performed in the *C. cellulovorans* medium. These results revealed that 0.5% acetate as the sole carbon source produced CH_4 only by cocultivating *C. cellulovorans* and MFMP. Furthermore, MFMP was only cultivated with 1% acetate or 1% methane as a carbon source after precultivated with 0.5% glucose medium for 12 h. As a result, methane productivity of MFMP with 1% methanol medium was approximately eight times higher than that with 1% acetate medium. Finally, next-generation sequencing (NGS) analysis of MFMP after cultivation with 1% acetate or 1% methane was carried out. Interestingly, *Methanofollis* (0.211%) belonging to H_2/CO_2 -using methanogens (CO_2 reduction pathway) was dominant in the 1% acetate medium for 72 h cultivation, whereas *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. are belonging to acetoclasts (acetoclastic pathway), methanol could promote to grow *Methanosarcina* spp. rather than acetate. Therefore, it seemed *Methanosarcina* spp. may play a key methanogenesis in MFMP. Thus, these results will provide important information for low cost biomethane production.

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

1. Introduction

Anaerobic digestion (AD) consists of a series of biochemical processes (i.e., hydrolysis, fermentation (acidogenesis), acetogenesis and methanogenesis) performed by various interacting microorganisms, including bacteria (i.e., acidogens, acetogens) and archaea (methanogens). It is also obvious 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, prediction of the magnitude of carbon loss as CO_2 or CH_4 is hampered by our limited knowledge of microbial metabolism of organic matter in these environments [2]. Genome-centric metagenomic analysis of

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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 such as 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, i.e., acetoclastic pathway and 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 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. In the corresponding coculture, 15mM lactate was consumed in approximately 2 weeks while methane was produced, indicating lactate fermentation by *S. multivorans* and H_2 transfer to *M. voltae* as 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*

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 is that the combination of the metabolic capacity of two or more microorganisms allows for the utilization of more complex substrates and the production of specific products [14]. In addition to treatment of waste-water, biodegradation of textile azo dye and dispose of contaminated soil, recently, cocultivation systems were also applied to produce biofuels, bulk chemicals, and natural products [15–26].

Cellulose is most abundant on the Earth and 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 which synergistically collaborates with non-complexed enzymes [28,29]. By the cocultivation of *C. cellulovorans* and *C. beijerinckii*, IBE fermentation was performed using mandarin orange wastes [30]. Moreover, methane was produced from sugar beet pulp [31] and mandarin orange peel [32] under cocultivation with *C. cellulovorans* and methanogens. 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 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

was by implementing nanotechnology in combination with *C. cellulovorans* through 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 that are the products from cellulose degraded by *C. cellulovorans* and acetate metabolized from glucose through 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 and Culture Condition

Clostridium cellulovorans 743B (ATCC35296) was grown anaerobically 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, Germany) and was cultivated with the JCM230 medium [35]. 0.5% (w/v) Glucose, 0.5% acetic acid (FUJIFILM Wako Chemicals, Japan), and 0.5% (w/v) cellobiose (Sigma, 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 on January, 2017 at Gifu in Japan [32]. *C. cellulovorans* (*C.c*) was precultured with 0.5% cellobiose for 12 h at 37 °C and *M. mazei* (*M.m*) and MFMP were done with 0.5% glucose for 12 h at 37 °C, respectively. Co-cultivation was performed as approximately 1000 RLU of *C.c* cells and approximately 20000 RLU of MFMP cells (*C.c* : MFMP=1:20) and approximately 1000 RLU of *C.c* cells and approximately 3000 RLU of *M.m* cells (*C.c*:*M.m*=1:3), respectively.

2.216. S rRNA Sequencing

Samples were crashed by Shake Master Neo (bms, Tokyo, Japan) and DNA was extracted by Fast DNA spin kit (MP Bio, CA, USA). iSeq 100 (Illumina, CA, USA) was used for sequencing under the condition of 2 × 150 bp. The 16S Metagenomics App performs 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 are: PCR1_Forward (50 bp): 5′–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3′ and PCR1_Reverse (55 bp): 5′–GTCTCGTGG GCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3′, respectively The 16S rRNA sequences of MFMP previously reported [31] has been deposited in the DDBJ database (accession no. DRR160954).

2.3. 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 (Shimadzu, Kyoto, Japan) with UV detector.

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 mm filter to remove the inhibitor for

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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 result suggested *C. cellulovorans* was able to grow in the 0.5% PM medium and acetate and butyrate were increased, while formate and lactate were decreased after once increased at 1 day cultivation (Figure 1). Total concentrations of acetate and butyrate at 14 days was approximately 2300 mg/L and 820 mg/L, respectively, resulting that PM would be an excellent biomass for methanogenesis.

3.2. Co-Cultivation 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 cocultivation of *C. cellulovrorans–M. mazei* was done with 0.5% cellobiose as the sole substrate. As shown in Figure 2A, the cell growth in each coculture was observed and 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, resulting that *M. mazei* could never use cellobiose for its growth (Figure 2B). These results suggested methanogenesis promotes not sugars such as glucose or cellobiose but acetate as the carbon source.

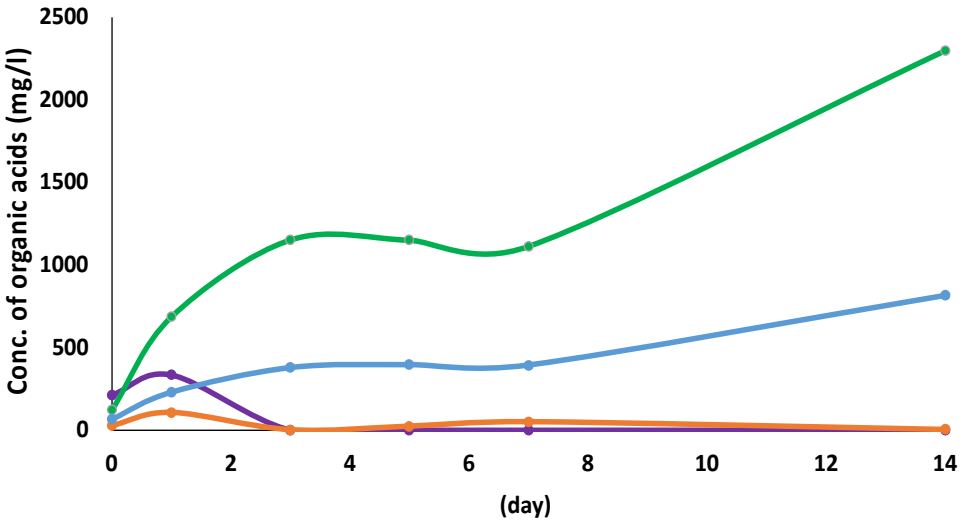

Figure 1. Measurement of organic acids from 0.5% pig manure (PM) cultivated by *C. cellulovorans*. Lines: orange, formate; purple, lactate, green, acetate; blue, butyrate.

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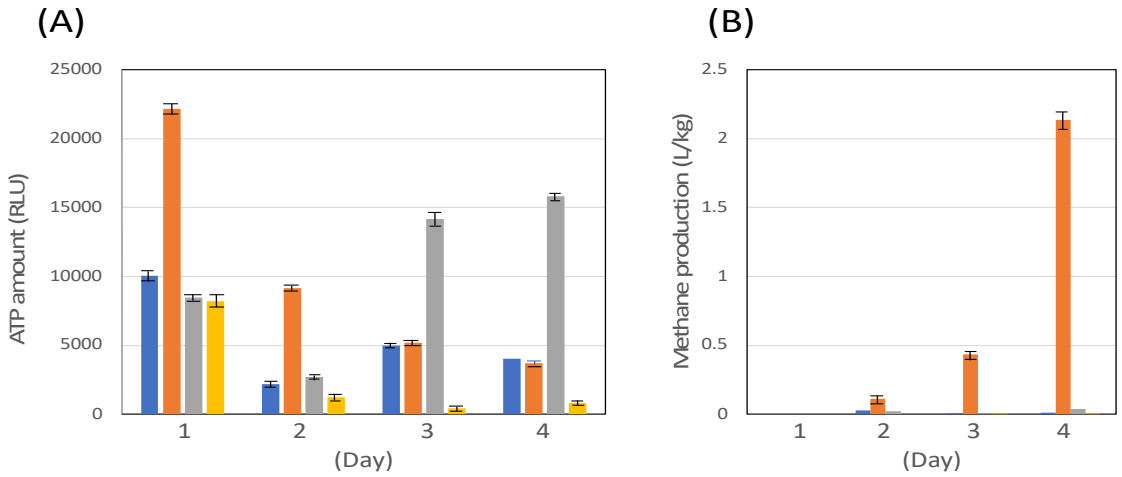

Figure 2. Measurement of ATP amount (RLU) and methane production (B) with cocultivation of *C. cellulovorans* and MFMP or *M. mazei*. Bars: blue, 0.5% cellobiose cultivated with C. cellulovorans and MFMP; orenge, 0.5% acetate cultivated with C. cellulovorans and MFMP; gray, 0.5% glucose cultivated with C. cellulovorans and MFMP; yellow, 0.5% cellobiose cultivated with C. cellulovorans and M. mazei.

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 a peak at 1 day, while that in the medium of 1.0% methanol was a 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 a 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 of metabolic pathway of methanogenesis in MFMP. These results indicated 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.

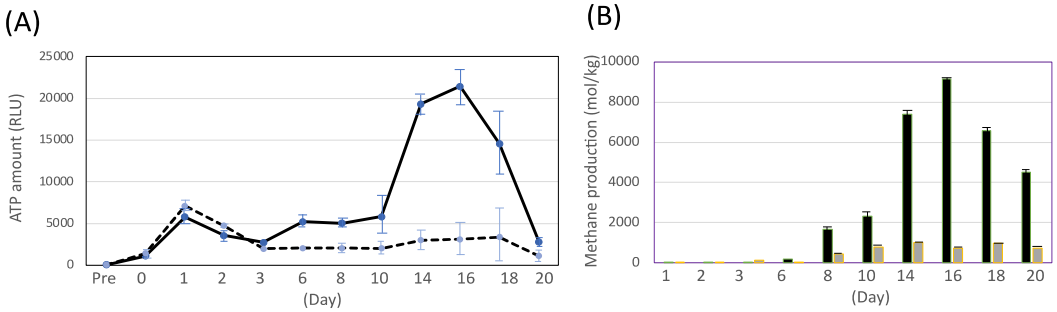

Figure 3. Measurement of ATP amount (RLU) (A) and methane production (B) in MFMP cultivation. (A) black line, 1% methanol; wavey line, 1% acetic acid. (B) black bar, 1% methanol; gray bar, 1% acetic acid.

3.4. Identification of Methanogens for Different Carbon Sources

MFMP was precultivated with 0.5% glucose medium for 12 h at 37°C and then 1,000 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 next generation sequencer. As shown in Table 2, *Methanofollis* was a majority of 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

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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 the growth of methanogens was dependent on the carbon sources and their growth trend of individual methanogens seemed remarkably different under the sole carbon sources.

Table 2. 16S rRNA analysis of archaea in MFMP after cultivated with the different carbon sources

1% Acetic acid				1% Methanol			
24 h		72 h		24 h		72 h	
Archaea	Ratio (%)	Archaea	Ratio (%)	Archaea	Ratio (%)	Archaea	Ratio (%)
Methanosarcina barkeri	0.011	*Methanofollis*	0.211	*Methanofollis*	0.007	*Methanosarcina siciliae*	1.178
Methanofollis	0.008	*Methanofollis ethanolicus*	0.076			*Methanosarcina barkeri*	0.571
Methanofollis ethanolicus	0.006	*Methanosarcina siciliae*	0.055			*Methanofollis*	0.490
Methanosarcina	0.004	*Methanosarcina barkeri*	0.015			*Methanosarcina*	0.244
Methanosarcina siciliae	0.002	*Methanosarcina*	0.006			*Methanofollis ethanolicus*	0.131
		Methanosarcina vacuolate	0.001			*Methanosarcina vacuolate*	0.027
						Methanosarcina mazei	0.004
						Methanofollis liminatans	0.001

4. Discussion

In Japan, around 25.31 million tons of food waste was generated in 2018 from food manufacturing, retail, and consumer households [36]. Appropriate food waste management practices should be implemented to minimize the environmental impacts and maximize social and economic benefits. Since recycling food waste as compost and animal feed is preferred in Japan, composting of food waste still presents high-quality demand by farmers, relatively low price, and a shortage of cropland for application [37–39]. Therefore, since the most successful application so far at the commercial scale has been anaerobic digestion (AD), which has been widely adopted for waste treatment, pig manure (PM) is a plentiful source of organic compounds that can be used as feedstock in AD. Namely, recycling food waste into fermented liquid feed (FLF) for pigs that contains several nutrients required for bacterial growth was considered a possible alternative for many years. Also, PM has a high buffering capacity, which possibly protects AD against failures due to the accumulation of volatile fatty acids (VFAs) [40–42]. It was reported that the effect of varying PM with food waste mixing ratio was evaluated on methane yield, suggesting that the feedstock composition of 60:40 (volatile solid basis) enhanced methane yield significantly [43]. On the other hand, the other group reported that using vegetable processing wastes as co-substrate with a feedstock ratio of 50:50 (dry weight basis) could improve methane yield up to 3-fold [44]. Thus, since several potential co-substrates have been examined to assess the effect of varying feedstock composition on increasing methane yield and improving the AD process performance, 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). As another possibility to efficient methane production, since the high ammonia concentration might inhibit bacterial activity in AD [45–49], PM was pretreated with 0.45 mm filtration before inoculation of *C.*

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cellulovorans in this study. Therefore, by adjusting the carbon-to-nitrogen (C/N) ratio, co-digestion of PM with organic waste containing high carbon dilute seemed to improve the inhibitory effect of ammonia and to enhance the macro and micronutrient balance in the feedstocks [50,51]. Besides, 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.

Pathways related to methanogenesis and relevant energy conservation systems were reconstructed in all archaeal the metagenome-assembled genomes (MAGs) [55]. The holistic microbial community activity could be evaluated by the average RPKM of genes in each KEGG module [56]. Thus, in order to maintain the methanogenic activity of the microbial community, a syntrophic behavior is needed 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. Besides H_2, also formate, similarly formed during fermentative metabolism, is an important electron carrier in e.g. syntrophic fatty acid-degrading methanogenic consortia [57]. In fact, formate was low concentration and immediately consumed in the PM medium (Figure 1). Therefore, other anaerobes may use both H_2 and formate as an electron donor for sulfate respiration or methanogenesis.

Clostridium coculture systems are typically used to produce biofuels such as H_2 and CH_4, solvents, and organic acids [58]. Because cellulosic materials are commonly found in nature [18], the specific metabolic capacities of cellulolytic strains and producers in coculture systems have attracted significant attention and offered many long-term prospects for development. Furthermore, since the combination of genome-centric metagenomics and metatranscriptomics 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]. 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 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, *Methanofollis* (0.211%) was dominant in the 1% acetate medium for 72 h cultivation, whereas *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in the 1% methanol medium for 72 h cultivation (Table 2). 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 [33,59]. 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 (Table 2), it seemed that *Methanosarcina* spp. may play a key methanogenesis in 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).

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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, with 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 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 of excellent biomass for methane production. On the other hand, methanol was a best carbon source for CH_4 production with MFMP. Regarding next generation sequence analysis of MFMP, *Methanofollis* (0.211%) was dominant in the 1% acetic acid medium for 72 h cultivation, whereas *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in 1% methanol medium for 72 h cultivation. Therefore, it seemed *Methanosarcina* spp. may play a key methanogenesis in MFMP.

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