Influence of C/N Ratio on Performance and Microbial Community Structure of Dry-Thermophilic Anaerobic Co-Digestion of Swine Manure and Rice Straw

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***Abstract*—Dry anaerobic digestion has several advantages over liquid anaerobic digestion. However, the digestion would be inhibited at low C/N ratio of substrate such as swine manure. In this study, we tried to prevent the inhibition by adding rice straw into swine manure. Swine manure was co-digested with rice straw in semibatch digesters at 8 (manure only), 20 and 30 of C/N ratio. In only C/N 8, NH_4^+ concentration exceeded threshold (3000 mg N kg w.w.$^{-1}$) of ammonia inhibition. Methane production of C/N 8 and C/N 20 decreased during incubation, while C/N 30 showed stable CH_4 production. Pyrosequencing results showed that the halophilic bacterial communities were altered to non-halophilic ones by co-digestion of manure with straw. In terms of archaea, acetoclastic methanogen was dominated in C/N 20 and 30. Therefore, adding rice straw into swine manure is effective for prevention of the inhibition and related to structure of microorganisms.**

***Index Terms*—C/N ratio, Dry-thermophilic anaerobic digestion, Rice straw, Swine manure**

I. Introduction

Anaerobic digestion is a method to decompose organic matter by variety of microorganisms under anaerobic condition. The end product of anaerobic digestion includes biogas (CO_2 and CH_4) and an organic residue rich in nitrogen [1]. Therefore, anaerobic digestion can treat organic waste as well as produce renewable energy and fertilizer. Conventionally, anaerobic digestion was conducted under less than 10% of total solid (TS) content (anaerobic liquid digestion). On the other hand, dry anaerobic digestion is characterized by the high solid content of the feedstocks, which is typically greater than 15%. Dry anaerobic digestion has advantages over liquid anaerobic digestion such as smaller reactor volume, lower energy requirements for heating and minimal material handling [2].

In dry thermophilic anaerobic digestion treating substrate with high ammonium concentration, a serious problem would be occurred by low C/N ratio because of inherent high ammonia concentration in a substrate such as swine manure, which leads to ammonia inhibition to methanogens [3]. On the other hand, co-digestion of various biomass is expected to increase C/N ratio and alleviate ammonium inhibition [4], [5]. Wu et al. demonstrated that co-digestion of swine manure with corn stalks at C/N ratio of 20 increased biogas production. Our previous study also revealed that controlling C/N ratio by mixing rice straw as a carbon source successfully produced CH_4 in dry-thermophilic anaerobic digestion of pig manure [6]. Therefore, inhibition by a low C/N ratio is potentially alleviated by increasing dose of rice straw as a carbon source.

The anaerobic digestion of cellulosic biomass is carried out by four group of microorganisms responsible for hydrolysis, acidogenesis, acetogenesis, and methanogenesis [7]. In previous study, presence of several kinds of microorganism has been reported. For example, *Bacteroidetes*, *Chloroflexi* and *Firmicutes* were major phyla in the wet and dry mesophilic anaerobic digestion [8]. Meanwhile, there has been a few report about dynamics of microorganisms in the dry thermophilic anaerobic digester affected by ammonia inhibition.

The aim of this study is to clarify influence of C/N ratio on performance and microbial community structure of dry-thermophilic anaerobic co-digestion of swine manure and rice straw. We conducted continuous dry thermophilic anaerobic digestion with swine manure and rice straw at different C/N ratio in the semibatch digesters. In order to analyze bacterial and archaeal community structures, pyrosequencing was performed.

Manuscript received October 30, 2014, revised January 23, 2015.

doi: 10.12720/jomb.5.1.11-14

II. Materials and Methods

A. Feedstock and Inoculum

Forage rice straw (Oryza sativa L. cv. Takanari) and mixture of swine urine and dung were used as a feedstock. The forage rice straw used in this study was harvested in 2012 in a rice field located at Ibaraki prefecture, Japan. The forage rice straw were air dried and cut into 10 cm length before feeding. Swine urine and dung was obtained from a pig farm located at Ibaraki prefecture, Japan. The urine and dung were mixed at 1.9:3.5 in weight before feeding.

Inoculum was obtained from a dry-thermophilic anaerobic digester of food waste, paper and/or wood/grass. Properties of inoculum, swine dung, urine and rice straw are shown in Table I.

B. Dry Thermophilic Anaerobic Digestion

Dry-thermophilic anaerobic co-digestion was performed in 20 L of semi-batch reactors. The reactors were connected to a gas sampling port and flow meter, and incubated at 55 ℃ in a constant-temperature oven. Sludge (inoculum and substrate) volume in each reactor was 10 kg. In order to evaluate biogas production at different C/N ratios of substrate, three mixing ratios of swine manure (mixture of urine and dung) and rice straw were studied as shown in Table II. In 30 of C/N ratio, water was added to adjust total solid (TS) content. At the beginning of incubation, sludge retention time (SRT) was 40 days. Sludge retention time was decreased in a step-wise manner periodically. The incubation was stopped when change in SRT resulted in decrease in biogas production.

During incubation, biogas and sludge samples were taken periodically. Biogas composition was analyzed by a gas chromatograph equipped with a thermal conductivity detector (TCD). Ammonium (NH_4^+) concentration of sludge were measured by an ion chromatograph after extraction with pure water.

TABLE I. Properties of Forage Rice Straw, Pig Urine, Dung and Inoculum

	Inoculum	Dung	Urine	Straw
TS (%)	21.4	25.5	4.6	92.3
VS[a] (%)	11.6	17.6	2.4	69.8
TC (mg kg^{-1})[b]	5.8	11.8	1.1	35.6
TN (mg kg^{-1})[b]	0.35	0.57	0.23	0.49
NH_4^+ (mg kg^{-1})[b]	2098	2004	3035	N.D.[c]
pH	8.7	8.4	7.8	N.D.

[a] Volatile solid
[b] Wet basis.
[c] Not determined.

TABLE II. Substrate Composition Of Each C/N Ratio for a Cycle of SRT.

C/N ratio	Manure (kg)	Straw (kg)	Water (kg)	TS[a] (%)
8	10	0	0	10.5
20	8.16	1.84	0	27.0
30	4.04	2.40	3.56	27.0

[a] Mixture of pig manure and rice straw.

C. Analysis of Bacterial and Archaeal Community Structures

Total DNA was extracted from sludge sample using the FastDNA SPIN Kit for Soil (QBIOgene, Carlsbad, CA, USA) according to the manufacturer's protocol.

For the bacterial and archaeal community analysis, the V6 hypervariable regions (bacteria and archaea) was amplified using primers targeting the regions between the 958 and 1048 regions of archaeal 16S rRNA. These amplifications were conducted using the primer set described by Crump et al. [9]. The resulting amplicons were sequenced using the Genome Sequencer FLX System (Roche, Basel, Switzerland). The sequences were clustered using CD-HIT. Representative sequences from operational taxonomic units (OTUs) were extracted and aligned using RDP Classifier software.

III. Results and Discussion

A. Methane Production

Fig. 1a shows NH_4^+ concentration in each digester. In C/N 8, NH_4^+ concentration was continued to increase during incubation and higher than 3000 mg N kg w.w.$^{-1}$, which is an inhibitory level for CH_4 production as previously reported [10]. In contrast, co-digestion of swine manure with rice straw (C/N 20 and 30) resulted in decrease in NH_4^+ concentration (Fig. 1a).

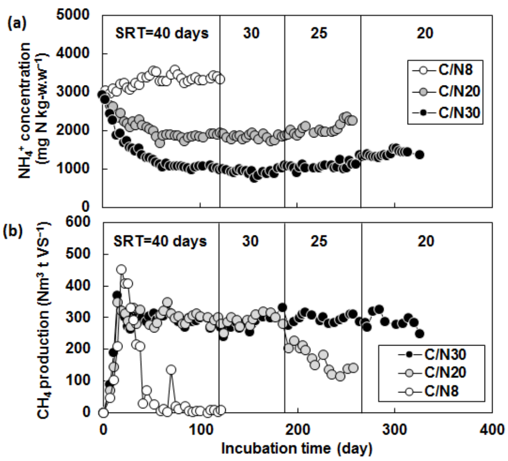

Figure 1. Temporal changes in (a) NH_4^+ concentration in the reactor and (b) CH_4 production at different C/N ratios.

During incubation, CH_4 concentrations in the headspace of the reactor were around 60%. Fig. 1b shows volume of CH_4 produced per ton of VS. Methane production of C/N 8 (pig manure only) decreased down to zero at 60 day after rapid increase (Fig. 1b). In C/N 20, CH_4 production was around 300 Nm^3 t VS^{-1} during SRT 40 and 30 days, while it decreased after changing SRT to 25 days. In C/N 20, accumulation of acetate and propionate was observed during 25 days of SRT (data not shown). This would be due to difference in degradability of rice straw and manure. In contrast to CH_4 production in C/N 8 and 20, C/N 30 showed stable CH_4 production even at SRT of 20 days of SRT. These results revealed that higher C/N ratio permitted more stable CH_4 production at shorter SRT.

B. Bacterial and Archaeal Community Structures

In inoculum, *Firmicutes* was the predominant phylum, accounting for 60%, followed by *Thermotogae*, *Synergistetes* and *Proteobacteria* at phylum level (data not shown). In the sludge of dry-thermophilic anaerobic digesters, predominant phylum was also *Firmicutes*, accounting for 79–88%, followed by *Proteobacteria*. In the sludge, abundance of *Thermotogae* was decreased and accounted for less than 1%. The dominance of *Firmicutes* has been also reported previously in the dry-mesophilic and dry-thermophilic anaerobic digester [7], [8]. The disappearance of *Thermotogae* with succession of the incubation has been also reported [7], [11].

For further understanding of the bacterial communities, relative abundance of each genus was evaluated (Fig. 2a). In Fig. 2a, only the 10 most abundant genera in each sample was presented and other genera were included in “Others”. In the inoculum, *Clostridium* (phylum *Firmicutes*), *Petrotoga* (phylum *Thermotogae*) and *Halothermothrix* (phylum *Firmicutes*) were accounted for 23% of the relative abundance. More than 60% of genera was included in “Others”. Dry-thermophilic anaerobic digestion of swine manure with or without rice straw for 120 days showed large increase in *Halothermothrix*, *Selenomonas* and *Halocella* affiliated in *Firmicutes* (Fig. 2a) without large difference among C/N ratios. On the other hand, in C/N 20 and 30, *Halothermothrix* and *Halocella* were reduced during 120–180 days, while *Clostridium*, *Moorella* and *Selenomonas* were increased (Fig. 2a).

Community structures of archaea at genus level are summarized in Fig. 2b. Archaeal communities were drastically altered by incubation at different C/N ratio. In the inoculum, *Methanoculleus* and *Methanothermobacter* were dominated. This trend was also found in C/N 8 and C/N 20 on 120 days. Meanwhile, *Methanosarcina* predominated in C/N 20 on 180 days and in C/N 30 on both 120 and 180 days.

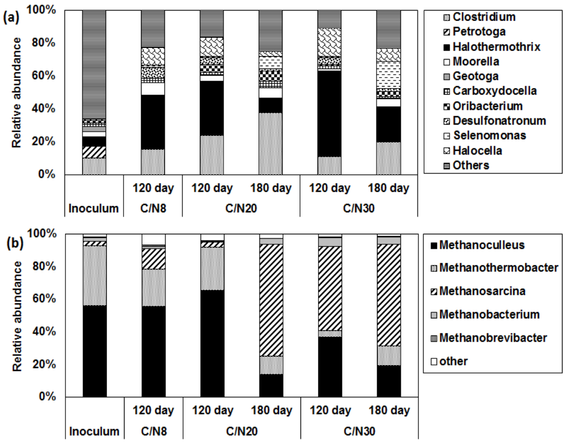

Figure 2. Community structures of (a) bacteria and (b) archaea at genus level in the inoculum and sludge of dry-thermophilic anaerobic digesters operated at different C/N ratios. In Fig. 2a, the 10 most abundant genera in each sample was presented and other genera were included in “Others”.

In this study, both C/N ratio of substrate and incubation time influenced community structures of bacteria and archaea (Fig. 2). Because C/N ratio of the substrate was adjusted by mixing swine manure and rice straw, different amount of organic matter and NH_4^+ would affect decomposition of substrates. *Halocella* has been detected in thermophilic anaerobic digester [12] and known to degrade cellulose and produce acid. Increase in this genus after beginning of incubation would be due to feeding rice straw or swine manure, which contain cellulose. *Halothermothrix* is a thermophilic, halophilic anaerobic bacteria and capable to produce hydrogen from glucose [13]. Interestingly, *Halothermothrix* and *Halocella*, which were once increased on 120 days, were decreased on 180 days in both C/N 20 and C/N 30, while *Clostridium* and *Selenomonas* were increased (Fig. 2a). It has been known that *Halothermothrix orenii* and *Halocella celluloytica* are halophilic anaerobic bacteria [13], [14]. Probably, bacterial communities would be altered from halophilic communities to non-halophilic ones by decrease in NH_4^+ concentration in C/N 20 and C/N 30 with time (Fig. 1a).

Archaeal community would be influenced by changes in bacterial community. During incubation, predominant archaea was shifted from *Methanoculleus* and *Methanothermobacter* to *Methanosarcina* (Fig. 2b). *Methanoculleus* and *Methanothermobacter* are known as hydrogenotrophic methanogen, while *Methanosarcina* is acetoclastic methanogen. Decrease in *Methanothermobacter* during operation has been also reported in dry-thermophilic anaerobic digester fed with garbage [11]. A large decrease in *Halothermothrix* would partially explain decrease in *Methanothermobacter* because it produces hydrogen as noted above. Decrease in *Methanothermobacter* would also affect decrease in *Thermotogae*. Yabu et al. pointed out that decrease in *Thermotogae*, which degrades acetate syntrophically with hydrogenotrophic methanogen [15], is influenced by decrease in *Methanothermobacter* [11]. In contrast, increase in *Methanosarcina* would be related to increase in bacteria capable to produce acetate such as *Moorella* [16]. Since *Moorella thermoacetica* produces acetate from carbon dioxide and hydrogen [16], hydrogenotrophic methanogens and other hydrogen producing bacteria were outcompeted for hydrogen. Therefore, source of the methanogenic pathway would be different between digestion of manure only and co-digestion of manure and rice straw. Thus, interaction between bacteria and archaea is influenced by C/N ratio in the dry-thermophilic anaerobic co-digestion of swine manure and rice straw.

IV. Conclusion

Dry-thermophilic anaerobic co-digestion of swine manure with rice straw could effectively alleviate ammonia inhibition. Continuous operation with reducing SRT suggested that larger amount of substrate can be treated under high C/N ratio. Similarly, bacterial and archaeal community structures were also affected by C/N ratio. Our data suggested that during incubation of manure (C/N 8) hydrogenotrophic methanogenesis is a main pathway of CH_4 production, while acetoclastic methanogenesis would be dominated in 20 and 30 of C/N ratio. The change in methanogenic pathway would be responsible for predominance bacteria. Therefore, prevention of ammonia inhibition by adjusting C/N ratio is closely related to performance and structure of microorganisms.

ACKNOWLEDGMENT

The research work was supported by the Environment Research and Technology Development Fund (1B-1103 and 1-1404) from the Ministry of the Environment, Japan.

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