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Anaerobic codigestion of pretreated wheat straw with cattle manure and analysis of the microbial community

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HIGHLIGHTS

- Wheat straw (WS) pretreated with H_2O_2 was codigested with cattle manure (CM).
- Methanogenic community was measured by the high-throughput sequencing technique.
- The optimal concentration of H_2O_2 for treating WS was 3%.
- A 40:60 ratio of H_2O_2-treated WS mixed with CM produced the highest methane yield.
- Methanogen shifted from acetoclastic to hydrogenotrophic population in digestion.

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ABSTRACT

Wheat straw (WS) was pretreated with four concentrations of H_2O_2 (1%, 2%, 3%, and 4%) and was anaerobically codigested with dairy cattle manure (CM) at various ratios from 100:0 to 0:100. Wet-state H_2O_2 pretreatment effectively enhanced the biodegradability and methane yield of the WS. The optimal concentration of H_2O_2 for treating WS was 3%. The methane yield was higher with the codigestion of CM and H_2O_2-treated WS than with untreated WS and higher than with H_2O_2-treated WS alone or CM alone. A 40:60 ratio of H_2O_2-treated WS mixed with CM produced the highest yield of methane (320.8 mL g volatile solid (VS)$^{-1}$). Results of high-throughput sequencing indicated that the methanogenic community shifted during the codigestion from the acetoclastic methanogens, *Methanosarcina*, to the hydrogenotrophic methanogens, *Methanosphaera* and *Methanoculleus*.

1. Introduction

Environmental problems caused by the burning of fossil fuels and the increasing depletion of resources during the last two decades have led to the development of renewable and sustainable energy sources. The production of biogas by anaerobic digestion (AD) is a cost-efficient and environmentally beneficial bioenergy technology and has thus received much attention (Amon et al., 2007). Lignocellulosic biomass, such as agricultural straw, stalks, waste sludge, and livestock manure, is currently the most common feedstock for AD. Lignocellulosic materials, however, are difficult to enzymatically or bacterially digest because they contain cellulose, hemicellulose, and lignin in complex and cross-linked structures; soluble compounds with low molecular weights available for anaerobic digestion are less abundant (Taherzadeh and Karimi, 2008). Economical and effective pretreatments are thus often needed to enable bacteria to degrade these materials.

Thermal, ultrasonic, chemical, and biological pretreatment can decompose celluloses and hemicelluloses into relatively readily biodegradable components (Laureano-Perez et al., 2005; Dewil et al., 2006; Fernández-Cegrí et al., 2012). Thermal pretreatment can improve biodegradability but requires a substantial amount of energy, and ultrasonic and biological pretreatments are very expensive (Lin et al., 2009). Previous studies have shown that chemical pretreatment is the preferred method for improving the biodegradation of lignocellulosic material (Pang et al., 2008; Guo et al., 2011). Of the chemical pretreatments, hydrogen peroxide (H_2O_2) is commonly used for pretreating agricultural residues because of its strong oxidizing properties. This method effectively digests lignocellulosic biomass, including paper-tube residuals (Teghammar et al., 2010) and various agricultural straws (Song et al., 2012). Most chemical pretreatments, however, presently soak substrates in large volumes of chemical solutions and water, which requires the recycling of chemicals, disposal of waste

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solutions, and sometimes high temperatures and thus requires high investments in facilities, high treatment costs, and potential environmental pollution. Pang et al. (2008) developed a "solid state" pretreatment to improve the biodegradability of corn stover, which used a limited amount of water and produced no waste chemical solutions. Solid-state pretreatment, however, requires long treatment times (three weeks) and has a low pretreatment efficiency. Wet-state pretreatment, in contrast, allows the straw to absorb water thoroughly and can maintain a complete saturating state without losing extra water, which avoids the generation of waste chemical solutions, shortens the pretreatment time, and enhances pretreatment efficiency (Zheng et al., 2009). Pretreatment studies for improving the biodegradability of lignocellulosic biomass have mainly focused on the digestion of single raw materials. The low pHs, suboptimal carbon/nitrogen (C/N) ratios, and poor buffering capacities of single lignocellulosic substrates greatly hinder their digestibility (Zeshan et al., 2012). The codigestion of mixed substrates for biogas production has consequently attracted interest because of its better C and nutrient balance during AD (El-Mashad, 2013).

Codigestion is defined as the digestion of mixtures of at least two waste materials for improving AD efficiency. Many successful codigestions of substrates have increased methane potential substantially compared to the separate digestion of the substrates (Teghammar et al., 2013; González-Fernández et al., 2011). For example, Zhou et al. (2012) reported that the codigestion of corn cover with cow manure increased biogas production by 29.1% relative to the digestion of corn cover alone. Xie et al. (2011) suggested that applying pig manure to grass silage at a ratio of 1:1 would produce a high specific methane yield with a short lag phase. These studies, however, have mostly focused on the technology, such as the effects of operational parameters on the biogas yield and the optimization of the substrate proportions to increase the digestion efficiency. Little information is available for the performance of microbial communities during the codigestion. The characterization of the composition of the microbial community is important for assessing and enhancing digestion efficiency, because the stability and efficiency of AD largely depends on the identity of the active microorganisms (Cho et al., 2013).

The present study evaluated the codigestion performance of H_2O_2-treated wheat straw (WS) with dairy cattle manure (CM) and determined the optimal proportion of the H_2O_2-treated WS and the CM for efficient methane production. The microbial community was also analyzed using the Illumina MiSeq platform, a high-throughput metagenomic sequencing method based on sequencing-by-synthesis technology, to investigate the microbial dynamics of the codigestion.

2. Methods

2.1. Raw material

Wheat straw (WS) was collected from local villagers near Northwest A&F University (Yangling, Shaanxi, China). Prior to use, the straw samples were air dried and cut with a grinder into 20–30 mm segments. CM was obtained from a livestock farm in Yangling. Inoculum was taken from the anaerobic digester treatingcattle manure in a local biogas demonstration village in Yangling, China. The substrates and inoculum were individually homogenized for further use. The chemical characteristics of the substrate and the sludge are shown in Table 1.

2.2. Pretreatment

H_2O_2, purchased from Sinopharm Chemical Reagent Co. Ltd, Beijing, China, was used as the pretreatment reagent. The H_2O_2 was mixed with distilled water to obtain concentrations of 1%, 2%, 3%, and 4% (w/w). Moisture contents of 70%-85% of the ground WS were tested before the pretreatments. The moisture content was calculated as:

$$\text{Mositure content } (\%) = 1 - \frac{\text{dry weight of straw}}{\text{dry weight of straw} + \text{water added}} \times 100\%$$

Preliminary tests indicated that a moisture content of 75% allowed the dried WS to absorb water thoroughly and to maintain a complete saturating state without the loss of water, known as a wet-state pretreatment. This method used a limited amount of water and produced no waste chemicals. Dried corn straw (500 g) was thus soaked in 1.5 L of the prepared H_2O_2 solutions in beakers to produce straw samples with 75% moisture. All beakers were covered with plastic film secured with a plastic ring and were then stored in a chamber at an ambient temperature of 25 ± 2 °C for 7 days. WS soaked in distilled water and stored as above but without chemical pretreatment was used as the control. The straw samples were then removed from the beakers, dried in an electronic oven at 80 °C for 48 h, and refrigerated until compositional determination and the AD experiments to investigate the effect of pretreatment on methane yield. Each pretreatment was conducted in triplicate.

2.3. Digestion experiments

Methane production was determined in two sets of experiments. In the first set, untreated and four H_2O_2-treated (1%, 2%, 3%, and 4%) samples of WS were digested anaerobically in batch flasks to investigate the effect of the pretreatment on the biodegradability and digestibility of the WS. In the second set, the most effective H_2O_2-treated WS from the first set of experiments was codigested with CM at mixed dry-weight ratios of 100:0, 90:10, 80:20, 70:30, 60:40, 30:70, 40:60, 20:80, 10:90, and 0:100. Untreated WS was codigested with CM at the same ratios as controls to investigate the effects of the pretreatments on the performances of the codigestions. The amounts of the substrates in the codigestions are shown in Table 2.

For both sets of experiments, the digestion tests were conducted in batch anaerobic Erlenmeyer flasks. The volume of each flask was 1 L, with a working volume of 0.75 L. The inoculum (200 g) was added to each digester, followed by deionized water to obtain a total solid (TS) content of 8%. The solutions were stirred

Table 1
Chemical characterization of substrate used in the digestion experiments.

	pH value	TS (%)	VS (%)	TC (%)	TN (%)	C/N
Wheat straw	NA	95.2 ± 2.2	86.7 ± 1.8	37.9 ± 1.1	0.43 ± 0.03	88.1 ± 4.5
Cattle manure	6.89 ± 1.0	13.7 ± 1.4	66.2 ± 2.9	17.6 ± 1.4	1.06 ± 0.08	16.6 ± 1.1
Sludge	7.80 ± 0.8	4.86 ± 0.5	67.4 ± 2.4	NA	NA	NA

Value are expressed as the mean ± deviation (n = 3). TS, Total solid; VS, volatile solid; % dry matter, TC, total carbon, % dry matter; TN, total nitrogen, % dry matter; NA = not applicable.

Table 2
The amount of substrate for codigestion.

Straw:manure	H_2O_2-treated/untreated straw (g)	Cattle manure (g)
100:0	185.1	0
90:10	166.6	33.8
80:20	148.1	67.6
70:30	129.6	101.3
60:40	111.1	135.1
50:50	92.6	168.9
40:60	74.0	202.7
30:70	55.5	236.5
20:80	37.0	270.3
10:90	18.5	304.0
0:100	0	337.8

The mixed ratio was based on the dry matter.

and placed in a thermostatic water bath under mesophilic conditions of 37 ± 1 °C for 35 days. All flasks were tightly sealed with rubber septa and screw caps and were gently mixed manually for approximately 1 min day^{-1} prior to the measurement of biogas volume to ensure mixing of the flask contents. The digestions with each pretreatment were performed in triplicate.

2.4. Analysis and calculations

The amount of biogas produced from each digester, determined by water displacement, was recorded daily. The methane content of the biogas was analyzed by gas chromatography (7890A, Agilent, USA). The total solid (TS) and volatile solid (VS) contents and the pH were determined using standard methods (APHA, 1998). The total C and N contents were determined by a total carbon analyzer (Liqui TOCII, Elementar, Germany) with an auto sampler. The C/N ratio was determined by dividing the total C content by the total N content. The volatile fatty acid (VFA) was analyzed using a colorimetric method (Chinese Academy of Sciences, 1984) and was expressed in terms of acetic acid content. The soluble fraction and cellulose, hemicellulose, and lignin contents were analyzed as described by Van Soest and Wine (1967).

2.5. Microbial-community analysis by high-throughput sequencing

2.5.1. DNA extraction, 16S rRNA gene amplification, and sequencing

Half a gram of digestion sludge was collected from digesters for DNA extraction on days 1, 7, 14, 21, 27, and 35. Total DNA was extracted as described by Rademacher et al. (2012). A fragment of the 16S rRNA gene, including the variable V4-V5 region, was amplified by PCR from the DNA using primers 515F (5′-GTGYCA GCMGCCGCGGTA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′) (Tamaki et al., 2011). Barcode sequences were attached to both primers as unique tags for sample identification. Two PCR reactions were conducted for each sample, and the PCR products in the replicate reactions were pooled. The details of the PCR reactions have been described by Li et al. (2013). The amplicons from each sample were pooled at equimolar concentrations and were sequenced with the Illumina MiSeq platform (Illumina Company, USA).

2.5.2. Processing of sequencing data

The raw sequences data were classified based on sample-specific barcode tags, and primer and tag sequences were trimmed from the sorted sequences. Raw sequences were denoised and processed using the QIIME pipeline (Caporaso et al., 2010). First, the ambiguous chimeric and short sequences with a length less than 250 nucleotides were removed using the UCHIME algorithm (Edgar et al., 2011). The number of sequences differed among the samples, so we randomly resampled the sequences to 5600 reads per sample for further analysis. Second, the sequences were then clustered by complete linkage clustering in the QIIME pipeline. The qualified sequences were clustered into operational taxonomic units (OTUs) using a cutoff of 97% identity of the 16S rRNA gene sequence for statistical analysis. Third, the Chao1 estimator and Shannon diversity index were calculated at 97% sequence identity in the Ribosomal Database Project (RDP) pipeline (http://pyro.cme.msu.edu/). The phylogenetic affiliation of each sequence was analyzed with the RDP classifier at a confidence level of 80%. To ensure the accuracy of the RDP classifier results, the representative sequences of dominant bacteria and archaea were subjected to BLAST homology searches against non-environmental sequences and non-metagenomes in the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov).

2.6. Data analysis

Statistical significance between means was tested by a one-way analysis of variance (ANOVA). Duncan's multiple range tests at a level of 5% were used to compare the means. All statistical analyses were performed using the SPSS 15.0 software package. Principal coordinates analysis (PCoA) conducted by CANOCO Software (Biometris, Netherlands) was used to evaluate the differences of microbial-community structures.

3. Results and discussion

3.1. Effect of pretreatment on the chemical composition and methane yield of WS

3.1.1. Chemical composition

Chemical pretreatment changes the complex physical and chemical roles of the components of lignocellulosic biomass. The pretreatment decompose celluloses and hemicelluloses into relatively readily biodegradable components, while, breaking the linkages between polysaccharides and lignins to make celluloses and hemicelluloses more accessible to bacteria. These changes contribute to the improvement of biodegradability and biogasification (Li et al., 2009). In our study, H_2O_2 pretreatment significantly decreased the total lignocellulosic composition relative to untreated WS (Table 3). Some of the lignocellulose was decomposed and converted to other components. The decomposition rates, however, differed; hemicellulose, cellulose, and lignin contents decreased by 12.5–45.2%, 9.3–30.2%, and 5.4–21.9%, respectively. More hemicellulose than lignin or cellulose was decomposed, perhaps because hemicellulose reacted more with H_2O_2. The soluble fraction increased by 30.5–77.3% after pretreatment, likely mainly from the decomposition of hemicellulose and partially from the decomposition of lignin and cellulose. The soluble fraction contained simpler chemical structures with lower molecular weights, which could be more readily biodegraded than lignin, cellulose, and hemicelluloses that have more complex chemical structures and higher molecular weights. The increased soluble fraction would contribute to the biodegradability and increase of methane yield of the WS. Furthermore, the hemicellulose, cellulose, and lignin contents were significantly lower and the soluble fraction was higher with the pretreatments with 3% and 4% H_2O_2 than with the pretreatments with 1% and 2% H_2O_2, suggesting that the 3 and 4% pretreatments were more effective in the biodegradation of the lignocellulosic structure. Pretreatment with H_2O_2 decreased C content from 37.9% to 31.5%, and the N content remained unchanged, decreasing the C/N ratio from 88:1 to 78.7.

3.1.2. Methane yield

H_2O_2-treated WS significantly improved the methane yield compared to untreated WS ($P < 0.05$) (Table 3). The methane yields

Table 3
Effect of pretreatment on the chemical composition (% dry matter) of wheat straw and methane yield.

H_2O_2	Cellulose	Hemicellulose	Lignin	Soluble faction	Total C	C/N	Methane yield (mL CH_4 g VS^{-1})
1%	43.3 ± 1.6 b	25.7 ± 1.5 b	6.9 ± 0.2 a	21.4 ± 1.5 c	34.4 ± 2.6 ab	86.6 ± 1.9 a	94.8 ± 8.4 c
2%	38.4 ± 2.4 c	20.8 ± 2.5 c	6.8 ± 0.3 a	24.7 ± 1.3 b	32.8 ± 0.8 b	84.9 ± 2.0 a	108.5 ± 10.9 bc
3%	34.3 ± 1.1 d	16.1 ± 1.4 d	6.0 ± 0.4 b	28.6 ± 1.2 a	31.5 ± 1.1 b	78.7 ± 2.4 b	128.4 ± 6.2 a
4%	33.9 ± 3.1 d	16.8 ± 2.3 d	5.7 ± 0.3 b	28.0 ± 2.0 a	32.1 ± 1.2 b	80.8 ± 2.2 b	118.7 ± 9.8 ab
Untreated	48.6 ± 3.7 a	29.4 ± 2.1 a	7.3 ± 0.3 a	17.9 ± 1.1 d	37.9 ± 1.1 a	88.1 ± 3.0 a	84.3 ± 5.1 d

Value are expressed as the mean ± deviation ($n = 3$). The One-way ANOVA was conducted to determine the differences among the pretreatments. Values with the same letters indicate no significant difference at $P < 0.05$.

were 94.8, 108.5, 128.4, and 118.7 mL g VS^{-1} for the 1%, 2%, 3%, and 4% H_2O_2-treated straw, respectively, representing increases of 12.5%, 28.7%, 50.3%, and 40.8%, respectively, over the untreated straw. Methane yield increased as lignocellulosic decomposition increased (Table 3), indicating that H_2O_2 pretreatment can significantly improve the biodegradability and biogasification of WS. The enhanced methane yield is likely associated with the increased soluble fraction that is available to anaerobic microorganisms. Methane yield increased significantly as the H_2O_2 concentration increased from 1% to 3% and 4% but did not differ significantly at 3% and 4%. Given that there was no significant difference between 3% and 4% on the biodegradation of lignocellulosic composition and methane yield, 3% H_2O_2 would be advisable as the pretreatment method before anaerobic digestion.

3.2. Performance of codigestion of H_2O_2-treated WS with CM

3.2.1. Methane yield

The codigestion of mixed substrates produced more methane than the digestion of WS alone. The codigestion of 3% H_2O_2-treated WS with CM at different mixture ratios was conducted to investigate the effect of pretreatment on the performance of the codigestion. Methane yield was significantly higher in the codigestion of H_2O_2-treated WS with CM than in untreated WS and CM at the various mixture ratios ($P < 0.05$) (Fig. 1). Codigestion of untreated WS with CM initially increased the methane yield, which then declined as the mixture ratio decreased. A 40:60 ratio of H_2O_2-treated WS to CM produced the highest yield of methane, at 320.8 mL g VS^{-1}. A similar trend was observed in the codigestion of the untreated WS with CM, which had a highest methane yield of 254.6 mL g VS^{-1} at a ratio of 30:70. These data indicated that codigestion of H_2O_2-treated WS with CM improved methane yield more effectively than the codigestion of untreated WS with CM. The higher digestibility of pretreated WS was due to the higher soluble component from the degradation of hemicellulose, cellulose, and lignin that became available to anaerobic bacteria.

The different optimal mixture ratios between treated and untreated WS are likely related to the C/N balance. N needs to be added during the anaerobic fermentation of the WS to adjust the C/N ratio, because straw has a low N content. In contrast, C needs to be added during the anaerobic fermentation of the CM to adjust the C/N ratio, because manure has a low C content (Zhou et al., 2012). The mixture of C-rich WS and N-rich CM as raw materials for fermentation balanced the C/N ratio, but pretreatment changed this balance and reduced the C content of the straw (Table 3), thereby decreasing the C/N ratio. More straw was thus needed during the codigestion of pretreated straw with manure to restore the optimal C/N ratio (between 20 and 30). Codigestion also produced more methane than the digestion of pretreated WS alone (100:0) and CM alone (0:100), indicating a synergistic effect of the codigestion.

3.2.2. C/N ratio

The C/N ratio represents the relationship between the amounts of C and N in the feedstock. Higher C/N ratios indicate rapid N consumption by methanogens, which leads to lower methane yields. Low C/N ratios imply the accumulation of ammonia and increased pH, which are toxic to methanogenic bacteria (Verma et al., 2002). A suitable C/N ratio is thus important for efficient anaerobic digestion. In the present study, the C/N ratios of the feedstock at different mixing ratio of WS and CM tended to decrease as the mixture ratios decreased for both codigestion treatments (Fig. 2), which may have be due to the lower C fraction caused by the lower amounts of straw and the increase in N content from the higher amounts of manure. The C/N ratios during the codigestion of untreated WS with CM ranged from 77.2 to 17.9, whereas the ratios for the codigestion of treated WS with CM ranged from

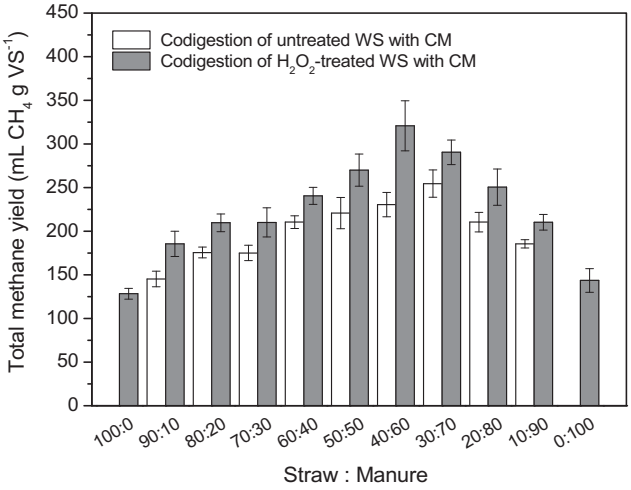

Fig. 1. Methane yield of two codigestions at different mixed ratio.

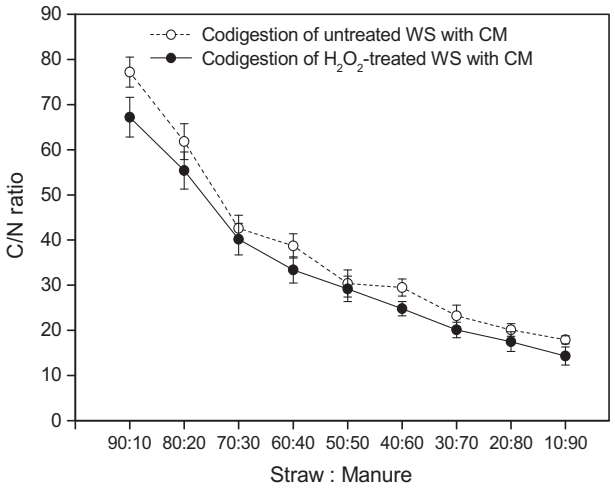

Fig. 2. C/N ratio of two codigestions at different mixed ratio.

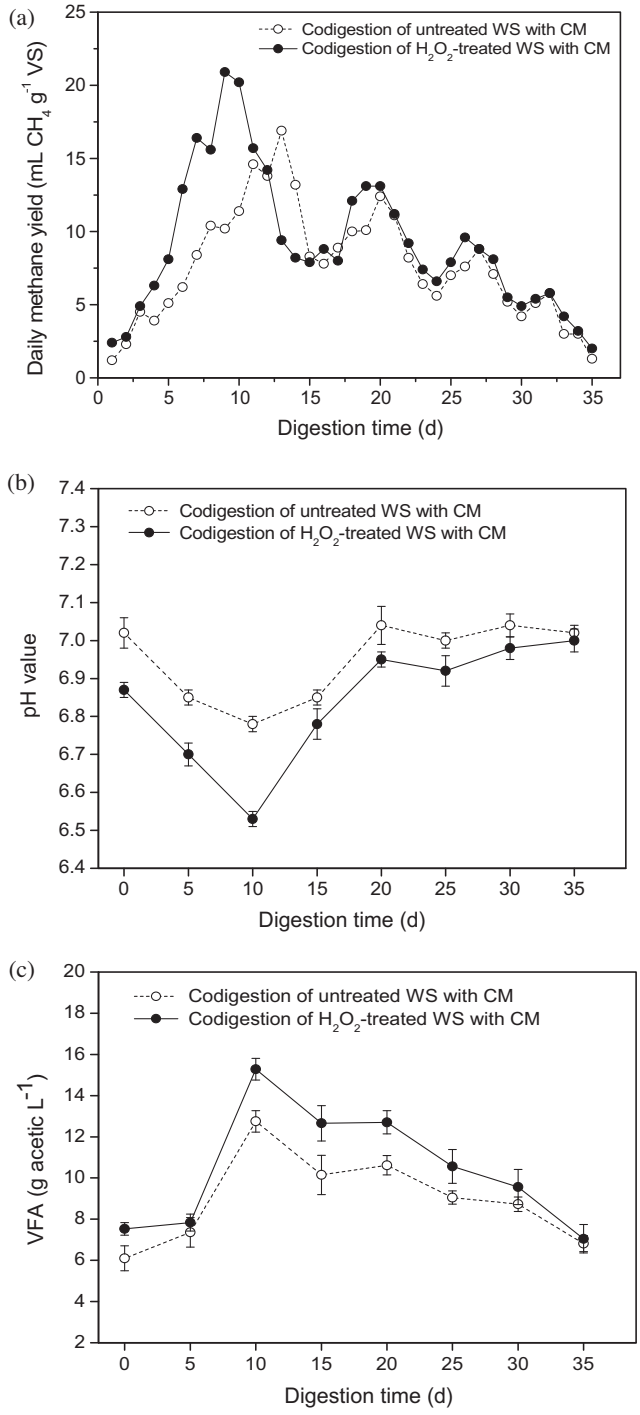

Fig. 3. Daily methane yield, pH values, and VFA content of two codigestions.

67.2 to 14.3. C/N ratios were significantly lower in the codigestion of the H_2O_2-treated WS with CM than in the codigestion of untreated WS with CM, indicating that pretreatment effectively reduced the C/N ratios of the anaerobic codigestion. Estevez et al. (2012) suggested that suitable C/N ratios ranged from 20 to 30. Similarly, the C/N ratios in our study were 24.8 and 23.2 for the highest methane yields from the codigestions of H_2O_2-treated WS with CM and of untreated WS with CM, respectively. The C/N ratios of the H_2O_2-treated WS codigested with CM, however, were lower than those with the untreated WS codigested with CM, because pretreatment of the WS degraded more of the C fraction, thereby decreasing the C/N ratio.

Table 4
Microbial diversity indices based on 97% identity of 16S rRNA gene sequences and 5325 reads per sample.

Sample	Chao1 estimator of richness	Observed species	Shannon diversity index
Day 1	673.35	483	6.11
Day 7	847.92	683	7.86
Day 14	736.38	542	7.38
Day 21	815.37	617	7.51
Day 28	662.23	552	6.24
Day 35	611.56	491	5.97

3.2.3. Daily methane yield, pH, and VFA content

The daily methane yields, pH, and VFA contents were analyzed for the codigestions. The codigestion of H_2O_2-treated WS with CM produced the highest methane yield and VS removal at a mixture ratio of 40:60, while the codigestion of untreated WS with CM produced the highest methane yield and VS removal at a mixture ratio of 30:70. These ratios were thus selected for the digestion analyses.

The daily methane yield is shown in Fig. 3a. The daily methane yields of the two codigestion treatments exhibited similar trends, initially increasing and subsequently decreasing. This phenomenon was related to the dynamics of the methanogen populations during the digestion, discussed below in Section 3.3. The peak methane yields and the times required to reach the peaks differed for the two codigestions. The methane yield of the codigestion of H_2O_2-treated WS with CM peaked (20.9 mL g VS^{-1}) on day 9, whereas the yield of the codigestion of the untreated WS with CM peaked (16.7 mL g VS^{-1}) on day 13. The earlier and higher peaks of the pretreated codigestion indicated that the pretreatment improved the digestibility of the WS and CM, thereby facilitating their consumption by hydrolytic bacteria and shortening digestion times (Li et al., 2009).

The pH and VFA are important indicators of anaerobic digestion because their dynamics reflect the changing conditions. As shown in Fig. 3b, the pH of the fermentation broths of both codigestion treatments remained <7.0 during the first 10 days, subsequently increased until day 14, and then remained stable until the end of the digestions. The initial decrease in pH may have been associated with the variation in VFA concentration, because the VFAs produced during AD reduce the pH. The highly concentrated acids in the manure led to a noticeable drop in pH. The pH decreased dramatically during the initial stage of the codigestion of H_2O_2-treated WS with CM, likely due to the production of various organic acids from the degradation of cellulose, hemicellulose, and lignin after pretreatment (Guo et al., 2011). Methanogens are active at pH between 6.2 and 8, with an optimal range of 7.0–7.2 (Poliafico et al., 2007). The pH of the two codigestion treatments at the optimal mixture ratios in our study ranged from 6.9 to 7.2 within 20 days. The codigestion of the pretreated WS with CM, however, produced a lower pH than the codigestion of the untreated WS with CM due to the release of various acids from the lignocellulose.

VFAs are intermediate organic acid products, and the total VFA concentration is an important indicator of metabolic status (aside from influencing the pH) during AD (Fernández et al., 2005). In contrast to the pH, the VFA contents of the two codigestion treatments increased during the first 10 days and subsequently decreased (Fig. 3c). The VFA content increased more sharply in the codigestion of the H_2O_2-treated WS with CM than in the codigestion of the untreated WS with CM, perhaps due to the higher organic acid content released by the hydrolysis of the hemicelluloses of the pretreated straw (Guo et al., 2011). Song et al. (2012) reported that chemical pretreatment of rice straw significantly improved the total VFA content because of the biodegradation of lignocellulosic structures. We observed a similar

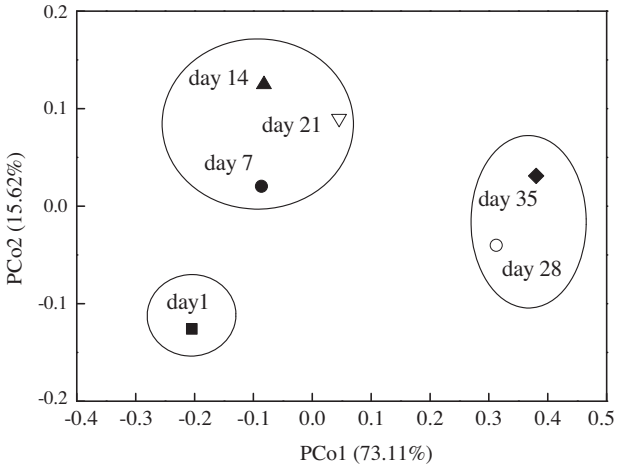

Fig. 4. The principal coordinates analysis (PCoA) of the microbial communities at genus level.

phenomenon: the average VFA concentration was higher during the codigestion with pretreated straw (10.1 g acetic acid L^{-1}) than during the codigestion with untreated straw (8.7 g acetic acid L^{-1}). VFA accumulation decreased the pH, thereby affecting the growth of methanogens during AD. The change in VFA content was in accordance with the daily methane yield (Fig. 3a), indicating a close relationship between VFA concentration and methane yield.

3.3. Sequence analysis of the microbial communities

DNA for the amplification and subsequent sequencing of the 16S rRNA gene was extracted from the sludge samples of the codigestion of H_2O_2-treated WS with CM at the mixture ratio of 40:60 to investigate the microbial performance of the codigestion. The sludge was collected on days 1, 7, 14, 21, 28 and 35. A total of 15 290 high-quality sequences were acquired and clustered to calculate the OTUs at a cutoff of 97% sequence identity. The Chao1estimator of richness, observed species, and Shannon diversity index of the microbial communities were higher on days 7, 14, and 21 than on days 1, 28, and 35, with the highest values on day 7

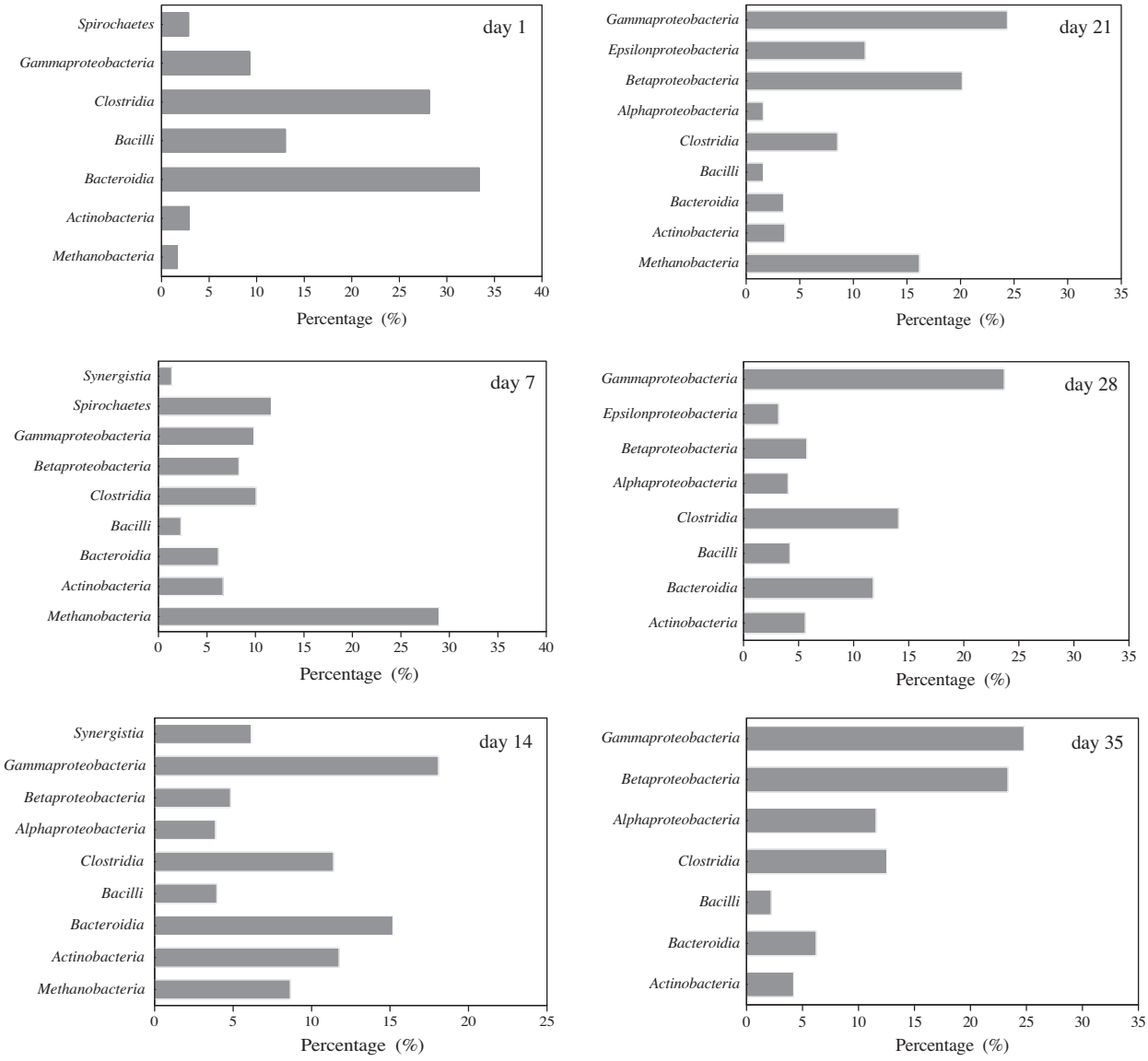

Fig. 5. Microbial community structure at class level in the codigestion.

(Table 4). The microbial community was more complex during the growing period than during the initiation and stabilization periods. The differences in the microbial communities between the stages of digestion were also supported by the PCoA (Fig. 4). The compositions of the microbial communities of the six samples could be clearly classified into three groups. The samples from days 7, 14, and 21 grouped together, the samples from days 28 and 35 grouped together, and the sample from day 1 was separate.

Microbial community composition varied during the digestion (Fig. 5). The community mainly consisted of six kinds of microbes, *Methanobacteria*, *Bacteroidia*, *Clostridia*, *Betaproteobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria*. As important methanogens closely related to methane production, *Methanobacteria* increased continuously to 28.7% by day 7, decreased slightly, and then increased over the following days. *Methanobacteria* composed 16.6% of the community on day 21 and decreased thereafter. This variation was in accordance with the change of methane production (Fig. 3a). The microbes that function in AD can be mainly classified as hydrolytic, fermentative, acetogenic, and methanogenic communities (Veeken and Hamelers, 1999). Complex molecules are transformed into simpler products by extracellular enzymes during the hydrolysis stage, so cellulolytic bacteria such as *Clostridium*, *Bacillus*, and *Bacteroides* (Lo et al., 2009) were common on day 1 of our study. Fermentative bacteria can adapt more quickly to new conditions due to their relatively high growth rates, while methanogens grow much slower. Carbon dioxide, hydrogen, and acetic acid were the main products of the fermentative and acetogenic bacteria. Because the metabolic capacity of methanogens was initially not sufficient to balance the increasing activity of the acetogenic bacteria, acetate and hydrogen were not consumed at the same rate at which they were produced, as indicated by the low methane concentration of the biogas on day 1. Methanogens increased greatly as the digestion proceeded and consumed acetate, carbon dioxide, and hydrogen to produce methane. The methanogens and methane yield gradually decreased, however, because the substrate concentration decreased with the consumption by the microbes.

The composition of the methanogens at genus level was further investigated to evaluate microbial performance (Fig. 6). Six genera of methanogens, *Methanosarcina*, *Methanosaeta*, *Methanoculleus*, *Methanosphaera*, *Methanobrevibacter*, and *Methanobacterium*, were identified in the system. Hydrogenotrophic methanogens are most common in the digestion of agricultural waste (Jaenicke et al., 2011). Acetoclastic methanogenesis may be a major pathway in certain systems, or both pathways were utilized for methanogenesis (Li et al., 2013). In the present study, acetoclastic methanogens, *Methanosarcina*, *Methanosaeta* and *Methanobrevibacter* accounted for 41.1%, 8.2%, and 5.3%, respectively, of the populations, and hydrogenotrophic methanogens, *Methanoculleus*, *Methanosphaera*, and *Methanobacterium* accounted for averages of 21.2%, 16.9%, and 7.3%, respectively, suggesting two methanogenetic pathways co-occurring for methane production during the codigestion of pretreated WS with CM. Cho et al. (2013) reported that hydrogenotrophic methanogens, *Methanosphaera*, were most abundant (400 sequences, 57.1% of total archaea) in the initial seed sludge of anaerobic digester sludge and that fewer clones affiliated with *Methanosarcina* were detected. In contrast, we found few *Methanosphaera* but abundant *Methanosarcina*. This discrepancy could be explained by the different microbial reactions to the AD substrates because the feedstock used by Cho was the food waste under the conditions of dry digestion and the digestion feedstock used in our study was the mixture of WS and CM. Furthermore, the methanogenic community was overrepresented by the *Methanosarcina* during the first 14 days, but which decreased gradually as digestion proceeded, whereas *Methanosphaera* and *Methanoculleus* gradually increased and were abundant from days 14 to 35. This shift indicated that the acetoclastic methanogens during the initiation and growth periods were displaced by hydrogenotrophic methanogens during the subsequent stabilization period. This result was consistent with founding by Yan et al. (2015) who reported that the acetoclastic methanogens in the early stage of the reactor start-up were replaced by hydrogenotrophic methanogens at the stabilization stage in the solid-state anaerobic digestion from rice straw. The decreased concentrations of VFA (especially acetate) caused by the digestion system seemed to create favorable evolutionary conditions for hydrogenotrophic methanogens, *Methanoculleus* and *Methanosphaera* to be dominant.

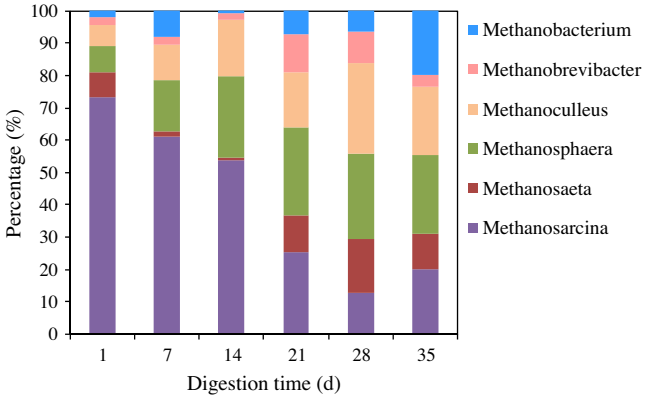

Fig. 6. Composition of methanogens at genus level in the codigestion.

4. Conclusions

Wet-state H_2O_2 pretreatment effectively improved the biodegradability and methane yield of WS. H_2O_2 at 3% was optimal for treating WS. The methane yield was significantly higher in the codigestion of CM and H_2O_2-treated WS than in the codigestion of CM and untreated WS and higher than in the digestion of H_2O_2-treated WS alone and CM alone. H_2O_2-treated WS and CM mixed at a ratio of 40:60 produced the highest methane yield of 320.8 mL g VS^{-1}. The methanogenic community shifted during the codigestion from the acetoclastic methanogens, *Methanosarcina*, to the hydrogenotrophic methanogens, *Methanosphaera* and *Methanoculleus*.

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