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Evaluation of Biogas Production Performance and Archaeal Microbial Dynamics of Corn Straw during Anaerobic Co-Digestion with Cattle Manure Liquid

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The rational utilization of crop straw as a raw material for natural gas production is of economic significance. In order to increase the efficiency of biogas production from agricultural straw, seasonal restrictions must be overcome. Therefore, the potential for biogas production via anaerobic straw digestion was assessed by exposing fresh, silage, and dry yellow corn straw to cow dung liquid extract as a nitrogen source. The characteristics of anaerobic corn straw digestion were comprehensively evaluated by measuring the pH, gas production, chemical oxygen demand, methane production, and volatile fatty acid content, as well as applying a modified Gompertz model and high-throughput sequencing technology to the resident microbial community. The efficiency of biogas production from fresh straw (433.8 ml/g) was higher than that of production from straw silage and dry yellow straw (46.55 ml/g and 68.75 ml/g, respectively). The cumulative biogas production from fresh straw, silage straw, and dry yellow straw was 365 l^{-1} g^{-1} VS, 322 l^{-1} g^{-1} VS, and 304 l^{-1} g^{-1} VS, respectively, whereas cumulative methane production was 1,426.33%, 1,351.35%, and 1,286.14%, respectively, and potential biogas production was 470.06 ml^{-1} g^{-1} VS, 461.73 ml^{-1} g^{-1} VS, and 451.76 ml^{-1} g^{-1} VS, respectively. Microbial community analysis showed that the corn straw was mainly metabolized by acetate-utilizing methanogens, with *Methanosaeta* as the dominant archaeal community. These findings provide important guidance to the biogas industry and farmers with respect to rational and efficient utilization of crop straw resources as material for biogas production.

Keywords: Anaerobic fermentation, biogas, corn straw, cow dung, methane, *Methanosaeta*

Introduction

Because of its significant agricultural production, China is rich in crop straw resources. In 2002, the annual output of crop stalks in China was 6.4 million tons, of which 3.7 million tons consisted of straw with the potential to be used for energy production; however, only 33% of this potential energy resources was utilized [8]. In Jilin Province alone, 0.6 tons of straw is wasted each year, most of which was discarded or burned, leading to serious environmental pollution, in addition to wasted economic potential [27]. Therefore, processes supporting comprehensive utilization of straw resources are an important research focus, because such methods will facilitate sustainable agricultural development and protection of the environment.

Anaerobic fermentation technology is an effective means of converting agricultural waste into clean energy [7]. Corn (*Zea mays*) is the primary crop utilized for biogas production

and is considered to have the highest yield potential of field crops grown in central Europe [16]. Straw resources vary with region, season, and year; therefore, effective and flexible storage methods are necessary to provide consistent raw material of high quality for biogas production.

A significant body of research exists on utilization of corn stalk resources. In a study of the methane production characteristics of corn straw silage, Herrmann *et al.* [9] found that silage increased the yield of methane from corn straw by 11%. Moreover, co-digestion of corn stover with manure increased biogas production by 29.1% relative to that of corn stover alone [31]. Ren *et al.* [19] demonstrated that the addition of cow dung was beneficial for anaerobic straw fermentation and found that silage straw produced more gas than dry yellow straw. Gao *et al.* [6] studied the methane production potential of corn silage at nine different harvest stages and found that mature corn straw silage produced the most methane. Amon *et al.* [1] found that the optimum time for corn silage is after suitable wax maturity and demonstrated that maximum biogas yield can be obtained with maize of maturity class FAO 500. The characterization of the composition of the microbial community is important for assessing and enhancing digestion efficiency, because the stability and efficiency of anaerobic digestion largely depends on the identity of the active microorganisms [2, 23]. Previous experiments showed that acetate could mainly be degraded by aceticlastic methanogenic archaea during anaerobic corn straw digestion [18].

The methane characteristics of silage corn straw and dry yellow corn straw have been compared, but few reports have compared the biogas production potential of silage corn straw, dry yellow corn straw, and fresh corn straw. Therefore, we assessed the biogas production potential of silage corn straw, dry yellow corn straw, and fresh corn straw by measuring the gas production, methane production, chemical oxygen demand (COD), pH, and volatile fatty acid (VFA) content, as well as by assessing the microbial community dynamics during biogas production through high-throughput sequencing using the Illumina Miseq platform, a sequencing method based on sequencing-by-synthesis. The goal of this study is to provide a theoretical basis for sustainable and economical utilization of corn stalk resources in a manner that reduces environmental pollution.

Materials and Methods

Materials

Fresh corn (whole plant) was cultivated at the Yanbian University Experiment Station in Jilin, China. The chosen corn varieties were obtained from the National Maize Improvement Center of China and were suitable for large-scale cultivation. The selected varieties were characterized by high yield and heavy grain ND13.

Harvest and Silage

Whole plant corn was harvested. During sampling, plants were chopped about 5 cm above the ground. The fresh samples were immediately broken into pieces that were made as small as possible and frozen at –20°C. Silage was prepared in pits (50 cm × 20 cm × 80 cm) lined with two layers of waterproof polyethylene plastic film. Silage packets were tightly wrapped in polyethylene plastic film to protect them from water and soil, placed in the silage pits, covered with soil, and labeled. The silage period was 180 days. Dry yellow corn, a natural straw, was cut into pieces approximately 3 cm in length for further use.

Fermentation Conditions

Biogas production and quality were analyzed using anaerobic digestion tests. Tests were performed in three CSTR anaerobic fermentation devices. The volume of each anaerobic fermentation device was 7 L, with a working volume of 6 L (Fig. 1). The solid content of the reactors was 6% total solids (TS). The organic load was 3 g TS l^{-1} d^{-1}. The reactors were incubated at 30°C in the fermentation devices for 50 days. The contents of each fermentation device were mixed twice every day (5 min each at 200 rpm). The activated sludge used to initiate the fermentation was dung from domesticated cows.

Biogas and Methane

Gas determination was performed by the consumption-by-drainage method [27]. The methane content was determined by a Biogas-5000 gas analyzer (British Geotech Co., England).

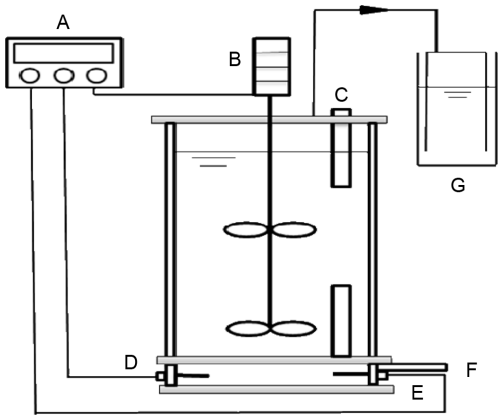

Fig. 1. Schematic diagram of the CSTR.
A: automatic control system; B: mixing system; C: feed port; D: temperature probe; E: heating rod; F: discharging port; G, gas collection.

pH and Chemical Oxygen Demand

The pH values of the influent and effluent were monitored daily with a pH meter (Shanghai Leici PHS-3C, China). The COD was tested by the potassium dichromate method [29]. COD was measured using a Rex COD-571 (Shanghai Leici, China) tester.

HPLC Analyses of Organic Acid Products

The high-performance liquid chromatography (HPLC) conditions were as follows: Hitachi La Chrom C18-AQ (5 μm) chromatographic column; column temperature, 25°C; mobile phase, 1 mmol/l H_2SO_4 and 8 mmol/l Na_2SO_4; mobile phase flow rate, 0.6 ml/min; sample volume, 10 μl; and acquisition time, 60 min. The samples were centrifuged for 10 min at 12,000 rpm in 2 ml centrifuge tubes, after which the supernatant was subjected to membrane filtration and used for the determination. Standard curves for formic acid, acetic acid, propionate acid, butyrate acid, and lactic acid were generated. Finally, a quantitative analysis of the sample was performed [27].

Straw Anaerobic Digestion Performance Evaluation

A modified Gompertz equation was used to fit the biogas and methane accumulation curves of the different treatments [12].

$$B = B_0 \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{B_0}(\lambda - t) + 1\right]\right\}$$

where B is the cumulative methane yield ($ml^{-1}\ g^{-1}\ VS_{added}$), B_0 is the ultimate methane yield ($ml^{-1}\ g^{-1}\ VS_{added}$), R_m is the maximum methane production rate ($ml^{-1}\ g^{-1}\ VS_{added}\ d^{-1}$), λ is the lag phase time (day), t is the digestion time (day), and e is a mathematical constant (2.718). R_m, B_0, and e were used to measure the gas production performance of the raw materials.

Microbiological Analysis

Digestion sludge (0.5 g) was collected from the digesters for DNA extraction on days 0 and 50. Total DNA was extracted as described previously [30]. A fragment of the 16S rRNA gene, including the variable V4-V5 region, was amplified by PCR from DNA using primers 349F (5′- GYGCASCAGKCGMGAAW-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) [4]. Barcode sequences were attached to both primers as unique tags for sample identification. PCRs were conducted for each sample. The PCR products of the replicate reactions were pooled. The details of the PCRs have been reported by Li *et al*. [11]. The amplicons from each sample were pooled at equimolar concentrations and were sequenced using the Illumina MiSeq platform (Illumina Co., USA).

Processing of Sequencing Data

The raw sequence data were classified based on sample-specific barcode tags, after which primary and tag sequences were trimmed from the sorted sequences. Raw sequences were processed utilizing the QIIME pipeline [15] First, ambiguous chimeric and short sequences shorter than 250 nucleotides were removed using the UCHIME algorithm. Because the number of sequences differed among the samples, 10,000 random reads per sample were used for further analysis. Second, the sequences were 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 with the 16S rRNA gene sequence for statistical analysis. Third, the Chao1 estimator and Shannon diversity index were computed according to 97% sequence identity in the Ribosomal Database Project 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 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).

Data Analysis

Statistical analyses were performed using the SPSS 17.0 software package, except for principal component analysis (PCA), which was conducted using CANOCO Software (Biometris, The Netherlands) to evaluate differences in microbial community structure.

Results

pH Changes in Corn Straw Stored Using Three Different Methods

The optimal pH range for anaerobic fermentation is between 6.0 and 8.0 [20]. When pH is too high or low, the normal physiological activities of microorganisms in the reactor are inhibited. Changes in the pH of corn straw stored using the three tested storage methods are shown in Fig. 2. The pH of fresh straw fluctuated during the first 7 days of storage. The lowest measured pH of the fresh straw occurred during the first 7 days of storage (pH = 6.1), after which the pH increased gradually as the reactor was operated, reaching a stable pH of around 6.5 after 40 days. The primary reason for the low early pH of the fresh straw was its reduced lignification, which rendered it susceptible to degradation by microorganisms during the early stage of storage, leading to organic acid accumulation and reducing pH [19]. The dry yellow straw showed little pH fluctuation, with a pH of 6.3 throughout the experiment. The relatively high degree of lignification of the dry yellow straw limited its microbial decomposition rate, which in turn limited acid production. The pH of silage straw fluctuated during the first 7 days of storage; the lowest pH was recorded after 7 days (pH = 6.28), after which it was stable (pH = 6.4). The ideal pH during the storage process was between 6.0 and 6.6, indicating that each of the storage methods produced a pH value within the acceptable range throughout the process.

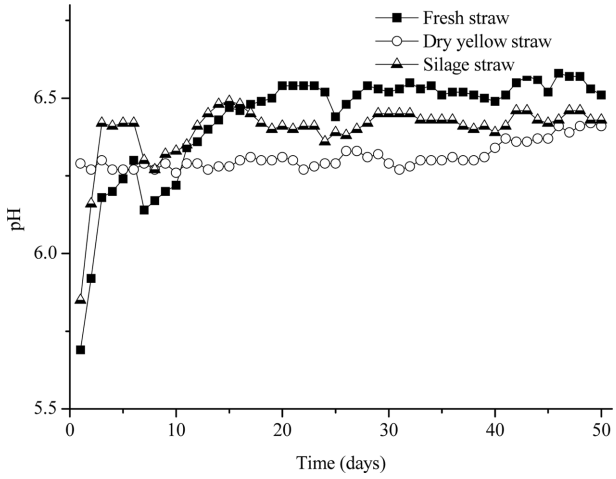

Fig. 2. Change in pH during the reaction process for three types of corn straw.

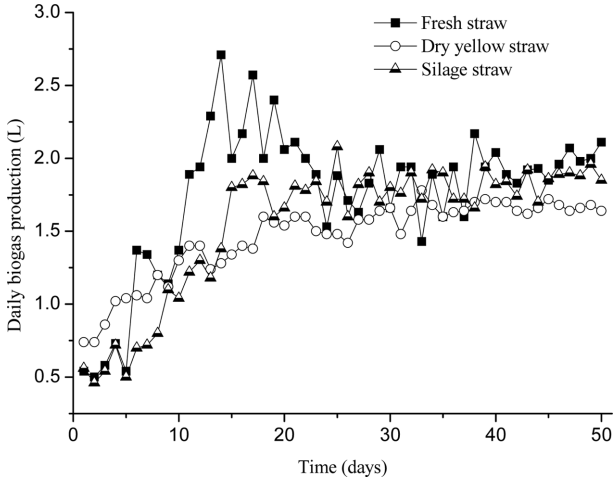

Fig. 3. Daily biogas production during the reaction process for three types of corn straw.

Daily Biogas Production and COD

Daily biogas production gradually increased during the operation of the reactor as anaerobic microorganisms adapted to the environment. The maximum daily biogas production rates of fresh straw, silage straw, and dry yellow straw were 2.71 L on day 14, 1.96 L on day 17, and 1.72 L on day 20, respectively. Daily biogas production from each type of straw stabilized at rates of 1.6–2.0 l/day after day 30. However, gas production was produced at the highest daily rate from the fresh straw, likely because the starch, crude protein, and other nutrients were susceptible to exploitation by microorganisms [16]. The organic acids produced by straw silage thus produce a competitive advantage for biogas producers by increasing total biogas production over the course of an entire year [12] .

The gas production rates per gram of material for the fresh straw, silage straw, and dry yellow straw were 433.8 ml/g, 387.25 ml/g, and 365.05 ml/g, respectively, which were higher rates than those reported in previous studies of corn straw biogas production [17, 22]. The practice of adding cow dung liquid to the reactor as a nitrogen source regulated the C/N ratio in the reactor and increased the diversity of the microbial communities present in the reactor, thus improving the microbial habitat and maximizing biogas production (Fig. 3).

The COD removal rate indicated that COD fluctuated during the initial stage (first 40 days) of storage; however, the COD removal rate was maintained at more than 70% throughout this stage. COD was stable after 45 days of operation, showing that the reactor was in stable operation. During reactor operation, the VFA content increased, indicating acceleration of microbial activity. The maximum COD removal rates for fresh straw, silage straw, and dry yellow straw were 90.43% on day 16, 85.95% on day 16, and 86.47% on day 21, respectively. Silage straw was better than fresh straw and dry yellow straw as a starting material for biogas production, because the nutrients of fresh straw are rapidly consumed, whereas the decomposition of remaining lignocellulose and metabolites in silage straw limits microbial consumption, leading to stable biogas production over an extended period (Fig. 4).

Methane Content

Methane production during reactor operation was primarily

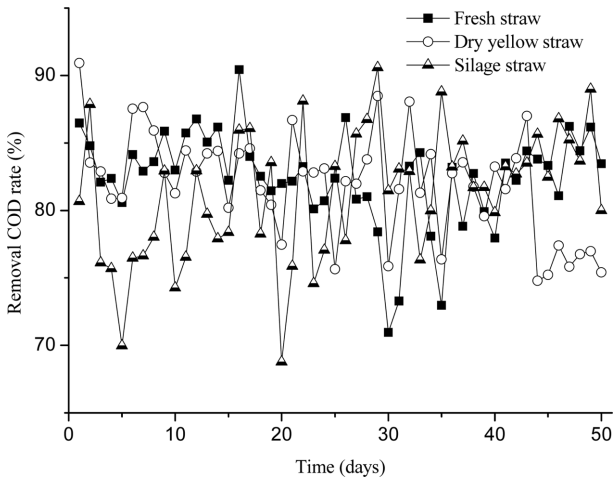

Fig. 4. COD removal rate during the reaction process for three types of corn straw.

attributed to the metabolic activity of methanogens present in the reactor. For fresh straw, the methane content was approximately 60%. For silage straw and dry yellow straw, the methane content was lowest after 9 days (52%) and 10 days (47%), respectively, after which it fluctuated until day 43 and was maintained at levels greater than 55% and 50%, respectively, for the remainder of the experiment. The methane content of the biogas produced from fresh straw was greater than that of gas produced from silage straw or dry yellow straw, in which the metabolic activity of methanogens was enhanced, whereas starch content, soluble sugar content, and soluble matter content were increased, while lignin content was reduced. In the process of silage, the utilization rate of nutrients is improved, because the process destroys the lignocellulose structure and improves the efficiency of acidification. The methane content of the biogas produced from the fresh straw, silage straw, and dry yellow straw was greater than 50% after day 40, showing that the reactor was stable (Fig. 5).

VFA Content

Changes in the VFA content of fresh straw, silage straw, and dry yellow straw measured during the experiment are shown in Fig. 6. The acetic acid content was highest on day 5 for fresh straw (11.95 g/l) and silage straw (2.61 g/l), indicating that these types of straw are more susceptible to digestion by microorganisms, which can produce a large amount of VFA and other intermediate products that change the pH and contribute to the growth of methanogens. Storage of dry yellow straw also led to VFA accumulation, but the change in pH was small, while the accumulated VFAs were completely exploited by the microorganisms after 20 days. The difference in VFA production from the

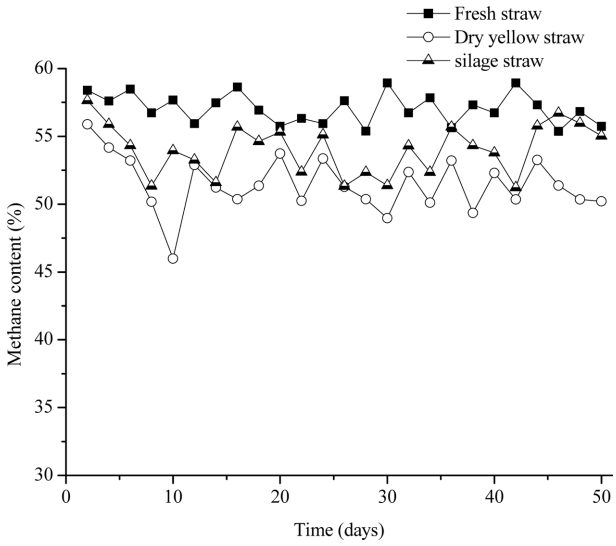

Fig. 5. Change in methane content during the reaction process for three types of corn straw.

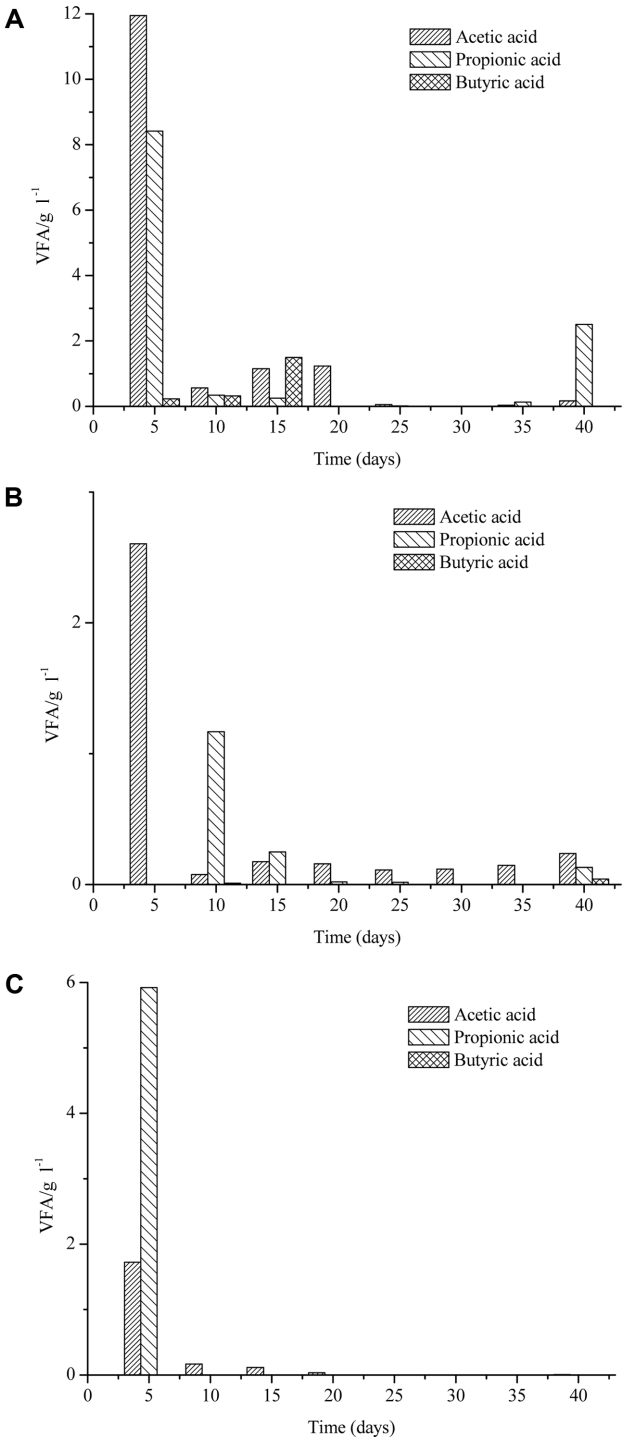

Fig. 6. Change in volatile fatty acid content during the reaction process for three types of corn straw.
(**A**) Silage straw, (**B**) fresh straw, (**C**) dry yellow straw.

Table 1. Characteristics of anaerobic corn straw digestion.

Samples	Cumulative biogas production				Cumulative methane production			
	B_0	μ	λ	R^2	B_0	μ	λ	R^2
Fresh straw	470.06	9.17	7.18	0.995	1,426.3	32.150	2.833	0.995
Dry yellow straw	451.76	7.35	7.23	0.997	1,286.1	28.891	2.715	0.996
Silage straw	461.73	8.32	9.54	0.998	1,351.4	30.192	2.766	0.995

dry yellow straw was a result of its greater lignin content, which prevented access to nutrients by microorganisms. The process of lactic acid conversion was increased by the corn straw silage process and was measured on days 10, 15, and 40.

Evaluation of Anaerobic Digestion of Different Types of Corn Straw

The modified Gompertz model can be used to predict methane production potential, maximum methane generation rate, and the lag period before raw material fermentation. The R^2 values for each type of straw were between 0.995 and 0.998, showing that the model was a good fit for the data. The maximum biogas and methane production rates of each type of straw were significantly different, with fresh straw showing the highest biogas and methane production rates, followed by silage straw, with dry yellow straw having the lowest rates of production. Fresh straw also had the highest λ value, followed by silage straw, with dry yellow straw having the lowest value, showing that fresh straw had the highest rate of methane generation. These results indicate that the use of fresh straw instead of silage straw or dry yellow straw in the process of biogas production should improve the rate of straw gas production, shorten the fermentation time, and improve the utilization of straw (greater efficiency) (Table 1).

Sequence Analysis of the Microbial Community in the Reactor

The methanogen composition in the reactor at the family level was investigated to evaluate microbial performance, resulting in identification of 11 methanogen families (Fig. 7): Archaea_unclassified, *Methanosaetaceae*, *Methanobacteriaceae*, Euryarchaeota_unclassified, Miscellaneous_Creanarchaeotic_Group_norank, *Methanospirillaceae*, *Metanomicrobia*_unclassified, WCHA2-08, Thermoplasmatales_thermoplasmatales_incertae_

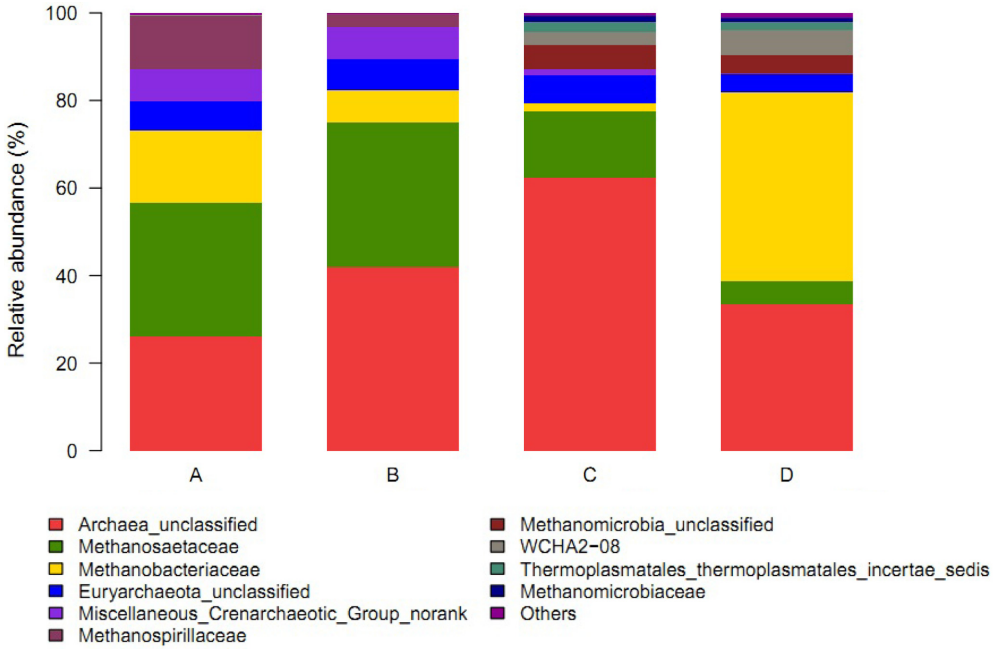

Fig. 7. Methanogen composition at the family level during anaerobic digestion.
A: silage straw; B: fresh straw; C: dry straw; D: seed sludge.

sedis, *Methanomicrobiaceae*, and Others. The families *Methanobacteriaceae*, *Methanospirillaceae*, and *Methanomicrobiaceae* are known to utilize H_2/CO_2 or formate [18, 29]. The families *Methanosaetaceae* and *Methanosarcinaceae* are known to include aceticlastic genera *Methanosarcina* and *Methanosaeta* [29]; however, *Methanosarcina* species were not detected in this study.

The abundance of Archaea_unclassified in silage straw, fresh straw, and dry straw was 36.50%, 20.61%, and 29.03%, respectively. The abundance of *Methanobacteriaceae* in silage straw, fresh straw, and dry straw was 26.47%, 35.86%, and 41.42%, respectively. The abundance of *Methanosaetaceae* in silage straw, fresh straw, and dry straw was 2.98%, 18.67%, and 28.25%, respectively. Several researchers have indicated that acetate is mainly degraded by aceticlastic methanogenic archaea during anaerobic corn straw degradation [18, 28]. The abundance of Miscellaneous_Crenarchaeotic_Group_norank in silage straw, fresh straw, and dry straw was 0.2%, 81.29%, and 99.05%, respectively. Previous studies indicated a close relationship between Miscellaneous_Crenarchaeotic_Group_norank bacteria, *Methanosaeta*, and acetate-utilizing methanogens in wastewater and marine sediments [3, 10].

Shannon's diversity index considers both richness and evenness. Rarefaction curves of the Shannon index were different from those of the OTUs as they approached the plateau from less than 10,000 tags per sample [25]. Fig. 8 illustrates that the Shannon-Weaver indices of the three tested types of corn were significantly dissimilar. The highest Shannon-Weaver index was 1.87 for fresh straw, whereas silage straw and dry straw had Shannon-Weaver indices of 9.6 and 17.11, respectively. The main reason for the difference in species diversity among the tested types of straw was the greater nutrient (starch, crude protein, crude fat, *etc.*) richness and availability of fresh straw in comparison with dry straw and silage straw. The hemicellulose and cellulose contents of fresh straw are relatively high; therefore, nutrients are readily utilized by microorganisms during anaerobic fermentation.

PCA demonstrated that the three types of straw could be separated using the different material. Archaeal microflora were enriched in silage straw and fresh straw (Fig. 9).

Discussion

Growing energy consumption and diminishing supplies of fossil fuels are significant concerns that have led to research into the use of renewable energy sources and development of new energy production processes [24]. Biogas production is of major importance as a means of achieving sustainable use of agrarian biomass as a renewable energy source [16]. Crop straw is a major raw material for biogas production; however, the straw harvest is seasonal [22], so the total harvest is greater than the immediate capacity for energy production from the crop. Therefore, corn straw storage is important because it must provide high-quality raw material suitable for use in sustainable biogas production. Fresh straw contains high levels of soluble sugars, starch, fat, carbohydrates, fiber, and hemicellulose, leading to a high biogas production rate; however, long-term storage of fresh straw is difficult and production is subject to seasonal restrictions, so it is not an ideal long-term anaerobic fermentation substrate. The biogas production potential of silage straw is reduced gradually during the storage process, when the abundance of some components (such as soluble protein and soluble solids) is decreased gradually as a result of microbial degradation and respiration, whereas crude fiber abundance is increased. Silage is the most common storage method used to improve the rate of nutrient utilization from straw and reduce fermentation losses, because it solves the problem of seasonal restriction. During storage, a large proportion of the potential CH_4 yield can be lost, as shown by reported losses of up to 37% and 52% during storage of grass and ryegrass, respectively, in laboratory studies, as well as reported losses of 17% and 41%, respectively, in boreal field studies of grass and ryegrass after 11 months [18]. In comparison with fresh straw and silage straw, dry yellow straw has a lower moisture content, higher lignin

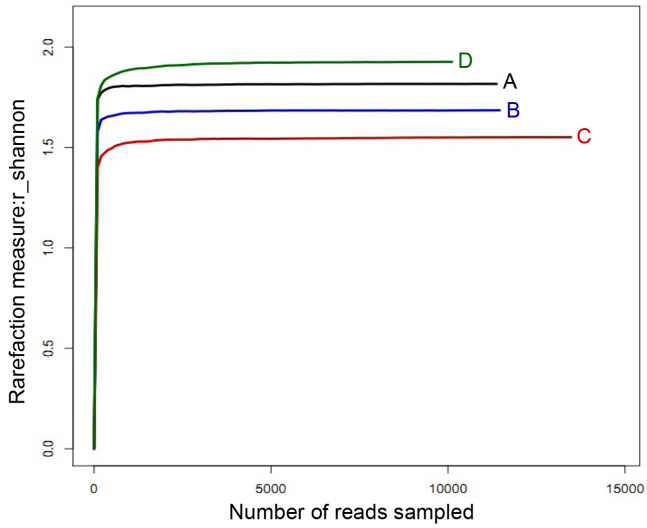

Fig. 8. Shannon-Weaver indices of the microbial community during anaerobic digestion.
A: silage straw; B: fresh straw; C: dry straw; D: seed sludge.

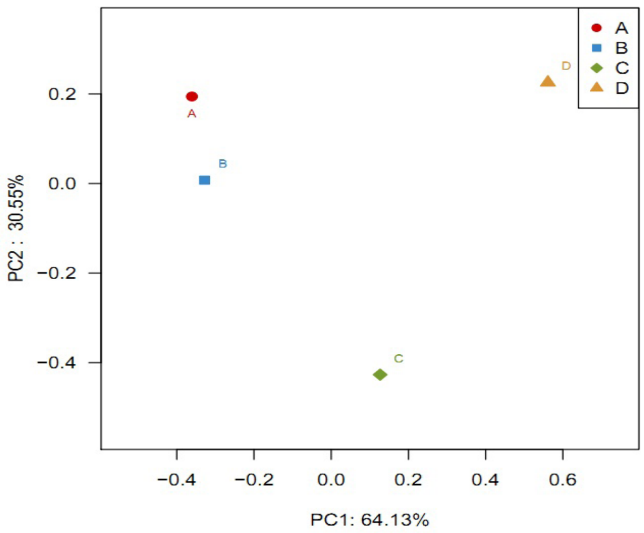

Fig. 9. Principal component analysis of the microbial community during anaerobic digestion.
A: silage straw; B: fresh straw; C: dry straw; D: seed sludge.

content, and reduced biogas production potential; however, dry yellow corn straw is easy to store. The cost of corn silage straw was 0.052 yuan RMB/kg [26]. The cost of converting biogas from dry yellow straw to natural gas (98% CH_4) was 0.013 yuan RMB/m^3, higher than that of biogas from silage straw [21]. Chinese law forbids the use of fresh straw as a raw material for biogas. Therefore, in northern China, dry yellow straw is often used as a raw material for biogas production.

As the present results show, the manner in which energy crops for CH_4 production are stored is an important issue, since with appropriate storage practices, CH_4 yield can be rather well maintained, and the previous studies suggest that the CH_4 potential (m^3/kg VS) of energy crops can in some cases be increased during storage, which thus acts as a pre-treatment step [17]. In our studies, the maximum daily biogas production of silage straw and dry yellow straw were 1.96 L on day 17 and 1.72 L on day 19, respectively. The previous study showed that the maximum daily biogas production of silage straw and dry yellow straw were 1.756 L on day 6 and 0.476 L on day 11, respectively [19], and that the CH_4 potential of stored whole crop maize (1.92 L) of silage straw [14] and the biogas potential of ensiled green pea shells increased by 9% [13]. This is consistent with the daily biogas production of silage straw being higher than the others, because of the VFA that can produce synergies by silage and increase the biogas production [24].

Acetate is mainly degraded by aceticlastic methanogenic archaea during anaerobic corn straw degradation [18]. The acetate levels of the silage straw and fresh straw were 11.87 g/l and 2.35 g/l, respectively (Fig. 6), whereas the proportions of *Methanosaeta* in the archaeal communities present in silage straw, fresh straw, dry straw, and seed sludge were 15.69%, 18.67%, 25.26%, and 28.25%, respectively (Fig. 7). A previous study reported that acetate accumulation could explain the dominance of *Methanosaeta* in the archaeal community during anaerobic production of biogas from corn straw [5].

In conclusion, total biogas production from the three types of tested corn straw ranged from 1.6 to 2.0 L after 30 days, with a methane content of approximately 55%. The biogas production rates per gram of fresh straw, silage straw, and dry yellow straw were 470.06 ml^{-1} g^{-1} VS, 461.73 ml^{-1} g^{-1} VS, and 451.76 ml^{-1} g^{-1} VS, respectively. *Methanosaeta* family bacteria dominated the archaeal community during biogas production from corn straw. A comprehensive economic benefit analysis indicated that dry yellow straw is suitable as a material for biogas production.

Acknowledgments

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