Enhancement of Biogas Production from Bundled Rice Straw Solid-State Fermentation by Adding Microbial Agents

Haoli Qu,[a] Jie Cao,[a,b] Yongsheng Chen,[a,*] Ruirong Li,[a] Pengjun Wang,[a] Mingjiang Chen,[a] Qiang Li,[b] Jing Du,[c] and Jing He [b,*]

Two contrasting garage-type solid-state fermentation experiments were designed at pilot scale to reveal the intensification process and effects of adding compound inoculants. Bundled rice straw and fresh pig manure were used as feedstock with a total solids concentration (w/w) of 20% under ambient temperatures of 26 to 35 ºC. The characteristics of biogas production and variations in bacterial community composition were investigated. The results indicated that using microorganisms shortened the start time of anaerobic solid-state fermentation. The target methane content in the biogas production reached 30% 7 days before the control group. A target of 50% was reached 8 days before the control group. Throughout the fermentation cycle, the cumulative gas production of the experimental group was 1340 m^3, which was 20.5% higher than the control group. Cumulative methane production increased by 45%. Clustering and principal coordinates analysis suggested that the addition of compound bacteria increased the diversity of the microbial community and stabilized its structure, thus improving gas production efficiency and methane purity. These findings can assist future bioaugmentation research pertaining to the application of composite microbial agents.

Keywords: Rice straw; Anaerobic digestion; Bioaugmentation; Bacterial community; Microbial ecology; 16SrRNA amplicon pyrosequencing; Prokaryotic community; Manure

*Contact information: a: Ministry of Agriculture, Nanjing Research Institute for Agricultural Mechanization, Nanjing 210014, People's Republic of China; b: Key Laboratory of Development and Application of Rural Renewable Energy, Ministry of Agriculture, Biogas Institute of Ministry of Agriculture, Chengdu 610041, People's Republic of China; c: Ministry of Agriculture, East China Scientific Observing and Experimental Station of Development and Utilization of Rural Renewable Energy, Nanjing 210014, People's Republic of China; * Corresponding authors: cys003@sina.com; hejing@caas.cn*

INTRODUCTION

Anaerobic solid-state fermentation generally refers to anaerobic fermentation with an initial total solids (TS) concentration (w/w) of 20 to 50%, and the process can produce clean energy and high-quality organic fertilizer (Bolzonella *et al.* 2003; Maritin *et al.* 2003). Unlike traditional anaerobic liquid-state fermentation, it offers the advantages of adaptability to different feedstocks, low water demand, low energy consumption, and reduced odor emissions (Cheng *et al.* 2012; Du *et al.* 2017). Thus, this process has become an important alternative for the disposal of organic solid waste in some countries.

Since the 1980s, Chinese researchers have studied anaerobic solid-state fermentation (Liu *et al.* 1995; Wu *et al.* 2005; Zhang *et al.* 2008; Zhu *et al.* 2011). Despite some achievements, many problems, such as long start-up time, incomplete fermentation, and low gas production efficiency, remain. These problems restrict the widespread

dissemination of the anaerobic solid-state fermentation process. As bioaugmentation technology is widely used in sewage treatment (Wang *et al.* 2002; Quan *et al.* 2004; Ma *et al.* 2009) and soil remediation (Grigg *et al.* 1997; Top *et al.* 2003), researchers have considered applying this technology to biogas production. They have thus attempted to solve the problems associated with anaerobic fermentation from the microscopic point of view. Bioaugmentation is a method of adding specific functional microorganisms to a system to increase its capacity or activity (Ritmann and Whiteman 1994). To date, some studies have focused on using biofortification technology to solve specific problems associated with anaerobic fermentation, such as shortening the start-up time of the fermentation system (Saravanane *et al.* 2001), increasing the feedstock utilization rate, shortening the recovery time of rancid systems, and reducing the inhibitory effect of toxic substances (Dhouib *et al.* 2010). However, most of the existing research has been at the laboratory scale (using batch assays), with a focus on characteristics of biogas production using bioaugmentation technology (Yang *et al.* 2007; Liu *et al.* 2012). On the other hand, studies on bioaugmentation are usually performed by relying on a single microbial component, characterized by a specific functional role. The focal point of the bioaugmentation process is the survival and activity of all functional microorganisms in the fermentation system. Therefore, it is important to understand how the application of bioaugmentation technology can be widened to include anaerobic fermentation projects.

The biogas microbial cocktail developed by the Biogas Institute of the Ministry of Agriculture (Chengdu, Sichuan Province, China) has been used for specific projects in the field in China, and it has achieved good results. In this study, a garage-type solid-state anaerobic fermentation system was used for a comparative study of the application of bioaugmentation technology in solid-state fermentation (Qu *et al.* 2015; Zhu *et al.* 2016). Waste rice straw and pig manure, which are common biowaste materials in southern China, were used as feedstock. The intensified process and effect of microbial consortia on the garage-type solid-state anaerobic fermentation were analyzed at a pilot scale. High-throughput 16S rRNA amplicon sequencing was used to analyze bacterial community changes. The objectives of this study were to characterize anaerobic solid-state fermentation of baled rice straw using bioaugmentation technology and to provide a microscopic explanation as to how this technology functions in anaerobic solid-state fermentation.

EXPERIMENTAL

Feedstock and Inoculums

Fresh rice straw was collected from an experimental field in Changshu, Jiangsu Province, China. It was cut to lengths of approximately 10 mm with a grinder and baled in 5 kg bundles. Fresh pig manure, taken from a pig farm in Changshu, was used as feedstock. The inoculated biogas slurry was collected from a continuously stirred tank of anaerobic digester to treat the pig waste. The parameters of the fermentation feedstock are shown in Table 1.

The microbial fortification agent developed by the Biogas Institute of the Ministry of Agriculture contained 1.5×10^8/g methane (CH_4) bacteria, 4.5×10^8/g cellulose decomposition bacteria, and 5.5×10^9/g fermentation bacteria. The microbial inoculants were also supplied by the Biogas Institute of the Ministry of Agriculture, and they contained eight microorganisms (Table 2). The total number of microbes was 6.1×10^9/g.

Table 1. Characteristics of the Raw Materials Used in the Experiments

Item	pH	TS (%)	VS (%)	COD (mg/L)	TC (%)	TN (%)	C/N
Swine Manure (SM)	7.30	23.5	73.24	11500	40.31	2.23	18.08
Inoculation Sludge (IS)	7.15	1.23	-	1970	0.13	0.07	1.86
Rice Straw (RS)	-	89.02	76.98	-	47.56	0.88	54

TS: total solids; VS: volatile solids; COD: chemical oxygen demand; TC: total carbon; TN: total nitrogen

Table 2. Characteristics of the Microbial Inoculants

Bacterial Strain	DSMZ Preservation Number[1]	Percent (%)
Pseudomonas alcaligenes	DSM 19550	15
Pseudomonas nitroreducens	DSM 14399	15
Smithella propionica	DSM 16934	15
Enterococcus aquimarinus	DSM 17487	15
Clostridium celerecrescens	DSM 5628	25
Methanosaeta concilii	DSM 2139	5
Methanosarcina mazei	DSM 2053	5
Methanocorpusculum sp.	DSM 4274	5

[1]Deutsche Sammlung von Mikroorganismen und Zellkulturen, the German Collection of Microorganisms and Cell Cultures, Leibniz Institute
Percent (%) refers to the bacterial percent, calculated as volatile solids in the total microbial inoculant.

Digester System

A modified biogas solid-state fermentation pilot system, namely the flexible roof membrane garage-type biogas solid-state fermentation system (GBFS), was used in this study. The overall design of the fermentation device is shown in Fig. 1a. The reactor contained two fermentation units, each with a total volume of 75 m^3 (Fig. 1b). The system controlled leachate recirculation (Fig. 1c), allowed real-time operational data collection, and was heated and insulated (Fig. 1c). The heat from solar energy combined with the biogas boiler pumped hot water into the water coil in the GBFS, which heats the feedstock by heat exchange. Leachate was extracted from the bottom leachate pool for recycling. Inlet door sealing and roof sealing solved the sealing problem of large-scale input and output.

Experimental Design

The pilot-scale anaerobic fermentation experiments in this study included a control experiment, CL (in the east section of the GBFS), and an experimental group, SL (in the west section of the GBFS). The treatments entailed single-stage solid-state fermentation (total solids (TS) = 20%). The fermentation period was 50 days. The fermentation temperature ranged from 26 to 35 ºC. The two groups were similar in every detail except that the experimental group contained an additional 2% of total dry matter in the fermentation feedstock.

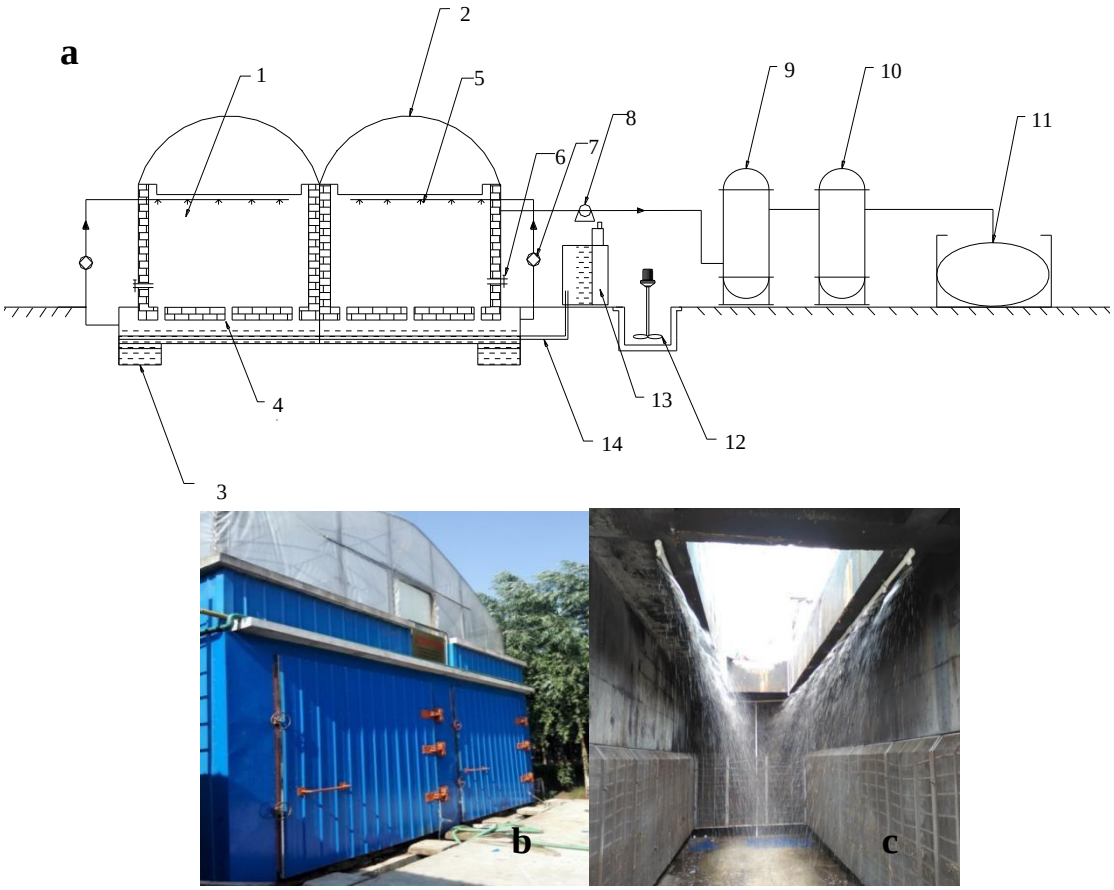

Fig.1. Flexible roof membrane garage-type solid-state anaerobic fermentation system. (a) Schematic diagram of the system. 1 - Anaerobic reactor, 2 - Flexible roof membrane, 3 - Leachate container, 4 - Leachate trough, 5 - Spray parts, 6 - Sample point, 7 - Reflux pump, 8 - Biogas flowmeter, 9 - Devulcanizing pan, 10 - Drain sump, 11 - Gasholder, 12 - Regulating reservoir, 13 - Constant temperature water-bath, 14 - Heating coil. (b) Outside view of anaerobic reactor; (c) Heating and insulation, and leachate recirculation.

During the experiment start-up, bundles of straw were placed in the GBFS in layers. Pig manure was placed between two layers of straw, and then the bioaugmentation bacterial product was added to one of the SL fermentation systems. The proportion of straw and pig manure was selected as 1:2 to adjust the initial C:N to about 25:1. Biogas slurry was added to regulate the moisture content. The pH was adjusted to 7.0 and the carbon-nitrogen ratio was adjusted to approximately 30:1. When anaerobic fermentation commenced, the reflux operation was conducted. The reflux operation began at day 0, and it was conducted twice a day in the GBFS, each time for 30 min. Gas samples were collected every day and leachate samples were collected after completion of the reflux operation. Leachates from each GBFS section were not mixed before recirculation. All the samples for microbial analysis were collected after the recirculation from the bottom deposit of the leachate.

Chemical Analysis

All chemicals were obtained from either Sinocem (Shanghai, China) or Fluka Chemical (Buchs, Switzerland). The CO_2, H_2, and N_2 were purchased from Nanjing Special Gases Factory (Nanjing, China). Biogas production was tracked with an ultrasonic gas flow

meter (BF-30008-160, Wuhan Sifang Company, Wuhan, China). Volatile solids (VS) and TS were determined in accordance with the standard methods of the American Public Health Association (APHA 1998). The temperature was monitored using a multi-channel temperature recorder (JWB/33, ColliHigh, Beijing, China). The pH and oxidation-reduction potential (ORP) were measured with a PHS-25 meter (Shanghai Leichi Instrumentation Factory, Shanghai, China).

The CH_4 concentration in the biogas was analyzed using a gas chromatograph (GC 9890A, Renhua, Nanjing, China) equipped with a TDC-01 column (ϕ 4 mm × 1 m, Shimadzu, Kyoto, Japan), using hydrogen as the carrier gas. The CH_4 volume fraction in the biogas was measured daily. The injector, oven, and detector temperatures were 100, 150, and 120 °C, respectively. The flow rate of the carrier gas was 50 mL/min, and the injection volume of the samples was 0.5 mL.

Microbial Community Analysis

Microbial DNA was extracted from the biogas slurry samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, Georgia, USA) according to the manufacturer's protocols. The V4 to V5 region of the bacteria 16S ribosomal RNA gene was amplified by the polymerase chain reaction (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min) using primers 515F 5'-barcode-GTGCCAGCMGCCGCGG)-3' and 907R 5'-CCGTCAATTCMTT-TRAGTTT-3'.

A library was constructed on the combined V4 region of the 16SrDNA district based on Illumina MiSeq Technology. The amplicon library was paired-end sequenced (2 × 250) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd.) according to standard protocols.

Statistical Analysis

All experiments and analytical measurements were conducted in triplicate, as a minimum. The standard deviations and fitted curves were analyzed using Microsoft Excel 2013 (Redmond, WA, USA) for Windows and Origin 9.0 (Northampton, MA, USA) for Windows, respectively. All bacterial community structure analyses used the Vegan package in R (Dixon 2015). The differences in bacterial community structures were evaluated using principal coordinates analysis (PCoA) based on Bray–Curtis distances using the relative abundances of OTUs without singletons as the input data.

RESULTS AND DISCUSSION

Characteristics of Biogas Production in Fermentation Processes

Daily biogas production and cumulative biogas production of the two groups are shown in Fig. 2. The experiments were conducted over 50 days. As can be seen in Fig. 2, the two groups show similar daily gas production trends. Daily gas production increased rapidly at the beginning of the trial, then decreased slowly, and gradually stabilized. The experimental group produced biogas on the second day after the start of the experiment. Daily biogas production peaked to 49 m^3 on Day 14 (the volume of gas production was 0.65 $m^3/(m^3 \cdot d)$). The control group began to produce gas on Day 5, with daily biogas production peaking at 43.9 m^3 on Day 18 (the volume of gas production was 0.59 $m^3/(m^3 \cdot d)$). The daily increase in gas production capacity of the experimental group was

noticeably higher than that of the control group. Its peak in gas production was 4 days ahead of the control group, and its maximum gas production volume was 9.2% higher than that of the control. During the test period, cumulative gas production of the experimental group reached 1340 m^3, 20.5% higher than that of the control group. These results indicate that the microbial agent effectively shortened the start-up time of anaerobic solid-state fermentation and increased the gas production. These results were consistent with the findings of Zhang *et al.* (2014).

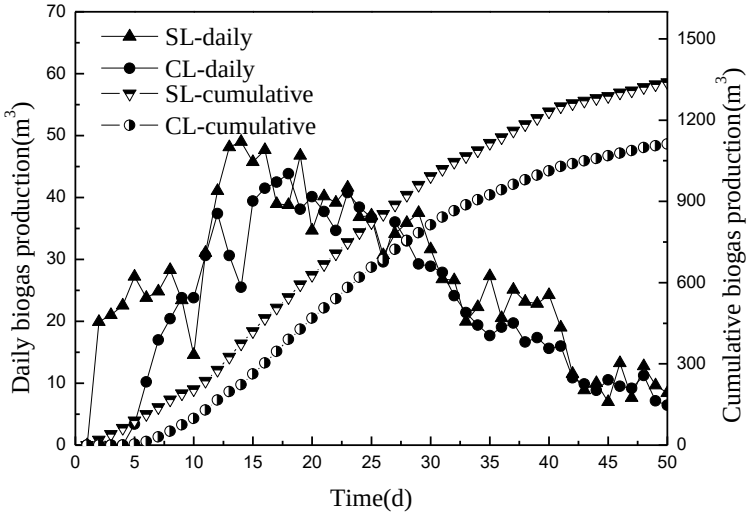

Fig. 2. Changes in daily and cumulative biogas yields for different treatments during the experiment (SL – experimental group, CL – control group)

The biogas was a mixed gas, mainly composed of CH_4 and CO_2, with small amounts of N_2, O_2, H_2, NH_3, and H_2S. The CH_4 volume fraction directly affected the quality of the biogas: the higher the CH_4 volume fraction, the higher the biogas quality. Therefore, the CH_4 concentration in gas production is a very important indicator for biogas projects.

The CH_4 volume fractions of the two groups are shown in Fig. 3. The two groups show similar trends in CH_4 content, which gradually increased and then stabilized. The CH_4 volume fraction of the experimental group reached 30% on Day 6 of the experiment and reached 50% on Day 11. No CH_4 was detected during the first 3 days of the experiment in the control group, but the CH_4 volume fraction reached 30% on Day 13 of the experiment and reached 50% on Day 19 (7 days and 8 days later than the experimental group, respectively).

In the start-up phase of anaerobic fermentation, the starting point and increasing range of the CH_4 volume fraction of the experimental group were higher than the corresponding values of the control group. Throughout the experiment, the average CH_4 volume fraction of cumulative gas production in the experimental group reached 52.8%, which was 20.8% higher than that for the control group (43.7%).

In summary, the microbial agent effectively shortened the start-up time of solid-state anaerobic fermentation, increased the CH_4 content in gas production, and improved biogas quality.

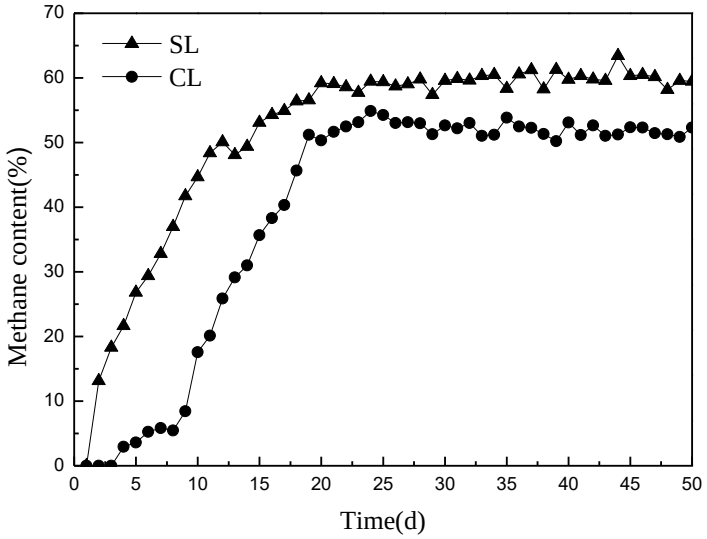

Fig. 3. Changes in methane content of the different treatments during the experiment

Figure 4 shows the pH of the experimental and control groups. After the experiment started, the pH of the two groups declined gradually. The lowest pH in the experimental group (6.78) was recorded on Day 9 after the start of the experiment. Then, the pH of the hydrolyzate rose slowly and gradually stabilized at approximately 7.3. For the control group, the minimum pH (6.21) was recorded on Day 8 after the start of the experiment. After a slight rise, the pH stabilized at approximately 6.3. After Day 27, the pH of the hydrolyzate gradually recovered to approximately 7.0. These results indicate that the microbial agent could effectively stabilize the pH of the hydrolyzate. These results were consistent with the findings of (Ghanimeh *et al.* 2013).

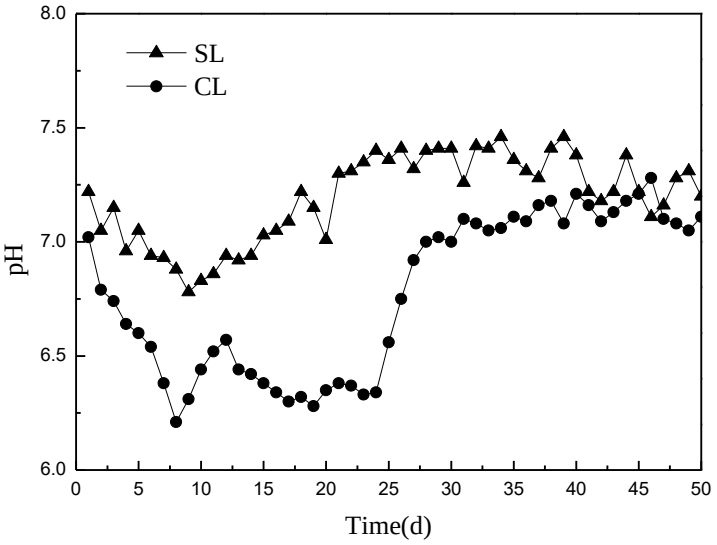

Fig. 4. Changes in pH of the different treatments during the experiment

Diversity and Structure of the Bacterial Communities

The variations of the bacterial community within 30 full-scale anaerobic digesters were characterized using barcoded amplicons, resulting in 1,209,348 chimera-free reads and 3,760 operational taxonomic units (OTUs) at a cut-off of 97% similarity. The 381 OTUs had an average relative abundance exceeding 0.01%.

Bacterial diversity indices varied across all the samples (Fig. 5). The number of OTUs detected in the experimental group (SL) was 1269 to 1992, and the Chao1 estimator of richness index was 1978 to 2423.

The number of OTUs detected in the control group (CL) was 1336 to 1781, and the Chao1 estimator of richness index was 1927 to 2930 (Fig. 5a). Figure 5a shows that the number of OTUs in each fermentation cycle of the experimental group exceeded that in the control group.

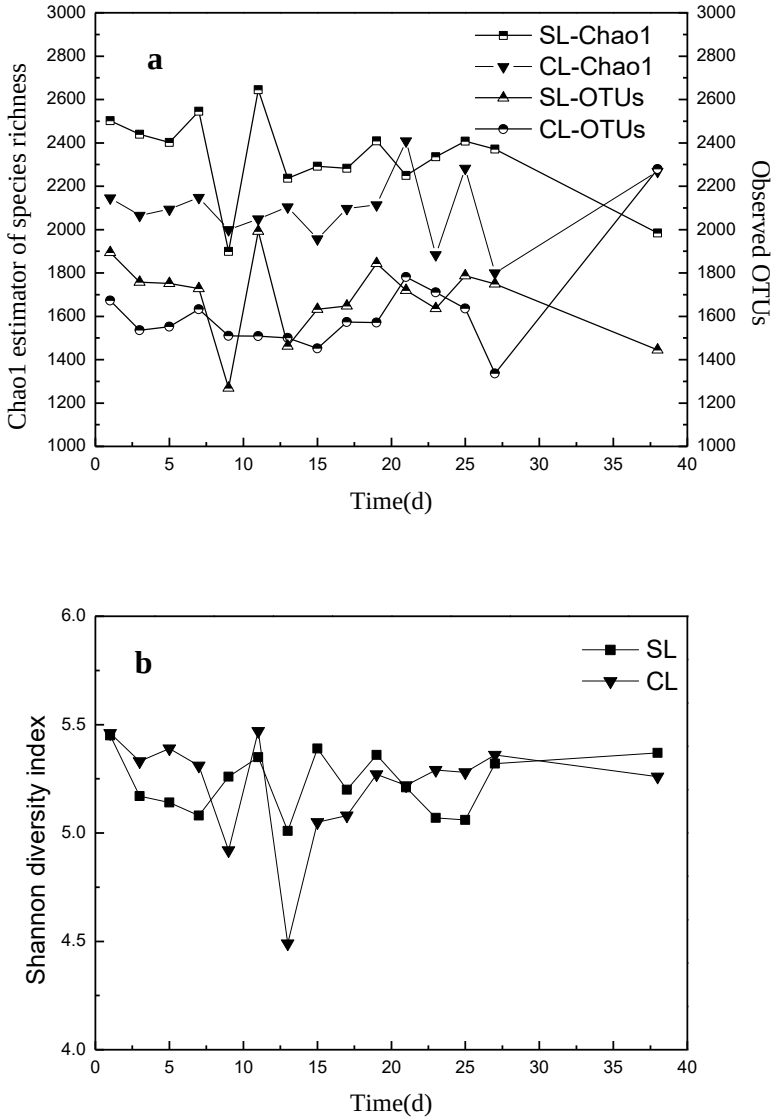

Fig. 5. Bacterial diversity indices at 97% 16Sr RNA sequence similarity for each sample. (a) Chao1 estimator of species richness and observed OTUs; (b) Shannon diversity index

Changes in the Shannon index are shown in Fig. 5b. At the initial stage of fermentation (1 to 5 days), the species index Chao1 and Shannon index of the experimental group were higher than those of the control group. This indicated that the addition of compound bacteria enhanced the diversity of the fermentation system and shortened the fermentation start-up time (by 4 days). The Shannon index for the mid-term fermentation of the fortified group changed, indicating changes in the diversity of the microbial community. With the introduction of fermentation into a number of gas-producing stages, the microbes increased their metabolism and reproduction. Figure 5b shows that the Shannon index started to decrease after 13 days for the experimental group, while the daily output of the macro index reached a maximum value on Day 14 of fermentation (Fig. 2). Microbial community diversity may be one of the most important internal factors leading to an increase in biogas production midway during fermentation.

Three potential clusters were observed in the PCoA analysis of the bacterial communities (Fig. 6). The clusters contained 30 samples originating from 2 anaerobic fermentation systems (CL1–CL38 and SL1–SL38). The other two clusters were from the added compound bacteria (ES0) and pig manure (EA0). Segregation of the bacterial communities was also observed. Clusters PCo1 and PCo2 explained approximately 55.5% and 17.02% of the total variation in the bacterial community structure of the two digesters. The intersecting part of the cluster consisted of eight samples from the two digesters, and the cluster was mainly in the early stage of fermentation. The two clusters were not intersect in the medium and late fermentation.

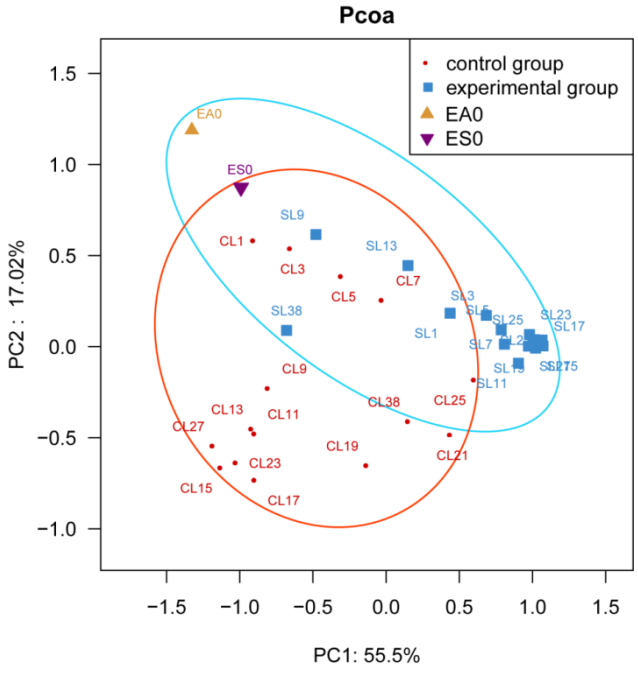

Fig. 6. Principal coordinates analysis of whole prokaryotic communities at the phylum level in the co-fermentation of rice straw and swine manure. CL - control group; SL - experimental group; 1, 3, and 5 represent sampling points during fermentation; ES0 - addition of compound bacteria; EA0 - pig manure sample

The results indicated that adding compound bacteria to the control group was likely to segregate the bacterial communities in the two anaerobic digesters. The microbial communities were relatively concentrated at the beginning of the fermentation process.

The microbial communities in the middle and late stages of fermentation differed between the two fermentation systems. The microorganism community during medium and late fermentation became more stable after the addition of compound bacteria. The proportional composition of the bacterial population tended to be more reproducible in the case of the control group (except in two of the cases). Thus, gas production of the experimental group increased by 20.5% and the CH_4 content of the biogas increased by 20.8%.

Characteristics of Community Composition in the Fermentation Processes

Approximately 32 samples were investigated for variations in bacterial community composition using high-throughput 16SrRNA amplicon sequencing. Thirty sludge samples were collected from the two fermentation systems (CL1–CL38 and SL1–SL38). The CL group represents the number of fermentation days in the control group in the east GBFS, and the SL group represents the number of fermentation days in the experimental group in the west GBFS. In addition, the added compound bacteria (ES0) and pig manure (EA0) were tested.

Approximately 99% of total reads were annotated at the phylum level. The bacterial communities in the two digesters consisted of Bacteroidetes, Firmicutes, Spirochaetae, and Proteobacteria according to high-throughput sequencing (Miseq) (Fig. 7).

During the fermentation period (before 38 days), the dominant microbes in the experimental group (SL) remained relatively stable (except sample SL13). The microbial abundance of the control group (CL) varied. The total abundances of Bacteroidetes and Firmicutes were not noticeably different between the two fermentation systems. The total relative abundance of the Bacteroidetes and Firmicutes was close to 80%. The former was stable from the initial stage of fermentation in the CL, and then it gradually decreased after the gas production peaked. Then, it gradually rose, and the abundance varied during the stationary period of gas production.

Compared with the abundance of Bacteroidetes, the abundance of Firmicutes showed the opposite trend. However, the relative abundance of Bacteroidetes in the experimental group was larger than that of Firmicutes, and the relative abundance showed a stable trend. The enrichment of Bacteroidetes had some advantages in ES0, while Firmicutes was relatively inferior in ES0.

Combined with the characteristics of biogas production during fermentation (Fig. 2), the above-mentioned changes in bacteria abundance during the fermentation process showed that the addition of compound bacteria enhanced the long-term stability of the dominant microbial community in the fermentation system, thus improving the fermentation efficiency of the system. The effective gas production time increased by 7 days, and the average CH_4 volume fraction increased by 20.8%. Studies have found that the bacteria in these processes typically belong to the phyla Firmicutes, Bacteroidetes, and Proteobacteria, which play a major role in anaerobic fermentation. These results were consistent with the findings of related studies (Hanreich *et al.* 2013; Li *et al.* 2014; Li *et al.* 2015).

Firmicutes is the dominant bacterial community in the hydrolysis and acidification stage of anaerobic fermentation (Luo and Angelidaki 2014; Rui *et al.* 2014; Kong *et al.* 2018). Improving the stability of Firmicutes flora may promote hydrolysis and increase the efficiency of biogas production.

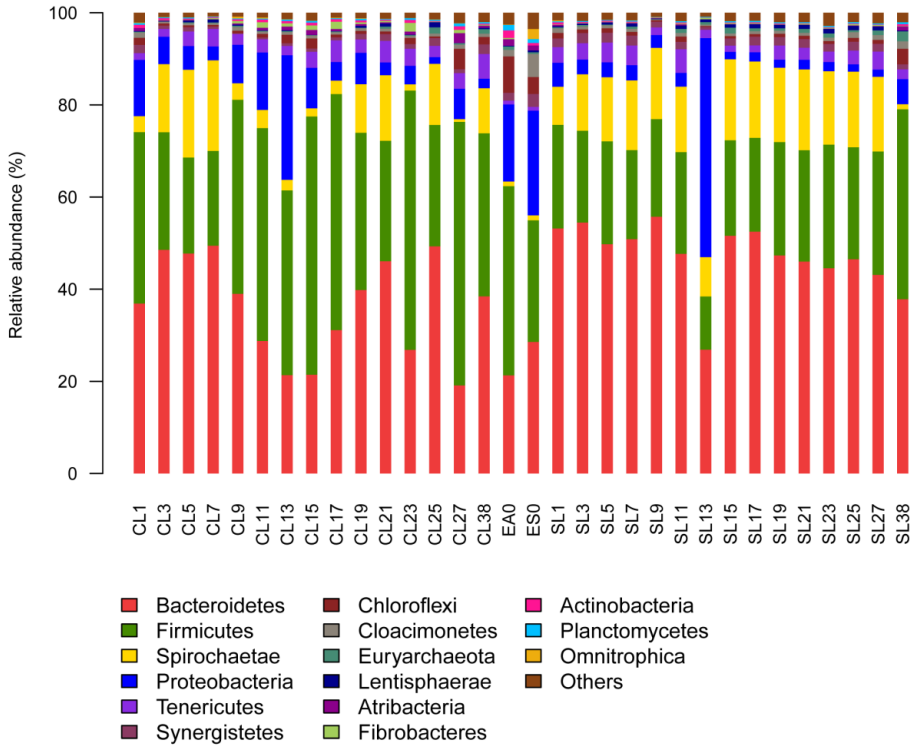

Fig. 7. Relative abundance of bacterial OTUs with 97% similarity at the phylum level. CL - control group; SL - experimental group; 1, 3, and 5 represent sampling points in the fermentation period; EA0 - pig manure sample; ES0 - addition of compound bacteria

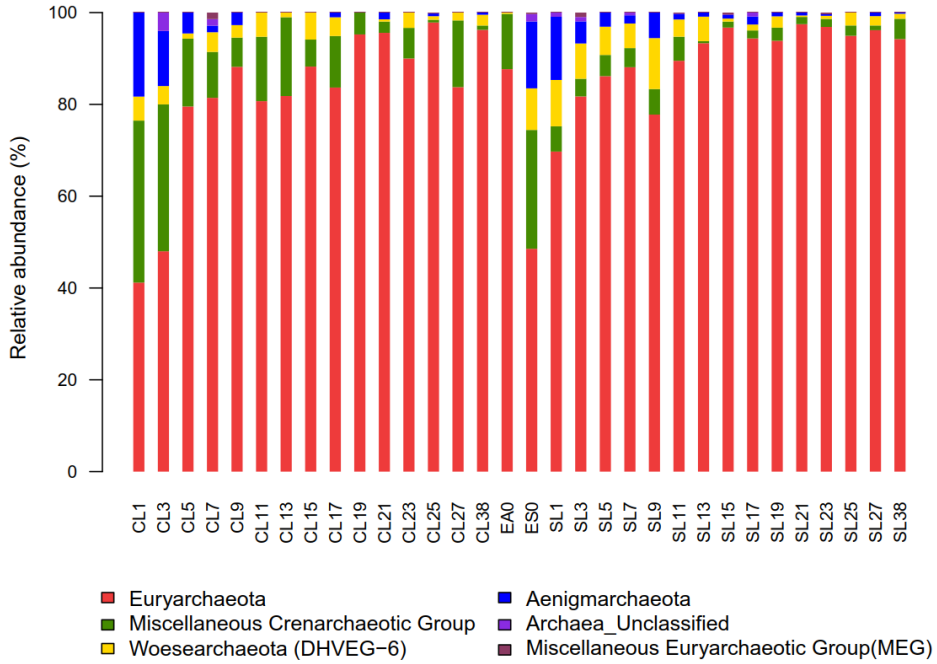

Fig. 8. Relative abundance of archaea OTUs with 97% similarity. CL - control group; SL - experimental group; 1, 3, and 5 represent sampling points in the fermentation period; EA0 - pig manure sample; ES0 - addition of compound bacteria

The dominant Archaea communities in the two fermentation systems were concentrated in Euryarchaeota, followed by the Miscellaneous Crenarchaeotic Group and Aenigmarchaeota, which were also distributed in the fermentation broth (Fig. 8). After the ninth day of fermentation, the relative abundance of Euryarchaeota reached more than 80%, and the proportion of relative abundance changes decreased in the medium and late stages of fermentation. Methanogens are the main members of Euryarchaeota. Thus, it can be inferred that the methanogenic relative abundance can be maintained at a relatively stable level in the medium and late fermentation stages by adding compound bacteria. The effective average CH_4 volume fraction increased by 20.8%.

DISCUSSION

Analysis of the degradation of the fermentation substrate showed that this microbial community could effectively increase the degradation rate of rice straw cellulose and hemicellulose. The degradation rate of straw cellulose and hemicellulose in the experimental group was 29% and 51.6%, which was 46.4% and 14.9% higher than that of the control group.

Identifying the most suitable process parameters for anaerobic solid-state fermentation of baling straw using microbial intensification technology has important practical value. In this process, the bundled straw does not need to be unbundled and comminuted, and it can be used for anaerobic fermentation directly. Doing so will reduce the cost of the straw biogas project. The experiment in this study was carried out at two 75-m^3 pilot plants. In the future, we plan to conduct an engineering test at another 240-m^3 solid-state fermentation unit in Yixing City, Jiangsu Province, China.

CONCLUSIONS

1. The results of the control fermentation experiments showed that the applied microbial cocktail could significantly shorten the starting time of anaerobic solid-state fermentation. The experimental group was 7 days and 8 days ahead of the control group in obtaining a CH_4 content of 30% and 50%, respectively. The cumulative gas production of the experimental group was 1340 m^3, which was 20.5% higher than that of the control group, and CH_4 production increased by 45%.
2. The bacterial communities in the two digesters consisted of Bacteroidetes, Firmicutes, Spirochaeta, and Proteobacteria according to high-throughput sequencing (Miseq). Changes in the core microbial communities showed that the addition of microbial agent enhanced the stability of the dominant microbial community in the fermentation system, thus improving the fermentation efficiency of the system.
3. Clustering and principal coordinates analysis (PCoA) suggested that the addition of the microbial agent not only increased the diversity of the microbial community but also enhanced the long-term stability of the core microbial community structure.

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