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Impacts of microaeration on the anaerobic digestion of corn straw and the microbial community structure

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HIGHLIGHTS

- The effect of limited oxygen daily supplied on the AD of corn straw was studied.
- Daily oxygen supplied could obviously improve the AD performance of corn straw.
- Specific methanogenic activity under microaerobic condition improved slightly.
- The microbial community structure shift could explain the better AD performance.

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ABSTRACT

Conventionally, oxygen is considered as inhibit factor of anaerobic digestion (AD). However, recent studies have demonstrated that AD performance could be enhanced by introducing limited amounts of oxygen (or air) directly into the anaerobic digester or during pretreatment step. In this study, impacts of microaeration on the anaerobic digestion of corn straw and the microbial community structure were investigated. Results showed that limited air introduced into fermentation system could improve the methane yield of corn straw. Maximum cumulative methane yield of 216.8 ml/g $VS_{substrate}$ and maximum VS removal efficiency of 54.3% were simultaneously obtained under microaerobic condition with the air load of 12.5 ml/L_R per day, which were 16.5% and 10.3% higher than those of sample under anaerobic condition, respectively. Compared to anaerobic condition, the relative abundances of phylum *Firmicutes*, class *Clostridia* and order *Clostridiales*, which associated with hydrolysis process of AD were raised under microaerobic condition. In addition, the relative abundances of oxytolerant *Methanosarcina* and *Methanobacterium* were both doubled under microaerobic condition. Accordingly, specific methanogenic activity (SMA) under microaerobic condition improved slightly. The microbial community shift might be the reason for improved AD performance under microaerobic condition.

1. Introduction

Anaerobic digestion (AD) has been widely applied in treating of organic waste as well as producing methane energy [1,2]. Due to the abundance and high carbohydrate content, corn straw has been demonstrated to be a potential substrate for methane production in AD [3].

Conventionally, AD is considered to be a four-step biological process. The solubilization of complex particulate organic compounds into simple soluble compounds such as volatile fatty acids (VFAs) was accounted as hydrolysis and acidification. They are followed by the acidogenesis step which converts VFAs to acetate and hydrogen gas that will in turn be consumed by methanogens to produce methane in the final step of the AD process [4,5]. During AD of cellulosic substrate like corn straw, hydrolysis is generally regarded as rate-limiting step [5,6]. Recent studies have demonstrated the hydrolysis of AD could be enhanced by introducing limited amounts of oxygen (or air) directly into the anaerobic digester or during a pretreatment step [7,8]. On the one hand, facultative bacteria have a quick grow rate, consequently, more cellulose and protease hydrolytic enzymes will be produced, which will lead to higher hydrolysis rate [9,10]. On the other hand, methanogens were demonstrated to have several mechanisms to survive and

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function under microaerobic condition with no or minor inhibitory effects [11–14].

Microaeration has been used during anaerobic digestion process in several studies. However, different results were obtained. During studying the effect of microaerobic condition on the degradation kinetics of cellulose, Diaz et al. [15] found limited oxygen supply did not substantially affect the maximum methane production and the hydrolysis constant. However, a shorter lag-phase was found in the microaerobic assays. Ramos et al. [16] also demonstrated oxygen supplied did not have a significant impact on the digestion performance of sewage sludge. During the anaerobic digestion of primary sludge, Johansen et al. [17] reported microaeration could only enhance primary sludge hydrolysis. Conversely, Mshandete et al. [18] reported nine hours of microaerobic pretreatment prior to AD of sisal pulp improved the methane yield for 26%. According to Jang et al. [19], using thermophilic aerobic digestion as biological pretreatment of sewage sludge significantly improved the total volatile suspended solid reduction and methane production rate. In AD of the compound of brown water and food waste, Lim and Wang [4] obtained 10–21% higher methane yield at oxygen load of 37.5 mL O_2/L_R per d during initial four days of AD. In our previous study, thermophilic microaerobic pretreatment before anaerobic digestion of corn straw at the oxygen loads of 5 mL/g $VS_{substrate}$ demonstrated 16.2% higher methane yield [20]. In addition, a secondary thermophilic microaerobic treatment at the 22th day of anaerobic digestion further improved the methane for 10.6% [21].

Microaerobic pretreatment has been proved to be an effective pretreatment method in several studies. However, the effects of continuously oxygen supplied during AD process on the AD performance of corn straw and the microbial community structure were less reported. In this study, the effects of continuous oxygen supplied during AD process on the AD performance of corn straw were investigated. In addition, the microbial community structures and specific methanogenic activities were also studied to reveal the reason for the improved AD performance of corn straw under microaerobic condition.

2. Material and methods

2.1. Substrate and inoculum

Corn straw collected from corn field of Pingdu (Shandong Province) was used as substrate. The TS (total solid) and VS (volatile solid) of corn straw were 91.9 ± 0.5% and 89.5 ± 0.5% (based on TS) (TS and VS were determined according to standard methods [22]), respectively. Before further use, corn straw was chopped and sieved to size of less than 1.0 cm by 1.0 cm sieve.

Active sludge with respectively TS and VS of 2.6 ± 0.3% and 52.7 ± 0.8% (based on TS) was collected from a local wastewater treatment plant (Tuandao Water Treatment Plant, Qingdao, Shandong Province, China). The collected active sludge was stored in refrigerator at 4 °C until further use.

2.2. Batch anaerobic digestion tests and oxygen supplied

Batch thermophilic (55 °C) anaerobic digestion tests were performed in duplicates. Before thermophilic anaerobic digestion, 5.8 g corn straw (wet weight) and 50 mL active sludge were mixed in bottles, and then nutrient solution was added to reach total volume of 0.2 L. The formula of nutrient solution was prepared according to Angelidaki et al. [23]. The bottles were flushed with N_2 for 5 min to replace the air and closed with rubber stoppers. Anaerobic digestion of corn straw was conducted in shaking water bath at 55 °C with 120 rpm.

Microaerobic conditions during thermophilic anaerobic digestion were attained by injecting air to the bottles with syringe. 0, 2.5, 5, 10, and 20 mL air at atmospheric pressure were injected daily into the bottles after biogas test to reach the air loads of 0, 12.5, 25, 50, and 100 mL/L_R-d (marked as T0, T1, T2, T3 and T4, respectively).

In this stage, biogas yield was measured daily by water replacement method. Methane concentration in biogas was also measured daily by gas chromatograph (SP 6890, Shandong Lunan Inc., China), equipped with a Porapak Q stainless steel column (180 cm long, 3 mm outer diameter) and a thermal conductivity detector. The temperatures of the injector, detector, and oven were 50, 100 and 100 °C, respectively. The carrier gas was argon.

2.3. Mathematical model analysis

In this study, the modified first order equation described as Diaz et al. [15] was used to estimate the hydrolysis constant (d^{-1}), which was written as:

$$P(t) = P_\infty \exp[1 - \exp(-k_H(t - L_p))]$$

where $P(t)$ cumulative methane yield (mL/g VS), P_∞ methane yield potential (mL/g VS), k_H is hydrolysis constant (d^{-1}), L_p is lag-phase time (d), t is elapsed time (d).

2.4. Specific methanogenic activity (SMA) tests

SMA tests were performed in triplicate in 300 mL bottles. During SMA tests, two substrates (sodium acetate and H_2/CO_2) were used for specific acetotrophic methanogenic activity (SAMA) and specific hydrogenotrophic methanogenic activity (SHMA) tests, respectively. During SMA tests, 160 mL nutrient solution and 40 mL fermentation broth were mixed in bottles to reach the working volume of 200 mL. Microaerobic condition during SMA test was obtained by daily 2.5 mL air injection to the bottles (marked as MO, using fermentation broth collected from T1 as inocula), SMA under anaerobic condition was marked as WO (using fermentation broth collected from T0 as inocula). For SAMA tests, each bottle was added with 0.6 g sodium acetate to reach the sodium acetate concentration of 3 g/L. The pH was adjusted to 7.0 with 2 M hydrochloric acid and 2 M sodium hydroxide. Then bottles were flushed with argon for 5 min to replace the air and closed with rubber stoppers. For SHMA tests, the pH was also adjusted to 7.0, and then bottles were closed with rubber stoppers and vacuumed, 100 mL H_2/CO_2 (4:1 v/v) was injected to each bottle. All the bottles were placed in a shaking water bath at 55 °C with 120 rpm. The methane yield was measured every 12 h with gas chromatograph (SP 6890, Shandong Lunan Inc., China) described as above.

2.5. Microbial community structure

10 mL of fermentation broth were collected from T0 and T1 with a syringe at the end of thermophilic anaerobic digestion and then stored in refrigerator (−80 °C) until further microbial community structure analysis.

Next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (Beijing, China). FastDNA® Spin Kit for Soil (CWBIO) was used for DNA extraction according to the manufacture's protocols. DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and DNA quality was checked on a 0.8% agarose gel. 5–50 ng DNA was used to generate amp icons using a MetaVx™ Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). A panel of proprietary primers was designed to anneal to the relatively conserved regions bordering V3 (variable V3 region

of 16S rRNA), V4 (variable V4 region of 16S rRNA), and V5 (variable V5 region of 16S rRNA) hypervariable regions. The V3 and V4 regions were amplified using forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGACTACNVGGGTWTCTAATCC". The V4 and V5 regions were amplified using forward primers containing the sequence "GTGYCAGCMGCCGCGGTAA" and reverse primers containing the sequence "CTTGTGCGGKCCCCCG YCAATTC". Besides the 16S target-specific sequence, the primers also contain adaptor sequences allowing uniform amplification of the library with high complexity ready for downstream NGS sequencing on Illumina Miseq.

DNA libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2×250 or 2×300 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) on the MiSeq instrument. The sequences were processed and analyzed by GENEWIZ. Taxonomy analysis was carried out on Qiime platform.

3. Results and discussion

3.1. The effect of microaerobic condition on the thermophilic fermentation performance of corn straw and mathematical model analysis

The methane production during thermophilic anaerobic digestion of corn straw was shown in Fig. 1. As can be seen, methane production started actively after incubation. At least 80% of the total methane yield was achieved within the first 12 days of AD. The average methane production rates of T0, T1, T2, T3 and T4 were 6.2, 7.2, 6.9, 6.8 and 6.3 mL/g $VS_{substrate}$ per day, respectively. The methane production rate of T1 was 16.1% higher than that of T0. Air introduced during AD process could obviously improve the cumulative methane yield of corn straw. When the air load was 12.5 mL/L_R-d, cumulative methane yield reached maximum, which was 216.8 mL/g $VS_{substrate}$ and 16.5% higher than that of sample under anaerobic condition. Improved methane yield from cellulosic substrate after pretreatment has also been reported by other authors [24–26]. Though the improvements were different, (this has something to do with the composition of the substrate and the different operational conditions [24]) these methods all can accelerate the hydrolysis process by destroying the substrate

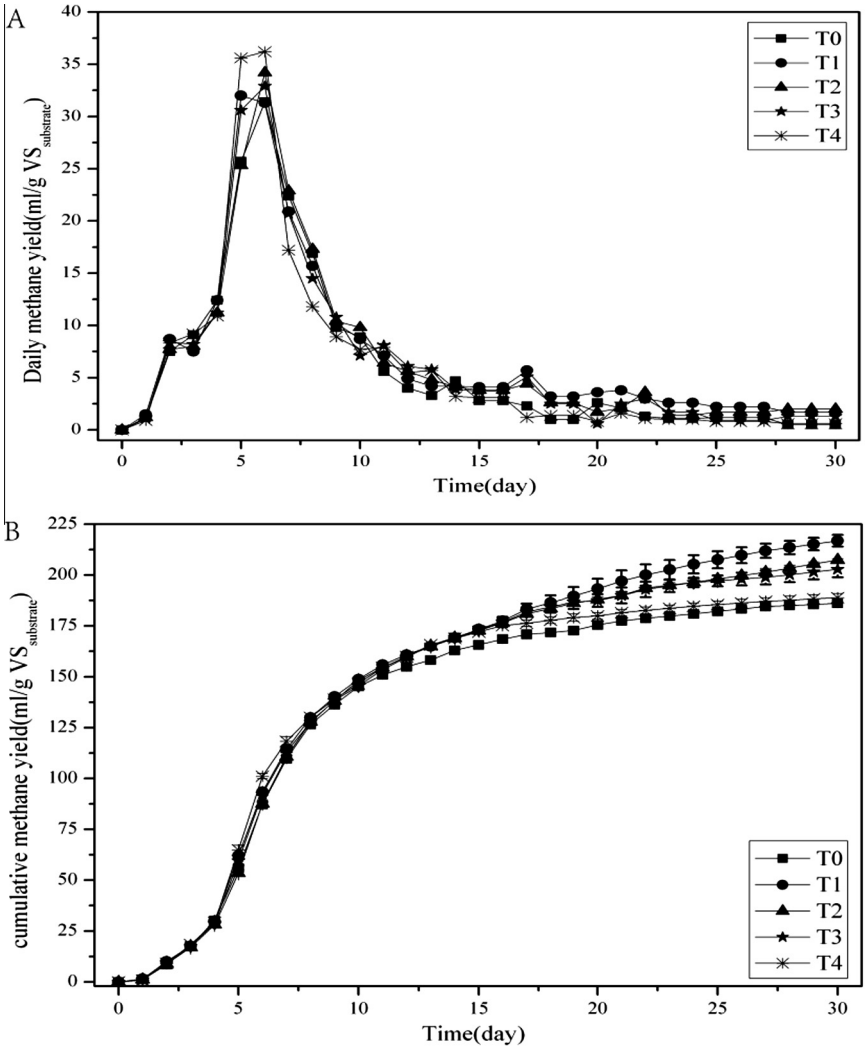

Fig. 1. Methane production during thermophilic anaerobic digestion of corn straw (A: daily methane yield, B: cumulative methane yield).

Table 1
Parameters of first order equation fitting experimental data.

Group	P_∞ (ml/g $VS_{substrate}$)	k_H (d^{-1})	Lp (d)	R^2
T0	194.3 ± 5.3	0.128 ± 0.012	1.122 ± 0.232	0.965
T1	227.7 ± 6.5	0.104 ± 0.009	1.080 ± 0.222	0.977
T2	216.5 ± 6.4	0.111 ± 0.010	1.160 ± 0.233	0.971
T3	213.1 ± 5.8	0.116 ± 0.010	1.120 ± 0.222	0.973
T4	196.7 ± 5.2	0.135 ± 0.012	1.092 ± 0.227	0.963

directly or improving the activity of extracellular enzyme. However, increasing air load did not lead to higher methane yield, which was very agreed with our previous study [20]. The same trend between oxygen load and methane yield was also reported by Mshandete et al. [18] and Botheju et al. [27]. The reason behind these could be inhibition of methanogens and substrate competition of facultative organisms [4,28].

At the end of thermophilic anaerobic digestion of corn straw, the VS removal efficiencies of T0, T1, T2, T3 and T4 were 49.3 ± 0.7%, 54.3 ± 1.5%, 53.3 ± 0.2%, 50.6 ± 1.4% and 50.1 ± 0.5%, respectively. The VS removal efficiency of T1 was 10.3% higher than that of T0. According to Appels et al. [29], the removal efficiency of VS expressed the degradation extent of substrate. Therefore, it could be concluded more substrate was used under microaerobic condition, which would be beneficial for the fermentation residue reduction.

The experimental data fitted with modified first order equation was shown in Table 1. The parameter k_H could represent the rate of hydrolysis process of AD, a lower k_H means a higher hydrolysis rate [15]. The k_H of T1, T2 and T3 were all lower than T0, which represented higher hydrolysis rate under microaerobic condition.

3.2. The effect of limited air supplied on the bacterial community structure

At the end of anaerobic digestion, the fermentation broth samples were collected from T0 and T1 for microbial community structure analysis. The 16S rRNA gene fragments of T0 and T1 were assigned to different taxa levels from phylum to order (as shown in Fig. 2). There were total 26 and 27 indentified bacterial phyla in T0 and T1, respectively. *Firmicutes* was the richest phylum in all samples. *Chloroflexi* and *Synergistetes* were the second and third dominant phylum. *Firmicutes* is known to produce extracellular enzymes (e.g. cellulase, lipase and protease), which mainly works

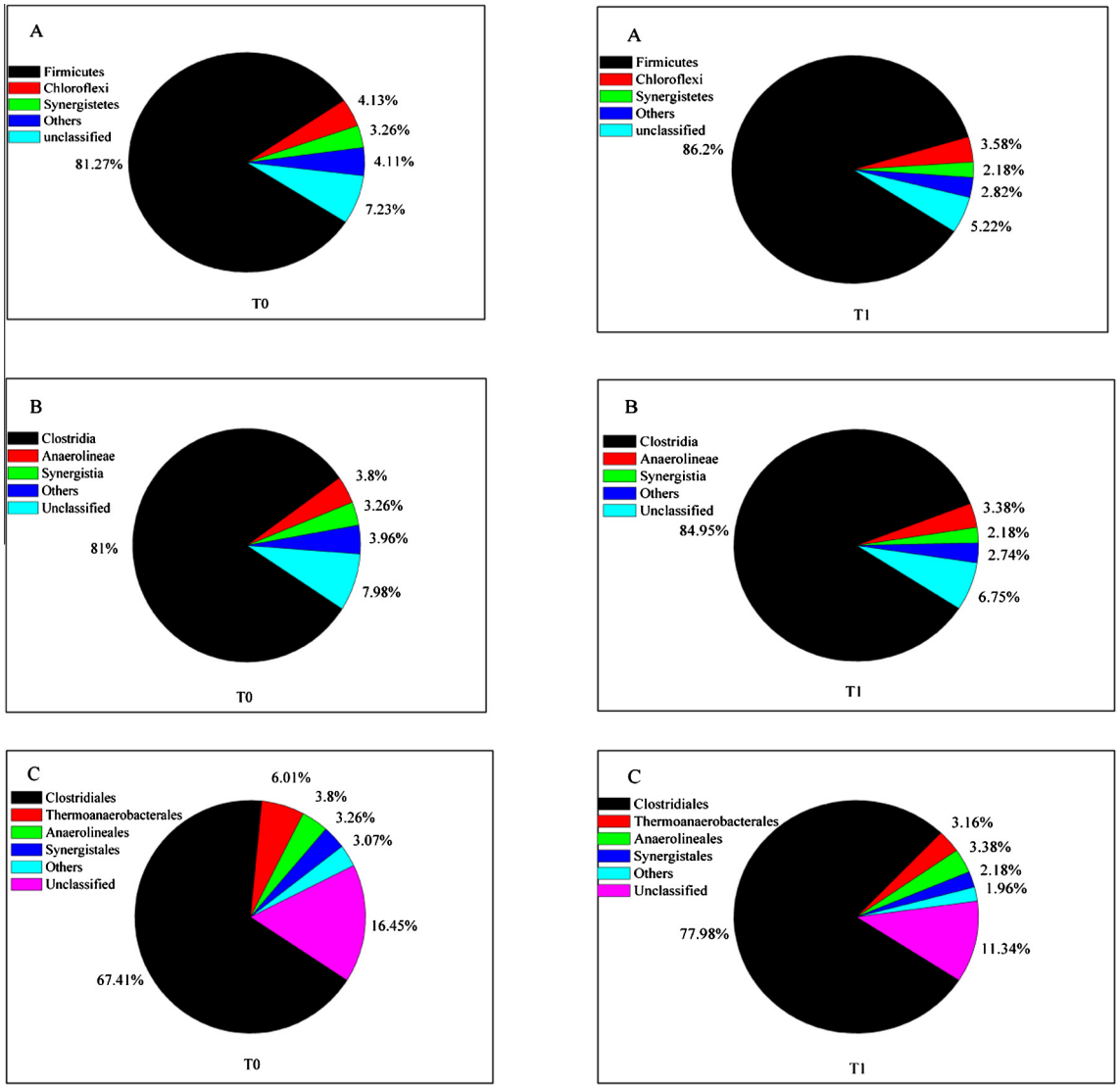

Fig. 2. Taxonomic classifications of the microbial communities from T0 and T1 at the (A) phylum, (B) class and (C) order levels. Phylum, class and order marking up less than 1% of the total sequences in each sample were classified as others.

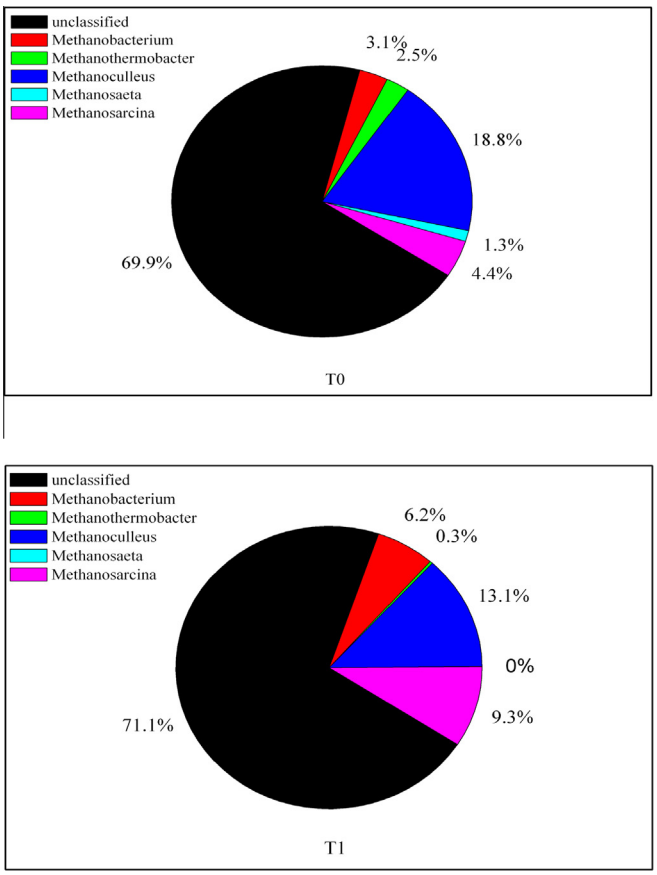

Fig. 3. Taxonomic classifications of the archaeal communities from T0 and T1 at the genus level.

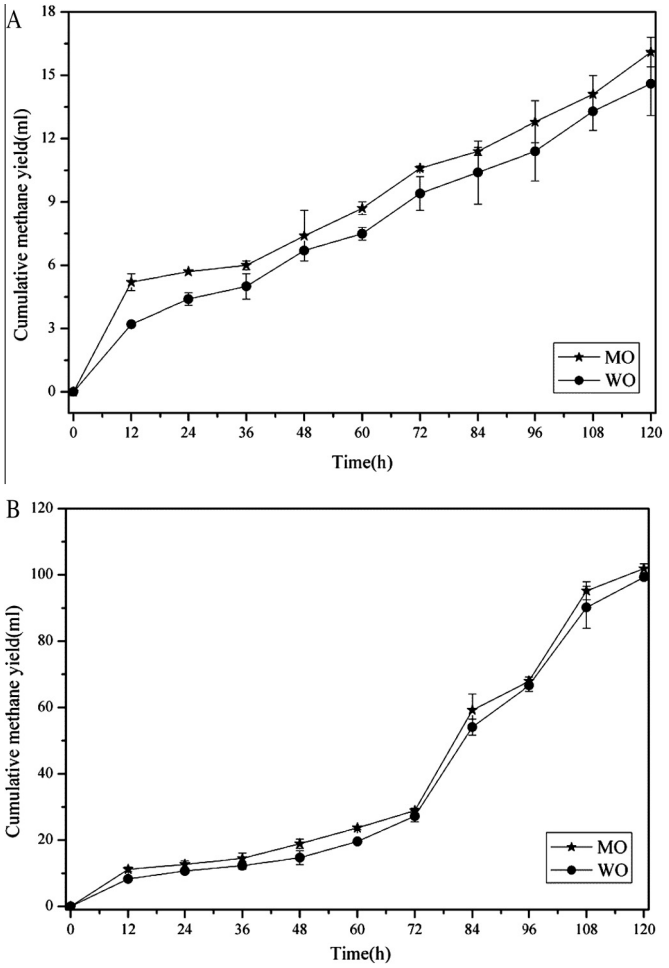

Fig. 4. Specific methanogenic activity under anaerobic and microaerobic conditions (A: specific acetotrophic methanogenic activity (SAMA), B: specific hydrogenotrophic methanogenic activity (SHMA)).

during the metabolism of cellulose, protein, lignin and lipids [30]. The relative abundance of *Firmicutes* in T1 was 6.08% higher than that of T0, which reflected the greater ability of T1 to metabolize complex substrate.

The microbial community structures of T0 and T1 at class and order level were illustrated in Fig. 2B and C. *Clostridia*, which affiliated to phylum *Firmicutes* was the most dominant, and followed by *Anaerolineae* and *Synergistia*. The relative abundance of class *Clostridia* in T0 and T1 were 81% and 84.95%, respectively, the most of which belong to the order *Clostridiales*. *Clostridia* are known to play a key role in biogas-producing process [31,32]. The high cellulolytic activity of most members belongs to order *Clostridiales* contributed to the breakdown of polysaccharide molecules, moreover, members of order *Clostridiales* could also ferment sugar to organic acids [33]. The relative abundance of order *Clostridiales* in T1 was 77.99%, which was 15.7% higher than that of T0. The higher abundance of order *Clostridiales* in T1 demonstrated the higher ability of T1 to break the polysaccharide molecules of substrate and ferment sugar to organic acids, which also meant higher hydrolysis under microaerobic condition.

During the anaerobic digestion of cellulosic substrate like corn straw, the hydrolysis process is widely considered as the rate-limiting step [6,34,35]. Compared to anaerobic condition, fermentation under microaerobic condition gave rise to the relative abundances of phylum *Firmicutes*, class *Clostridia* and order *Clostridiales*, which associated with hydrolysis. The fermentation under microaerobic condition therefore was able to metabolize complex substrates, which led to higher hydrolysis rate.

3.3. The effect of limited air supplied on the archaeal community structure and specific methanogenic activity (SMA)

3.3.1. The archaeal community structure change under microaerobic condition

Methanogenic microorganisms are conventionally thought to be strictly anaerobic, tiny oxygen exposed may be lethal to the activity of methanogenic microorganisms [11,36]. However, it was reported methanogenic microorganisms belong to *Methanobacterium* and *Methanosarcina* all showed tolerance to limited oxygen [11,12]. The archaeal community structure was shown in Fig. 3. *Methanoculleus* was the most dominant genus, which was also reported by Wirth et al. [33] to be the richest genus in the fermentation system with maize silage and pig manure slurry as substrate. Beside *Methanoculleus*, the genus *Methanosarcina* and *Methanobacterium* were also dominant genus. Compared with T0, the relative abundance of genus *Methanosarcina* and *Methanobacterium* all doubled in T1. As for the genus *Methanoculleus*, the relative abundance in T0 was 43.5% higher than that of T1. It could be concluded that under microaerobic condition the archaeal community structure was changed to acclimatize the microaerobic condition. The rise in relative abundance of oxytolerant methanogens ensured the efficient running of methanogenesis process under microaerobic condition.

3.3.2. Specific methanogenic activity under anaerobic and microaerobic conditions

In order to investigate the effect of daily air supplied on the specific methanogenic activity, specific acetotrophic methanogenic activity (SAMA) and specific hydrogenotrophic methanogenic activity (SHMA) under anaerobic and microaerobic conditions were tested. Results were shown in Fig. 4. For WO, the SMA tests were performed under anaerobic condition throughout the process. For MO, 2.5 mL air at atmospheric pressure was daily supplied, which was same with T1. The specific methanogenic activity could be characterized by the methane production rate [37]. During the five days tests, the average SAMA of WO and MO were 2.9 and 3.2 mL/d respectively and the average SHMA of WO and MO were 19.9 and 20.4 mL/d, respectively. SMA at the air load of 12.5 mL/L_R-d improved slightly (10.3% higher for SAMA, 2.5% higher for SHMA). The improved SMA under microaerobic condition could be due to the rise in the relative abundance of oxytolerant methanogens.

4. Conclusions

Daily air supplied at the load of 12.5 ml/L_R per day improved the methane yield and the VS removal efficiency of corn straw obviously. Under microaerobic condition, the relative abundance of bacteria associated with the hydrolysis raised obviously, the hydrolysis was therefore improved. In addition, the relative abundance of oxytolerant methanogens also raised significantly, accordingly, the SMA under microaerobic condition also improved. The microbial community structure shift might be the reason for better AD performance of corn straw under microaerobic condition. During anaerobic digestion of cellulosic substrate like corn straw, limited oxygen introduced to the anaerobic system could accelerate the hydrolysis process, therefore improved the anaerobic digestion efficiency.

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