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1 **Simultaneous biogas and biogas slurry production from co-digestion of pig manure**

2 **and corn straw: performance optimization and microbial community shift**

3 **Jing Ning^{a,b}, Mingdian Zhou^{a,b}, Xiaofang Pan^a, Chunxing Li^c, Nan Lv^{a,b}, Tao Wang^{a,b},**

4 **Guanjing Cai^a, Ruming Wang^{a,b}, Junjie Li^{a,b}, Gefu Zhu^{a*}**

6 ^a*Key Laboratory of Urban Pollutant Conversion, Institute of Urban Environment, Chinese*

7 ^a*Academy of Sciences, Xiamen 361021, China*

8 ^b*University of Chinese Academy of Sciences, Beijing 100049, China*

9 ^c*Department of Environmental Engineering, Technical University of Denmark, DK-2800*

10 ^c*Lyngby, Denmark*

*Corresponding author.

E-mail: gfzhu@iue.ac.cn; Phone: 86-592-6190790; Fax: 86-592-6190790

Abstract: Anaerobic co-digestion (AcoD) is proved as an effective approach to solving a bottleneck problem of the low biogas yield in agricultural biomass waste treatment with anaerobic digestion (AD) technology. The present study investigated the effect of C/N ratio, organic loading rate (OLR) and total solids (TS) contents on reactor performance in AcoD of pig manure and corn straw for simultaneous biogas and biogas slurry production. It was found that the highest biogas production was obtained at C/N ratio of 25, while the best biogas slurry performance was achieved at C/N ratio of 35. And high OLR and TS resulted in good performances in both biogas production and biogas slurry. At last, the microbial community analysis suggested that *Bacteroidetes* played a significant role in AcoD process. Acetoclastic methanogenesis was the main pathway for methane production in the stable system. And changing operational parameters could transform and shift the microbial community.

Keywords: Agricultural biomass waste; Anaerobic co-digestion; Biogas; Biogas slurry; Microbial community shift.

1 Introduction

With the development of agricultural economy, a large amount of agricultural biomass waste is produced, and now the annual output of livestock manure and crop straw (two major types of agricultural biomass waste) are 1.04 and 3.80 billion tons, respectively, in China. Nowadays, many environmental problems, such as deterioration of water quality, odor problem and eutrophication, have been occurred due to the irrational utilization of wastes (Nasir et al., 2012). So, it is necessary to suitably utilize the agricultural waste,

which can reduce pollution and alleviate global resource shortage. At present, there are several common agricultural biomass waste treatment methods including composting, making building materials and producing chemicals (like ethanol, ethylene, acrylic acid) (Debertoldi et al., 1983; Sawatdeenarunat et al., 2016). However, there were many problems occurred during applying these methods for disposal of biomass waste, like secondary pollution, insufficient utilization and difficulties in operation and management (Onwosi et al., 2017). Thus, it is necessary and urgent to seek appropriate technology to well make use of agricultural biomass waste.

Anaerobic digestion (AD) has the dual advantage in pollution control and resource recovery. The organic pollutants can be degraded through the metabolism of anaerobic microorganisms to solve environmental pollution problems. In addition, the produced biogas can be applied as clean renewable energy, and biogas slurry containing abundant nutrients including nitrogen, phosphorus and potassium, owned huge potential for liquid fertilizer preparation (Sawatdeenarunat et al., 2015). And up to now, the AD technology has been widely applied in many countries for waste treatment (Akhiar et al., 2017).

In terms of agricultural biomass waste treatment with AD technology, there is a bottleneck problem of the low biogas yield, which is mainly led by the imbalance of carbon to nitrogen ratio (C/N). It was reported that the optimum C/N ratio value ranged from 20 to 30 in AD process (Sawatdeenarunat et al., 2015). As the crop straw containing high content of lignocellulose with a relative high C/N ratio, it can easily cause the accumulation of volatile fatty acids (VFAs) when utilized as sole substrate in AD process.

And livestock manure has a lower C/N ratio due to its high protein content. The accumulation of ammonia can inhibit the growth of methanogenic microorganisms (Chen et al., 2008). According to the above two problems, researchers tried to balance C/N ratio of substrates via anaerobic co-digestion (AcoD) for the agricultural biomass waste treatment to obtain better performance. Lehtomäki et al. (2007) found that the volumetric methane production increased by 65%, 58% and 16% in manure AcoD with sugar beet tops, grass and straw, respectively. Zhao et al. (2018) reported that the cumulative methane yield in AcoD of cow manure and oat straw was 26.64% higher than that of oat straw alone. In recent years, the research mainly focused on the effect of operation parameters on biogas production, such as C/N ratio, organic loading rate (OLR), total solids (TS) contents and so on. Aboudid et al. (2016) found that specific methane production reached the highest value at C/N ratio of 23.5: 1 in AcoD of sugar beet byproduct and cow manure. Li et al. (2015b) reported the maximum biogas production rate was obtained at OLR of 6 g VS·L⁻¹·d⁻¹ in AcoD of rice straw and cow manure. However, most of these studies were conducted in batch experiment, little work was investigated on semi-continuous or continuous reactor operation, which had higher guidance for the practical project. And also, many researchers paid much attention to the impact of TS on AD process, while only a few articles studied its effect on AcoD of livestock manure and crop straw.

In terms of another important product of biogas slurry obtained from AD process, the nutrient concentration in biogas slurry is generally too low to be utilized as fertilizer directly (Fan et al., 2014; Li et al., 2013). And technology of concentration and nutrient

recovery, such as ammonia stripping, ion exchange and vacuum evaporation, are commonly applied to improve the fertilizer efficiency of biogas slurry (Li et al., 2016). But these techniques are difficult to be widely applied due to the high expensive capital expenditure. Therefore, maximizing nutrient concentration of biogas slurry should be considered in AcoD to make full use of resources. So far, there is no literature on this subject. Furthermore, if high biogas yield and high nutrient concentration (N, P and K) from biogas slurry can be simultaneously achieved during AcoD, it will construct an efficient process for sufficient resource utilization of agricultural biomass waste.

Based on the above, this study constructed a simultaneous biogas and biogas slurry production process from AcoD of livestock manure and crop straw. The effect of different operational parameters of C/N ratio, OLR and TS on biogas production and nutrient concentration of biogas slurry were studied in semi-continuous experiment to optimize the operational process. Moreover, the characteristics of the microbial community structure involved in the AcoD were revealed by high-throughput 16S rRNA pyrosequencing. All in all, this study provided detailed technical parameters and practical guidance for the exploration of highly efficient technology for simultaneous production of biogas and biogas slurry from agricultural biomass waste.

2 Material and methods

2.1 Substrates and inoculum

The basic characteristics of the substrates and inoculum were shown in [Table 1](#). The inoculum was obtained from an internal circulation anaerobic reactor (IC) which used food

starch as feedstock under mesophilic condition (37 ± 1 °C). Prior to use, the inoculum was acclimated using glucose at 37 °C for 1 week. The pig manure was collected from a pig farm of Lesen Ecological Co. Ltd in Xiang'an city, Fujian province, China. The sample was stored at -18 °C for use. It was diluted to desired feeding TS concentration and blended in a high-speed blender before inoculation. The corn straw was obtained from Chenghua Abrasive Co. Ltd in Jimei city, Fujian province, China, with particle size less than 1mm. To improve its digestion efficiency, the corn straw was pretreated as its high lignocellulosic content was difficult to be decomposed and utilized by microorganism (Zheng et al., 2014; Kumar et al., 2009). In this experiment, NaOH was used to pretreat the corn straw in a glass bottle with a rubber stopper (Chandra et al., 2012). The corn straw was soaked in 5% NaOH solution (g NaOH/g TS) with a solid to liquid ratio of 1: 10 (g: ml) at ambient temperature for 5 days and manually mixed twice a day. After pretreatment, the corn straw was washed to neutral with deionized water for seal storage before dried in vacuum drier at 60 °C.

Table 1

2.2 Experimental design

All the AD experiments were performed in three continuous stirred tank reactors (CSTR) with working volume of 8 L (Fig. 1). The temperature of the CSTR was maintained at 37 ± 1 °C by a hot water jacket system, and stirred by a mechanical agitating equipment at $130 \text{ rpm} \cdot \text{min}^{-1}$. The prepared feed was stored in a stirring tank with volume of 5 L, which was controlled at 6 °C by a water jacket system. The biogas produced were

collected using the gas bag.

Fig. 1

In this experiment, the pig manure with OLR of $0.5 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ and inoculum with $200 \text{ g} \cdot \text{L}^{-1}$ (based on dry matter) were injected into three CSTR reactors (CSTR I, CSTR II and CSTR III). And the OLR was gradually increased to 1, 1.5 and $2 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ to startup of the reactors, until system reached steady state. CSTR I was applied to investigate the effect of different C/N ratios (13.45, 20, 25, 30, 35 and 300) on AcoD. TS of feed substrates and OLR remained constant, which were $2 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ and 5% respectively throughout the fermentation process. Then, the evaluation of OLR (2, 3, 4, 5, 6 and $8 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$) and TS (5%, 8%, 12%, 15%, 20% and 25%) on the performance of AcoD of pig manure and corn straw were conducted in CSTR II and CSTR III applying the optimal C/N ratio. TS was maintained at 5% by shortening hydraulic retention time (HRT) in CSTR II. And TS was gradually increased with a constant OLR ($2 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$) in CSTR III. Table 2 shows the operating parameters in all systems under various conditions. Composition and volume of the biogas and nutrient concentration of biogas slurry including total nitrogen (TN), total phosphorus (TP) and total potassium (TK) were analyzed to evaluate the efficiency of AD.

Table 2

2.3 Analytical methods

The alkalinity, pH, concentration of total ammonia nitrogen (TAN) and TN, TS and VS were determined according to Standard Methods (APHA, 2012). C, N and C/N ratio

were analyzed by elemental analyzer (Vario Macro CHNS-O-/CL, Elementar, Germany). Samples were centrifuged at $12,000\text{ r}\cdot\text{min}^{-1}$ for 4 min. And the supernatant was acidized to $\text{pH} < 2$ using $3\text{ mol}\cdot\text{L}^{-1}$ HCl and filtered through a $0.22\text{ }\mu\text{m}$ membrane for analyzing VFAs including acetic acid, lactic acid, propionic acid, butyric acid, pentanoic acid and formic acid. VFAs were measured by ion chromatography (ICS-3000, Dionex, America) equipped with analytical column (PacTM AS11-HC, $4\times 250\text{ mm}$) and protect column (Ion PacTM AG11-HC, $4\times 50\text{ mm}$). The flow phase was KOH eluent and column temperature was $35\text{ }^{\circ}\text{C}$. Daily accumulative biogas production was collected using the gas bag. The specific biogas production rate (SBP) was calculated according to Eq. (1).

$$SBP = V_{day}/VS_{add} \quad (1)$$

Where V_{day} was the daily accumulative biogas production in the reactor, $\text{mL}\cdot\text{d}^{-1}$; VS_{add} was the daily feed added, g.

Biogas was analyzed using gas chromatography (GC9790 II, Fuli, China), equipped with a thermal conductivity detector TCD with argon as carrier gas. Temperature of thermal conductivity, column and detector were $160\text{ }^{\circ}\text{C}$, $120\text{ }^{\circ}\text{C}$ and $160\text{ }^{\circ}\text{C}$, respectively, and the model of packed column was TDX-1 (2 m long and a 3 mm inner diameter). TP and TK were measured by inductively coupled plasma - atomic emission spectrometry (Optima 7000DV, PerkinElmer, America).

2.4 DNA extraction and pyrosequencing analysis

The digestate samples were collected on the last day of each phase of the reactor to analyze their microbial communities via high-throughput 16S rRNA pyrosequencing. The

DNA was extracted from three samples from each stage and mixed for the high-throughput 16S rRNA pyrosequencing. And then the DNA was extracted by Fast DNA TM SPIN Kit for Soil (MP Biomedicals, USA). The V4 region of 16S rRNA gene was amplified by PCR using the primers 515FmodF (GTGYCAGCMGCCGCGGTAA) and 806RmodR (GGACTACNVGGGTWTCTAAT). Sequencing was performed on an Illumina Hiseq2500 PE250 platform accomplished by Novogene (Beijing, China).

3 Results and discussion

3.1 Effect of C/N ratio on production of biogas and biogas slurry

3.1.1 Analysis of the stability of the reactors at various C/N

The pig manure with C/N ratio of 13.45 was served as sole feedstock during startup period of reactor I. Results showed that pH was 6.95 - 7.00 (Fig. 2a), which located in the optimal range of AD of 6.80 - 7.20 (Hagos et al., 2017). The concentrations of TAN and VFAs were 367.66 mg·L⁻¹ and 11.56 mg·L⁻¹, respectively (Fig. 2a & 2b), which were lower than the inhibitory concentration during AD process (3860 mg·L⁻¹ and 3000 mg·L⁻¹) (Li et al., 2015a; Benabdallah et al., 2009). So, the system had sufficient buffering capacity and operated stably in this stage. Subsequently, pig manure and corn straw were fed with C/N ratio of 20 for AcoD. The concentration of TAN reduced gradually at the beginning, and then tended to be a constant of 247.76 mg·L⁻¹ finally. And as C/N ratio continues to go up, the concentration of TAN fell and reached a value of 160.71 mg·L⁻¹ at C/N ratio of 35 (Fig. 2a). This clearly showed that AcoD can prevent the possible adverse effect of high TAN on system stability during AD of pig manure. The change of alkalinity also declined

180 from 2610.92 to 1666.67 mg $\text{CaCO}_3 \cdot \text{L}^{-1}$ in AcoD (Fig. 2a), which was agreed with
181 previous results that alkalinity was mainly provided by TAN and reflected buffer capacity
182 during AD process (Nasir et al., 2012; Benabdallah et al., 2009). The pH also dropped from
183 6.92 to 6.65 which was lower than the optimum range of AD (Fig. 2a). Nevertheless, pH
184 still levelled off at each stage, and no VFAs accumulation occurred (no more than 20 mg·L⁻¹,
185 Fig. 2a) in the reactor. SBP also indicated that the reactor had normal operation
186 performance (Fig. 3a) at the C/N ratio of 20, 25, 30 and 35.

187 When pure corn straw (C/N ratio = 300) was fed in the reactor, TAN concentration
188 and alkalinity significantly decreased and dropped to 52.81 mg·L⁻¹ and 873.37 mg
189 $\text{CaCO}_3 \cdot \text{L}^{-1}$, respectively (Fig. 2a). In AD process, alkalinity of 1000 mg $\text{CaCO}_3 \cdot \text{L}^{-1}$ is
190 needed to resist CO_2 and VFAs, so as to maintain pH level and the stability of system (Ren
191 and Wang, 2004). Due to the low alkalinity, pH failed to maintain invariable and
192 significantly reduced to 6.18. VFAs was also accumulated up to 1661.74 mg·L⁻¹, consisting
193 primarily of propionic acid (1601.91 mg·L⁻¹) (Fig. 2b). Reports confirmed that high VFAs
194 concentration can inhibit AD, especially propionic acid (Akhiar et al., 2017; Dang et al.,
195 2016). Wang et al. (2009) explored the effect of different VFAs on AD that the
196 methanogenic microorganisms were inhibited with propionic acid concentration reaching
197 900 mg·L⁻¹. Obviously, propionic acid in this stage significantly exceeded the considered
198 inhibitory concentration. And the ratio of VFAs to alkalinity is used to determine the
199 stability of the AD system (Callaghan et al., 2002). When the value of VFAs/alkalinity is
200 less than 0.4, the system is considered to be stable (Callaghan et al., 2002). And it is

unstable with higher than 0.4. The high VFAs/alkalinity of 1.92 in the reactor demonstrated that the system was unstable, which were consistent with the SBP (Fig. 3a) in AD process with corn straw as sole feedstock.

Fig. 2

3.1.2 Effect of C/N ratio on performance of biogas production and biogas slurry

In addition, the SBP and CH₄ production was tested to characterize the effect of different C/N ratio on the biogas potential. The results meant that it was important to ensure that C/N ratio was not too high or too low, as this led to a low SBP and CH₄ production in AcoD. The maximum SBP was obtained at C/N ratio of 25 with 514.75 mL·g VS_{add}⁻¹·d⁻¹ on AcoD (Fig. 3a), which was consistent with the optimal C/N ratio mentioned in many literatures (Hagos et al., 2017; Zhang et al., 2016). Li et al. (2015a) reported that the biogas yield from AcoD of pig manure and rice straw reached the highest value at C/N ratio of 23.4. With the C/N ratio of 20, 25 and 30, the CH₄ proportion was 63.28%, 62.17% and 66.08%, respectively (Fig. 3b). The CH₄ proportion reduced significantly to 58.35% with C/N ratio of 35. Because methanogenic microorganisms are more sensitive to pH than acidogenesis microorganisms (Hagos et al., 2017). Growth of methanogenic bacteria slightly reduced at pH values of 6.65 below 6.80 in this phase. CH₄ production was 277.68, 320.02, 320.70, and 273.01 mL·g VS_{add}⁻¹·d⁻¹, respectively, at the four C/N ratios (Fig. 3b). Highest CH₄ production was obtained at C/N ratios of 25 and 30. Considering SBP and methane content, the AcoD system obtained the optimum biogas production performance at C/N ratio of 25.

Compared with AcoD, SBP was significantly inhibited during mono-digestion of corn straw, which was $46.00 \text{ mL} \cdot \text{g VS}_{\text{add}}^{-1} \cdot \text{d}^{-1}$ (Fig. 3a). And its proportion and production of CH_4 also reduced dramatically to 47.35% and $21.42 \text{ mL} \cdot \text{g VS}_{\text{add}}^{-1} \cdot \text{d}^{-1}$ due to the accumulation of VFAs (Fig. 3b). Many previous literatures also indicated that the imbalance C/N ratio of crop straw could lead to accumulation of VFAs causing lower biogas yield even system collapse (Sawatdeenarunat et al., 2015). Xie et al. (2011) reported that accumulation of VFAs inhibited AD process of single silage straw, and the system eventually failed. The results showed that the AcoD could effectively solve the problem of acid inhibition. Zhao et al. (2018) have also proven that AcoD of straw and nitrogen-rich substrate is beneficial to the stable operation of the reactor. And system with mono-digestion of pig manure was stable and SBP ($493.00 \text{ mL} \cdot \text{g VS}_{\text{add}}^{-1} \cdot \text{d}^{-1}$) was greater than that of AcoD with C/N ratios of 20, 30 and 35. Li et al. (2015b) also got the same result in AcoD of rice straw and cow manure. This may be due to the lower biodegradability and bioaccessibility of corn straw compared with pig manure. In AcoD with OLR of $2 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$, pig manure accounted for a small mass proportion of the feedstock. However, if mono-digestion of each feedstock of AcoD process was performed individually (Li et al., 2015a; Zhao et al., 2018), $\text{SBP}_{\text{Total, mono}}$ can be calculated according to Eq. (2), 347.03, 284.97, 243.41 and $212.43 \text{ mL} \cdot \text{g VS}_{\text{add}}^{-1} \cdot \text{d}^{-1}$ respectively. Compared with that, SBP increased by 26.45%, 80.63%, 99.38% and 120.25% respectively in AcoD. It can be seen that the AcoD obviously optimized the biogas production performance of ingredients.

$$\text{SBP}_{\text{Total, mono}} = R_{\text{PM}} \cdot \text{SBP}_{\text{PM, mono}} + R_{\text{CS}} \cdot \text{SBP}_{\text{CS, mono}} \quad (2)$$

Where $R_{pig\ manure}$ and $R_{corn\ straw}$ were respectively the proportion of pig manure and corn straw at different C/N ratio (based on VS).

Fig. 3

The results of nutrient concentration in biogas slurry indicated that the concentration of TK was the highest, followed by TN and TP was the least (Fig. 4a). And similar result was also reported by previous study (Jin et al., 2011). The total nutrient concentration was 809.96, 691.28, 660.26, 711.37, 749.29 and 518.92 $\text{mg}\cdot\text{L}^{-1}$, respectively, at C/N ratio of 13.45 (pig manure as mono-substrates), 20, 25, 30, 35 and 300 (corn straw as mono-substrates) (Fig. 4a). It was implied that the AcoD significantly enhanced the nutrient concentration (mainly in terms of TK), compared to AD of corn straw. Jin et al. (2011) analyzed the nutrient contents of 16 large-scale biogas project in Jiangsu province, and reported that concentration of TN, TP and TK in biogas slurry of pig farms was in the ranges of 400 - 900 $\text{mg}\cdot\text{L}^{-1}$, 30 - 100 $\text{mg}\cdot\text{L}^{-1}$ and 100 - 500 $\text{mg}\cdot\text{L}^{-1}$, respectively, which fitted with the relevant ranges in the present study. The lower concentration of TN and higher concentration of TK in other stages were caused by the reduction of pig manure and the increase of corn straw in the substrate. Total nutrient concentration in the reactor reached the highest value at C/N ratio of 35.

In general, the AcoD effectively solved the problem of low SBP and biogas slurry nutrients in mono-digestion and maximized the resource utilizing potential of corn straw.

Fig. 4

3.2 Effect of OLR on production of biogas and biogas slurry

3.2.1 Analysis of the stability of the reactors at various OLR

Organic nitrogen is converted to ammonia which is an essential nutrient substance required for microorganism during AD. With OLR from 2 to 6 $\text{VS}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, TAN gradually reduced from 322.87 to 283.42 $\text{mg}\cdot\text{L}^{-1}$ due to the consumption of nitrogen sources by methanogens, and further resulted in decrease in alkalinity from 2637.18 to 2400.97 $\text{mg}\cdot\text{CaCO}_3\cdot\text{L}^{-1}$ (Fig. 2c). As the OLR increased, the pH value edged down to 6.75 from 6.89 (Fig. 2c). Many researches have indicated that the increase in OLR could lead to a reduction of pH value (Sinbuathong et al., 2010). While, the pH was maintained at the suitable scope for methanogenic microorganism due to good buffering capacity at every stage. VFAs was consumed by syntrophic-methanogenic associations with maximum value of no more than 30 $\text{mg}\cdot\text{L}^{-1}$ and no acid inhibition occurred at the OLR of 2 - 6 $\text{g}\cdot\text{VS}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ (Fig. 2d). At the end of the experiment, the AcoD was operated at OLR of 8 $\text{g}\cdot\text{VS}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. During the first five days, TAN, alkalinity and pH value held constant. While, the sharp decline of pH to 5.93 after the fifth day was not conducive to the growth of methanogenic microorganism. The VFAs were up to 1423.58 $\text{mg}\cdot\text{L}^{-1}$ significantly including propionic acid (616.17 $\text{mg}\cdot\text{L}^{-1}$) and acetic acid (554.10 $\text{mg}\cdot\text{L}^{-1}$), which was below the inhibitory concentration. But the VFAs/alkalinity of 0.58 was higher than 0.4, indicating the unstable of the system. This was also corresponded to low SBP observed at the OLR of 8 $\text{g}\cdot\text{VS}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ (Fig. 3c). Obviously, VFAs/alkalinity is more reasonable as an indicator of system stability, compared with VFAs concentration.

3.2.2 Effect of OLR on performance of biogas production and biogas slurry

The SBP and CH₄ production in the CSTR reactor under different OLRs are listed in Fig. 3c and Fig. 3d, respectively. In this study, it was obviously observed that SBP and CH₄ production from AcoD declined with OLR increased from 2 to 3 g VS·L⁻¹·d⁻¹. While, they gradually increased after OLR going up to 5 g VS·L⁻¹·d⁻¹. It might due to that microorganisms obtained a higher ability of resist impact load after operating in increasing OLR system for a long time. The highest SBP and CH₄ production were achieved at OLR of 2 g VS·L⁻¹·d⁻¹ with 499.00 and 281.84 mL·g VS_{add}⁻¹·d⁻¹. Comino et al. (2010) found that the optimal OLR was 5.15 g VS·L⁻¹·d⁻¹ with highest SBP of 477.2 mL·g VS_{add}⁻¹·d⁻¹ in AcoD system of cow manure and energy crops. Kaoutar et al. (2015) reported that SBP reached the maximum in AcoD of sugar beet byproduct and pig manure with OLR of 4.2 g VS·L⁻¹·d⁻¹. Nevertheless, Li et al. (2015a) reported that there was no impact on biogas production with OLR of 3 - 8 g VS·L⁻¹·d⁻¹ in the AcoD of pig manure and rice straw. These indicated that the optimal OLR was different in various anaerobic systems. As OLR went from 2 to 6 g VS·L⁻¹·d⁻¹, volumetric biogas production rate (VBPR) significantly increased, which were 1.00, 1.31, 1.83, 2.29 and 2.61 L·L_{reactor volume}⁻¹·d⁻¹, respectively (Fig. 3c). VBPR is an indicator of the efficiency of the reactor. High VBPR improves the volume utilization of the reactor and reduces the operating cost, which is of great significance in practical engineering (Li et al., 2015a). It was obviously observed that SBP reached the maximum at low OLR, while high VBPR was obtained at high OLR. However, SBP and CH₄ production reduce under high OLR of 6 g VS·L⁻¹·d⁻¹. And when it further

raised to $8 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$, AcoD was inhibited with significantly decrease in SBP and VBPR. Because the growth of methanogens was inhibited by the high VFAs concentration resulted from the metabolic imbalance between acidogenesis microorganisms and methanogens (Ye et al., 2013). On the other hand, the short HTR caused a large loss of microorganisms at high OLR. Meanwhile, the slow growth and reproduction of methanogens was not conducive to timely supplement of microorganisms, which leads to the decline of the digestive capacity in the system. Previous studies also suggested that the system would deteriorate at excessive OLR in AD process. Kaoutar et al. (2015) and Li et al. (2015a) found that the system was inhibited at OLR of $12 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$. Chen et al. (2016) said that reactor could be operated stably when the OLR was lower than $1.8 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$. This may be due to the difference of substrates and reactors in AD.

Total nutrient concentration increased first and then decreased with obtaining the highest value of $974.30 \text{ mg} \cdot \text{L}^{-1}$ at OLR of $5 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ (Fig. 4b). It was mainly contributed by the increase in TN concentration. TK concentration fluctuated a little and TP concentration fell slightly as OLR elevated. It was conducive to the improvement of total nutrient concentration in biogas slurry with OLR lower than $5 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$. But the high OLR had negative effect on the production of total nutrient concentration of biogas slurry. Considering the biogas production and biogas slurry nutrients, the OLR of $5 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ could be a better operating parameter for better performance in SBP, CH_4 production and total nutrient concentration.

3.3 Effect of TS on production of biogas and biogas slurry

3.3.1 Analysis of the stability of the reactors at various TS

The performance of AcoD of pig manure and corn straw was investigated at different TS of 5%, 8%, 12%, 15%, 20% and 25%, respectively. TAN raised from 269.59 to 396.69 $\text{mg}\cdot\text{L}^{-1}$ along with TS gradually increasing from 5% to 15% (Fig. 2e). However, as TS further grew, TAN only presented a small decline. TAN was viewed as an important factor affecting the performance of anaerobic reactor. It has been reported that excessive TAN could inhibit the methanogens growth to decline biogas production, even caused the instability and failure of the system (Chen et al., 2008). TAN concentration in this system was kept at a low level, which was much lower than the inhibition level of 3860 $\text{mg}\cdot\text{L}^{-1}$ (2015). The change trend of alkalinity ranging from 2000 to 4000 $\text{mg CaCO}_3\cdot\text{L}^{-1}$ was consistent with that of TAN (Fig. 2e). Literature suggested that the alkalinity of the steady-state anaerobic system ranges between 1000 - 5000 $\text{mg CaCO}_3\cdot\text{L}^{-1}$ (Ren and Wang, 2004). So, the alkalinity in the system was suitable for AD process. With the increase of TS from 5% to 20%, the pH value stabilized at 6.90 - 7.00 (Fig. 2e). And when TS raised to 25%, the pH reduced gradually to 6.70 and remained steady due to good buffering capacity. And total VFAs concentration was kept at a low level, with a maximum of 20 $\text{mg}\cdot\text{L}^{-1}$ in this system (Fig. 2f). Obviously, there was no accumulation of VFAs and TAN with increasing TS from 5% to 25% in this system. Riya et al. (2018) reported that VFAs accumulation was observed in reactor with TS more than 28%. In this experiment, the pH presented a marked decline and CH_4 proportion also declined significantly at TS of 25% (Fig. 3f).

Further research is needed on whether pH will further reduce and VFAs accumulate with the continuous increase of TS.

3.3.2 Effect of TS on performance of biogas production and biogas slurry

TS of substance is another important factor effecting biogas production during AD process. The results of this study showed that the SBP were 499.23, 435.54, 482.11, 538.33, 549.82 and 461.43 mL·g VS_{add}⁻¹·d⁻¹ (Fig. 3e) with TS of 5%, 8%, 12%, 15%, 20% and 25%, respectively. As TS raised from 8% to 20%, SBP and CH₄ proportion gradually increased (Fig. 3e&3f). While, SBP and CH₄ proportion fell immediately when TS was 25%. Previous studied also revealed that CH₄ production was inhibited at high TS levels (Fan et al., 2014). Study suggested that the reduction of CH₄ production was possibly due to mass transfer limitation caused by the decrease of free water at high TS (Abbassi-Guendouz et al. 2012). Li et al. (2013) reported that CH₄ yield declined on AcoD of corn stover and chicken manure with TS range of 5.1% - 22.4%. But in these studies, the change in TS accompanied a change in OLR. In batch experiments, a few papers investigated the effect of variable TS on CH₄ production with a constant OLR and obtained the same phenomenon with this experiment. An et al. (2017) found CH₄ production increased firstly and then declined at TS of 2%, 4%, 6%, 8% and 10% in batch mesophilic AD. While there is no literature so far focusing on the effect of TS on CH₄ production in semi - continuous mesophilic AD. Xu et al. (2014) proposed a mass diffusion-caused hydrolysis inhibition mechanism to explain the phenomenon. And they revealed that there was a TS threshold between 15% and 20% for AD of lignocellulosic biomass. When TS is lower than the

threshold, CH₄ production tends to increase with the increase of TS, and falls after exceeding this threshold. It could be saw that TS threshold was around 20% in this experiment. And the highest SBP and CH₄ production were observed at TS of 20%.

The total nutrient concentration in the reactor were 660.26, 755.64, 922.78, 1144.38, 1372.64 and 1889.30 mg·L⁻¹ with TS of 5%, 8%, 12%, 15%, 20% and 25%, respectively.

Total nutrient concentration in biogas slurry increased significantly as TS raised (Fig. 4c).

When TS was 25%, the total nutrient concentration was about three times of that in TS of

5%. This was because the reduction of water content resulted in the concentration of

nutrients in biogas slurry as TS went up. Obviously, compared with C/N ratio and OLR, TS

had a relatively significant influence on total nutrient concentration. AcoD with high TS

not only improved the production of biogas, but also obtained biogas slurry with high

nutrient concentration. It was also suggested from the experiment that the high TS

substrate reduced the addition of additional water during AcoD and lowed the volume of

the biogas slurry.

In summary, it can be observed that the high SBP and high nutrient concentration (N,

P and K) from biogas slurry are not necessarily obtained simultaneously in AcoD system

with different C/N, OLR and TS. Therefore, in practical engineering, the selection of

operating parameters can be determined according to the economic calculation. In addition,

it is unilateral to evaluate the properties of biogas slurry only considering nutrient

concentration of N, P and K. In order to obtain more comprehensive information about

biogas slurry, some plant hormones, including indoleacetic acid, abscisic acid and

gibberellin, and safety indexes, such as antibiotics and heavy metals, are also required to be determined. Although the concentrations of indoleacetic acid, abscisic acid and gibberellin were detected in this study, the values were quite low (no data showed).

3.4 Microbiological analysis during biogas and biogas slurry co-production process

3.4.1 Dynamics of microbial community richness and diversity

The microbial diversity parameters, including Shannon, Simpson, Ace and Chao 1, were estimated at different stages of the reaction systems (Table 3). It could be observed that community diversity and population richness showed a decrease trend with increase of C/N ratio and OLR in stable phases of AcoD system. This could be explained by an increase in the relative abundance of microorganisms which performed major functions in the system (Fig. 6). Interestingly, when the system was in disorder (at C/N ratio of 300 and OLR of $8 \text{ g VS} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$), the diversity index plummeted. This suggested that community diversity in stable phase was significantly greater than that in deteriorative phase. The diversity indexes may be considered as indicators of process stability. While, Li et al. (2015) reported that the diversity indexes of both bacteria and archaea had no significant differences between stable and deteriorative phases in AD of food waste. This could be explained by the difference of other factors such as substrate in AD process. And it also was found that the diversity indexes had no significant differences between 5% and 8% at low TS. At TS of 12%, 15% and 20%, the same phenomenon was observed and the diversity index significantly dropped compared to that at low TS. A significant decrease was observed when TS continued to be up to 25%. It indicated that the microbial

community diversity of AcoD with high TS was lower than that of AcoD with low TS.

Table 3

3.4.2 Dynamics of bacterial populations at the phylum and genus level

For the bacterial community, the most abundant bacterial population phyla were *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, *Chloroflexi* and *Cloacimonetes* in all AcoD reactors (Fig. 5). The phylum *Bacteroidetes* was the dominant and the second predominant phylum was *Firmicutes* in AcoD of pig manure and corn straw process. However, many studies indicated that the *Firmicutes* predominated in AD or co-digestion of agricultural waste (Jiménez et al. 2016). Only Jiménez et al. (2016) investigated AcoD of pig manure and rice straw using clay as additive, and reported that *Bacteroidetes* was the dominant microorganisms in AD. When corn straw (C/N ratio = 300) as mono-substrates was digested, the relative abundance of *Firmicutes* increased obviously, which became the dominant microorganism in this stage. Zheng et al. (2015) also found that *Firmicutes* had a higher relative abundance during the AD of switchgrass alone in comparison with AcoD. This could be explained by the tolerance of *Firmicutes* with low pH (Zheng et al. 2015). At this stage, VFAs accumulation occurred and the pH went down significantly. In addition, it might also be resulted from the composition of substrate. *Firmicutes* is cellulose-degrading bacteria and have been reported as the dominant community in digester of lignocellulosic biomass in many papers (Xu et al. 2018). With the increase of TS, *Bacteroidetes* was gradually enriched and the relative abundance of *Firmicutes* dropped. Until TS was up to 25%, the relative abundance of *Bacteroidetes* had

a slight decrease. This was consistent with previous studies. Yi et al. (2014) also found the *Bacteroidetes* was enriched in the reactor with the increase of TS from 5% to 20% in the AD of food waste. And it was seen that the relative abundance of *Bacteroidetes* and *Firmicutes* was bound to change in the reverse direction when the operating parameter changed. Researchers had reported that there was strong negative correlation of the relative abundance between *Bacteroides* and *Firmicutes* (Chen et al. 2016). Studies have noted that the ratio of *Firmicutes* to *Bacteroidetes* (F/B) might have potential utility as an indicator of process stability, and dropped significantly during process disturbance and rebounded after process recovery (Chen et al. 2016). This finding was in agreement with the F/B variation in the system operated by different OLR, while F/B increased sharply when the system was disrupted in system of C/N with 300. Therefore, the rationality of F/B as an indicator of process stability of AD remains to be verified.

Fig. 5

On the level of genus, *Rikenellaceae* belonging to *Bacteroidetes* played an important role in in all AcoD reactors (Fig. 6). With increase of C/N ratio and TS, the relative abundance of *Rikenellaceae* was on the rise. *Rikenellaceae* was observed to be predominant and also showed an increase trend with the elevating of TS in other anaerobic digesters (Yi et al. 2014). *Rikenellaceae* had a very small fluctuation at OLR of 2 - 6 g VS·L⁻¹·d⁻¹ (Fig. 6c). While, it was dramatically enriched when OLR was up to 8 g VS·L⁻¹·d⁻¹, which was corresponded to the increase of acetic acid and propionic acid in reactor, since the main end-products of *Rikenellaceae* fermentation were acetic acid and propionic

acid (Yi et al. 2014). In addition, it could be observed that *Roseimarinus* belonging to *Bacteroides* remained at a level of relative abundance with 5.76%, which might due to the reactor had a pH of 5.93. *Roseimarinus* was observed to grow at pH 6.00 - 8.50 with optimum growth at pH 7.50 - 8.00 (Liu et al. 1999). *Bacteroidetes* (relative abundance of 13.70%) as the main genera in *Bacteroidetes*, can ferment carbohydrates, including glucose, to product of acid (mainly acetic and succinic acids) in the AD of corn straw (Fig. 6a). Compared to AcoD at different C/N ratios, *Acetivibrio*, *Turicibacter*, *Clostridiales*, *Saccharofermentans* and *Ruminococcaceae* belonging to *Firmicutes* were significantly enriched in AD system of corn straw serving as sole substrate, which accounted for 7.83%, 6.53%, 6.43%, 5.62% and 4.03% in relative abundance respectively. They could improve the production of short-chain fatty acids, among which *Ruminococcaceae* had a great capacity on cellulose and hemicellulose degradation of corn straw (Yi et al. 2014). As mentioned above, VFAs concentration in the reactor was relatively high at this stage, mainly propionic acid. Consistent with that, a lower proportion of *Smithella* (relative abundance of 0.65%), a propionate-oxidizing bacterium, could be observed (Rosenberg et al. 2014). Conversely, propionic acid was not the main acid of VFAs unlike corn straw as mono-substrates for AD at OLR of 8 g VS·L⁻¹·d⁻¹. Similarly, *Smithella* had high relative abundance.

Fig. 6

3.4.3 Dynamics of methanogens populations at the genus level

Considering the methanogens, *Methanosaeta* which use only acetate to produce

methane, had been dominant genus in all stable systems, indicating that acetoclastic methanogenesis played important roles in CH₄ production (Fig. 6). The relative abundance of dominant methanogens did not fluctuate at C/N ratio of 20, 25 and 30. Along with the C/N ratio increased to 35, the relative abundance of *Methanosaeta* went up. However, whether this was caused by the increase of C/N ratio still needs to be further investigation. With the increase of TS, the contribution of *Methanosaeta* was gradually enriched from 65.71% to 83.74%. And with the change of OLR, the relative abundance of *Methanosaeta* reduced at first and then increased. *Methanosaeta* accounted for more than 70% in relative abundance with OLR was 5 and 6 g VS·L⁻¹·d⁻¹ (Fig. 6d). However, when OLR was 8 g VS·L⁻¹·d⁻¹, *Methanoregula* became the dominant methanogens accounting for 60.44%, indicating that the dominant methanogenesis pathway shifted from acetoclastic methanogenesis to hydrogenotrophic methanogenesis. Some studies also observed hydrogenotrophic methanogens became more dominant in AD system under higher OLR condition (Ros et al. 2017). This was because that hydrogenotrophic methanogens have better tolerance to the environment, especially for low pH condition (Ros et al. 2017). Although high VFAs was accumulated during AD of corn straw, the dominant methanogenesis pathway was still acetoclastic methanogenesis. This might be due to the substrate composition was different between reactor I and II. The most abundant hydrogenotrophic methanogens were *Methanobacterium*, *Methanospirillum* and *Methanobrevibacter* in all stable systems (Fig. 6). Obviously, OLR had great influence on relative abundance of *Methanobacterium*. In general, the microbial community in AcoD

shifted with the variation of operating parameter, which was related to the change of SBP and reactor performance.

4 Conclusions

In this study, the optimal engineering parameters were obtained through investigating the effects of C/N ratio, OLR and TS on performance of biogas and biogas slurry from AcoD of pig manure and corn straw. It was found that the high SBP and high nutrient concentration from biogas slurry are not necessarily obtained simultaneously in AcoD. In practical engineering, the selection of process parameters can be determined according to the project requirement. The microbial community analysis indicated that the diversity and structure of microbial community were closely related to the operation parameters, which affect the operation state of the reactor.

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645

Figure Captions

Fig. 1 Experimental apparatus for anaerobic digestion.

Fig. 2 Reactor performance on all digestion: (a) pH, Alkalinity and TAN at various C/N ratio, (b) VFA at various C/N ratio, (c) pH, Alkalinity and TAN at various OLR, (d) VFA at various OLR (e) pH, Alkalinity and TAN at various TS, (f) VFA at various TS.

Fig. 3 Reactor biogas performance on all digestion: (a) SBR at various C/N ratio, (b) CH₄ proportion and CH₄ production at various C/N ratio, (c) SBR at various OLR, (d) CH₄ proportion and CH₄ production at various OLR (e) SBR at various TS, (f) CH₄ proportion and CH₄ production at various TS.

Fig. 4 Reactor biogas slurry performance on all digestion: (a) at various C/N ratio, (b) at various OLR, (c) at various TS.

Fig. 5 Taxonomic compositions in each sample at the phylum level of bacteria (a) at various C/N ratio, (b) at various OLR, (c) at various TS.

Fig. 6 Taxonomic compositions in each sample at the genus level of bacteria and methanogens (a) bacteria at various C/N ratio, (b) methanogens at various C/N ratio, (c) bacteria various OLR, (d) methanogens at various OLR, (e) bacteria at various TS, (f) methanogens at various TS.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

690

Table 1 Characteristics of the substrates and inoculums

691

Parameters	Pig manure	Corn straw	Pretreated corn straw	Inoculum
*C (%)	41.09±0.06	50.12±1.84	47.10±0.21	31.21±0.94
*N (%)	3.06±0.01	0.22±0.01	0.16±0.01	3.40±0.13
C/N ratio	13.45±0.02	227.80±8.34	294.38±5.6	9.18±0.07
TS (%)	30.07±0.87	90.33±0.07	99.02±0.07	15.83±0.18
VS (%)	81.06±0.64	86.02±0.79	86.09±0.83	62.12±0.15

Note: *based on dry matter.

692 Table 2 Design of operation parameters during anaerobic digestion

	C/N ratio	*PM/CS	TS (%)	OLR (g VS·L ⁻¹ ·d ⁻¹)	HTR (day)	#PM (g)	#CS (g)	Water (g)
CSTR I	13.45	—	5	2	20.31	65.64	0	328.21
	20	2.19	5	2	20.55	44.36	6.15	338.73
	25	1.22	5	2	20.74	35.05	8.77	341.87
	30	0.84	5	2	20.87	28.75	10.54	344.00
	35	0.63	5	2	20.97	24.19	11.82	345.54
	300	—	5	2	21.50	0	18.79	353.3
CSTR II	25	1.22	5	2	20.74	35.05	8.77	341.87
	25	1.22	5	3	13.83	52.58	13.15	512.80
	25	1.22	5	4	10.37	70.11	17.54	683.74
	25	1.22	5	5	8.30	87.63	21.92	854.67
	25	1.22	5	6	6.91	105.16	26.31	1025.61
	25	1.22	5	8	5.19	140.21	35.07	1367.48
CSTR III	25	1.22	5	2	20.73	35.35	8.77	341.87
	25	1.22	8	2	33.19	35.35	8.77	196.94
	25	1.22	12	2	49.78	35.35	8.77	116.59
	25	1.22	15	2	62.22	35.35	8.77	84.45
	25	1.22	20	2	82.96	35.35	8.77	52.31
	25	1.22	25	2	103.69	35.35	8.77	33.03

693 Note: *based on dry matter; # based on wet matter; PM means pig manure; CS means corn straw.

694

695 Table 3 Bacterial community richness and diversity during anaerobic digestion process.

Parameters	Taxonomy	Shannon	Simpson	Ace	Chao1
C/N ratio	20	7.5944	0.9845	1778.3015	1704.7609
	25	7.4119	0.9807	1933.1946	1960.6667
	30	6.9850	0.9732	1760.1241	1763.5412
	35	6.6482	0.9690	1791.9377	1761.3906
	300	6.1742	0.9567	1178.7112	1162.2264
OLR (gVS·L ⁻¹ ·d ⁻¹)	2	7.5278	0.9772	1426.2058	1499.1416
	3	6.5463	0.9455	1310.1586	1318.3778
	4	6.7986	0.9679	1386.2455	1413.9130
	5	6.4734	0.9647	1090.7276	1117.8594
	6	6.5194	0.9643	1094.5125	1106.7982
	8	5.8310	0.9400	956.1223	958.2376
TS (%)	5	7.0539	0.9754	1179.3714	1207.1667
	8	7.3329	0.9834	1332.5258	1316.8214
	12	6.6695	0.9729	978.6194	991.3628
	15	6.7691	0.9733	1096.8892	1103.1290
	20	6.6742	0.9735	1006.0719	976.0880
	25	6.2359	0.9658	802.9063	842.2340

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