Simultaneous Enhancement of Methane Production and Methane Content in Biogas from Waste Activated Sludge and Perennial Ryegrass Anaerobic Co-digestion: The Effects of pH and C/N Ratio

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Abstract It is necessary to find an appropriate strategy to simultaneously enhance the methane production and methane content in biogas produced from waste activated sludge (WAS) and grass co-digestion. In this study an efficient strategy, i.e., adjusting the initial pH 12 and C/N ratio 17/1, for simultaneous enhancement of methane production and methane content in biogas produced from WAS and perennial ryegrass co-digestion was reported. Experimental results indicated that the maximal methane production was 310 mL/gVS_{add} at the optimum conditions after 30-d anaerobic digestion, which was, respectively, about 1.5- and 3.8-fold of the sole WAS and sole perennial ryegrass anaerobic digestion. Meanwhile, the methane content in biogas was about 74%, which was much higher than that of sole WAS (64%) or sole perennial ryegrass (54%) anaerobic digestion.

Keywords: Waste activated sludge; Perennial ryegrass; Anaerobic co-digestion; Methane production; Methane content

1. Introduction

Huge amounts of waste activated sludge (WAS) were generated every year in wastewater treatment plants (WWTPs), which must be effectively disposed to avoid its secondary pollution to the environment. Anaerobic treatment is one of the most energy-efficient and environmental friendly technologies for sludge stabilization and sludge harmlessness and resource recovery. Nevertheless, anaerobic digestion of WAS as sole substrate has some disadvantages, such as low efficiency of organic conversion, long retention time and low yield of methane.

Anaerobic co-digestion of WAS with other organic matters has become a hot topic in recent years, for it is a feasible option to overcome the drawbacks of WAS mono-digestion and to improve the economic viability of anaerobic digestion plants due to higher methane production (Mata-Alvarez et al., 2000). WAS is characterized by relatively low C/N ratio and high buffer capacity (Astals et al., 2013), therefore, it is able to tolerate co-substrates with large amounts of easily biodegradable organic matter and with low alkalinity values. Meanwhile, green waste (e.g., grass) has long been used as principal livestock forage and is considered as an important source of biomass for anaerobic digestion (Prochnow et al., 2009). For example, co-digestion of grass silage with maize silage is

common in agricultural biogas plants of Germany (Weiland, 2006). In recent years, many investigations have evaluated the potential of WAS and grass co-digestion (Hidaka et al., 2013; Wang et al., 2014; Jia et al., 2013); the substantial increase in the use of grass as a co-substrate is due to its high biodegradability and associated biogas production rates compared to organic fraction of municipal solid waste (OFMSW) (Mata-Alvarez et al., 2000).

Previous studies showed that the addition of shredded grass in WAS and grass co-digestion system decreased the methane content in biogas even though the methane production was enhanced (Hidaka et al., 2013; Wang et al., 2014). The similar phenomenon was also found in WAS and food waste co-digestion system (Dai et al., 2013). Further, for biogas is primarily composed of methane and carbon dioxide, containing smaller amounts of hydrogen sulfide, ammonia and water vapor, biogas must be upgraded and dried before utilizing to prevent damage of the gas utilization units and the costs are relatively high since they need either high pressure or addition of chemicals. Thus, it is necessary to find an appropriate strategy to simultaneously enhance the methane production and methane content in biogas produced from WAS and grass co-digestion.

To improve the methane content in biogas produced from organic substrates, some researchers indicated that the configuration of reactor can affect the methane content in biogas (Jung et al., 2000; Sawyer et al., 1994); other investigations showed that the high methane content in biogas was partly because of the passing of biogas through a water filter which allowed dissolution of some of the carbon dioxide (Nizami and Murphy, 2011). Moreover, most of previous studies focused on providing enough hydrogen for the anaerobic digestion system via different ways, such as electronics industry (Kim et al., 2013) and simulated coke oven gas (Wang et al., 2013), can promote the interspecies H_2 /formate transfer pathway to enhance methane content in biogas. However, the current strategies can only improve the methane content in biogas and the energy consumptions are seldom considered, therefore a new strategy to simultaneously enhance methane production and methane content in biogas from anaerobic co-digestion system should be established. It has been reported in previous literature that when sludge was anaerobically treated under alkaline conditions the methane production was significantly enhanced (Zhang et

al., 2010), indicating that pH value affected the methane production and methane content in biogas from anaerobic digestion system; the synergistic impacts between sludge and grass appeared when they were digested together because the co-digestion of sludge and grass balanced the C/N ratio of anaerobic digestion system, which affected the hydrolysis, acidogenesis, acetogenesis and methanogenesis (Jia et al., 2013; Zhang et al., 2016). One method that can be used to simultaneously enhance the methane production and methane content in biogas from anaerobic digestion system, we think, is to combine the alkaline pretreatment and the synergistic impacts of sludge and grass co-digestion, by which the activities of key enzymes and microorganism community may be improved to digest the organics in the anaerobic co-digestion system.

The objectives of this study are as follows (**Figure S1**). First, this study aims to determine the impacts of C/N ratio and initial pH on methane generation and methane content in biogas produced from WAS and perennial ryegrass co-digestion. Second, this study aims to reveal the impacts of initial pH and C/N ratio on the consumptions of fermentation substrates and synergistic effects of main organic compounds in WAS and perennial ryegrass on methane production. Third, this study aims to evaluate the impacts of initial pH and C/N ratio on variations of process pH and total alkalinity. Finally, this study attempts to explore the shift of microbial community in anaerobic co-digestion system using the fluorescence in situ hybridization (FISH) technique and 454 pyrosequencing method. The activities of certain key enzymes that are associated with the anaerobic co-digestion process, such as protease, α -glucosidase, cellulase, pyruvate-ferredoxin oxidoreductase (POR) and acid-forming enzymes like phosphotransacetylase (PTA), acetate kinase (AK), were also considered.

2. Materials and methods

2.1. WAS and perennial ryegrass

The WAS used for anaerobic fermentation was obtained from the secondary sedimentation tank of a municipal wastewater treatment plant (WWTP) in Shanghai, China, which was operated with a traditional activated sludge process. After settling at 4°C for 24h, the sludge was concentrated, and the main characteristics (average data plus

standard deviations in duplicate tests) are shown in **Table S1** (Supplementary Data). The experimental perennial ryegrass, which were collected from Tongji University in October, 2014, were cut into the size of 1cm long and stored in a sealed bottle at -4°C temperature before feeding into the reactors. The main characteristics of perennial ryegrass were shown in **Table S1** (Supplementary Data).

2.2. Batch co-digestion experiments

To evaluate the methane production and methane content in biogas produced from sludge and perennial ryegrass anaerobic co-digestion, a series of identical reactors, with working volume of 1.0 L each, were used in the trials. All reactors, maintained at $35 \pm 1^\circ\text{C}$, are made of Plexiglas and stirred at 120 rpm (rotations per minute) to mix the contents. All the experiments were conducted in triplicate, and one way analysis of variance (ANOVA) at the 0.05 level was used to analyze the data.

The anaerobic co-digestion experiments to study the impacts of initial pH and C/N ratio on methane generation and methane content in biogas were operated in 52 reactors. The total volatile solids (VS) of each reactor were kept as the same amount 8 g/L. The reactors 1-2, without pH adjustment, were set as the blank tests: one filled with only WAS and the other filled with only perennial ryegrass. The other 50 reactors were conducted over a range of five C/N ratios (9/1, 12/1, 15/1, 17/1 and 20/1), while the initial pH were controlled at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0, respectively, by adding 2 M sodium hydroxide (NaOH) (**Table S2**, Supplementary Data) or 2 M hydrochloric acid (HCl). These 52 reactors were sealed with rubber stoppers and then fermented for 30 days. Every two days, the pH, total alkalinity, methane generation and methane content in biogas were monitored. The gas produced from these anaerobic reactors was collected using the water displacement method, and the composition of the gas was investigated by gas chromatography (Agilent 6890N, USA) with a thermal conductivity detector (TCD) and nitrogen as the carrier gas.

2.3. Semi-continuous-flow experiments to determine the key enzyme activities and microbial community

Three semi-continuous-flow reactors made of Plexiglas with working volume of 2.0 L each, internal diameters of

100mm and heights of 250mm were operated to investigate the key enzyme activities and microbial community.

Reactors 1-2 were set as the blank tests: one filled with only sludge and the other filled with only perennial

ryegrass. Besides, Reactor 3 was conducted with the optimum mixture of sludge and perennial ryegrass (C/N 17/1,

initial pH 12). The temperature was maintained at $35 \pm 1^\circ\text{C}$ and the agitator speed was controlled at 120 rpm.

Every day 100 ml amount of fermentation substrates were manually poured out from each reactor and the same

amount of new substrates were added, which resulted in the hydrolytic retention time (HRT) of 20d. When

methane production, pH, total alkalinity became stable after 100-d anaerobic digestion, the key enzymes activities

and microbial community were analyzed.

2.4. Experiments of the synergistic effect of main organic compounds in WAS and perennial ryegrass on methane production

Protein, polysaccharide, cellulose and hemicellulose were the main organic compounds of WAS and perennial

ryegrass. To investigate their roles in methane production, the batch experiments were operated with dextran, BSA

(bovine serum albumin), cellulose and xylan as model polysaccharide, model protein, model cellulose and model

hemicellulose. 6 anaerobic reactors with synthetic solution contained the following organic compound (g COD/kg

TS): 587 BSA (Reactor-1), 89 Dextran (Reactor-2), 587 BSA + 89 Dextran (Reactor-3), 510 Cellulose + 343

Xylan (Reactor-4), 587 BSA + 89 Dextran + 510 Cellulose + 343 Xylan (Reactor-5) and 587 BSA + 89 Dextran

+ 510 Cellulose + 343 Xylan (Reactor-5) + initial pH 12 (Reactor-6). The COD concentration in each reactor was

kept as the same amount 10 g/L. These organic matters were dissolved into 200 mL synthetic wastewater of each

bottle, containing (mg/L) 405 NaHCO_3 , 155 $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 50 CaCl_2 , 100 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 25 FeCl_2 , 10 NaCl , 5

$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 5 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.5 AlCl_3 , 15 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 5 H_3BO_3 , 5 $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 5 $\text{CuCl}_2 \cdot 5\text{H}_2\text{O}$, 5 ZnCl_2 (Ren

et al. 2008). Then 30 mL inoculums collected from the above anaerobic reactors (C/N 17/1 and initial pH 12)

was divided equally into each anaerobic reactor purged with nitrogen for 5 min in order to provide the anaerobic

conditions. All the 6 anaerobic reactors were then placed in an air-bath shaker (120 rpm) at $35 \pm 1^\circ\text{C}$ for 30 d. The

methane production was assayed.

2.5. Analytical methods

The determinations of pH, TS, VS, protein, carbohydrate and total alkalinity were the same as described in the previous publications (Dai et al., 2013; Zhang et al., 2010). The contents of hemicelluloses, cellulose and lignin in perennial ryegrass were determined by detergent extraction method (Goering and Van soest, 1970). The elemental compositions, including C (carbon), H (hydrogen), and N (nitrogen), of the sludge and perennial ryegrass were measured using an elemental analyzer (Elemental Analyzer NA 2500).

Protease, α -glucosidase, cellulase, pyruvate-ferredoxin oxidoreductase (POR) and acid-forming enzymes like phosphotransacetylase (PTA), acetate kinase (AK) were determined in this study. To determine the activities of protease, α -glucosidase, cellulase, POR and the acid-forming enzymes, the fermentation substrates (25 mL) was removed from the different anaerobic digestion reactors and then washed and suspended in 10 mL sodium phosphate buffer solution (100 mM). The suspension was sonicated at 20 kHz (4°C for 30 min) to break down the cells and then centrifuged at 10,000 rpm (4°C for 30 min) to remove the waste debris. The extracts were kept at -20°C before they were used in the enzymes activities assays. The analysis of PTA activity was according to the methods of Andersch et al. (Andersch et al., 1983) using acetyl-CoA as substrate. The activity of AK was assayed according to the method of Allen et al. (Allen et al., 1964) with potassium acetate as substrate. The activity of POR was detected according to Yakunin et al. (Yakunin and Hallenbeck, 1998). Activity of cellulase was determined by filter paper assay of Ghose (Ghose, 1987). The activities of protease and α -glucosidase were measured according to Miron et al. (Miron et al., 2000). One unit of enzyme activity was defined as the amount of enzyme necessary to catalyze 1 μ mol of substrate per minute. The specific enzyme activity was defined as the unit of enzyme activity per gram of VS.

The FISH (fluorescence in site hybridization) technique with 16S rRNA-targeted oligonucleotide probes was employed to monitor the difference of microbial community. The oligonucleotide probes used in this study were as follows: EUB338 for *Bacteria* (Alm et al., 1996), CHIS150 for *Acetogenic bacteria* (Franks et al., 1998), MS1414

for *Methanosarcinaceae* (Raskin et al., 1994), ARC915 for *Archaea* (Stahl et al., 1988), MX825 for *Methanosaetaceae* (Raskin et al., 1994). The detailed information about these probes is listed in **Table S3** (Supplementary Data). Hybridizations were performed at 46°C for 10 h with hybridization buffer containing 5 ng of each labeled probe/mL. The hybridization stringency was adjusted by adding formamide to the hybridization buffer (Zhang et al., 2011). After hybridization at 46°C for 10 h, the samples were stained with 4', 6'-diamidino-2-phenylindole (DAPI) (1 mg/mL). Then, the sections hybridized with the probes were observed with a confocal laser scanning microscope (CLSM, Leica TCS, SP2 AOBS). 454 high-throughput pyrosequencing methods are detailed in the Supplementary Information.

3. Results and Discussion.

3.1. Effects of C/N ratio and initial pH on methane production and methane content in biogas produced from WAS and perennial ryegrass co-digestion

Figure 1 shows the effects of C/N ratio and initial pH on methane production (a) and methane content (b) produced from WAS and perennial ryegrass co-digestion. It can be observed in **Figure 1a** that the methane generation presented a trend that it increased first and then decreased with the pH increased at a certain C/N ratio, but the pH of maximal methane generation at each C/N ratio was different. Furthermore, at each given pH value, the methane production was increased and then decreased with the C/N ratio improved. It seems that the optimum conditions for maximal methane generation were initial pH 12 and C/N ratio 17/1. Under such conditions the maximal methane production was 310 mL/gVS_{add}. When the pH was not controlled, the pHs of the WAS and grass digestion alone were 6.8 ± 0.2 and 5.9 ± 0.1 , and the methane productions of sole WAS (C/N 7/1) and sole ryegrass (C/N 26/1) were 200 mL/gVS_{add} and 80 mL/gVS_{add}, respectively. Obviously, the methane generation was significantly enhanced by controlling the initial pH 12 and C/N ratio 17/1, which was around 1.5- and 3.8- fold of the sole WAS and sole perennial ryegrass respectively. The above results indicated that the methane generation was improved at a suitable range of pH and C/N ratio, but extremely high pH value or C/N ratio would inhibit the

methane production. As shown in **Figure 1b**, the methane content in biogas decreased with the C/N ratio between 9/1 and 20/1 at a given pH value, which was in consistent with the previous publication (Dai et al., 2013; Hidaka et al., 2013). However, when the C/N ratio was constant, the methane content in biogas was increased first and then decreased with the pH increased from 4.0 to 13.0. The maximal methane content in biogas was 80% at initial pH 11 and C/N ratio 9/1, while the methane production of this condition was only 236 mL/gVS_{add}, which was much lower than that of initial pH 12 and C/N ratio 17/1. The pH and C/N ratio also effect the anaerobic digestion time of peak biogas production. The digestion time of peak biogas production was longer when the pH increased in a certain range, for the alkaline fermentation inhibited the methanogens and promoted the hydrolysis of WAS and perennial ryegrass. When the C/N ratio increased, the digestion time of peak biogas production was also longer, for the perennial ryegrass was not easy to be disrupted and digested. Apparently, the suitable fermentation conditions for maximal methane content in biogas were pH 12 and C/N ratio 17/1. Under such conditions the methane content in biogas was 74%, which was enhanced greatly compared with sole WAS (64%) or sole perennial ryegrass (54%).

Fig. 1 - Effects of C/N ratio and initial pH on methane production (a) and methane content (b) produced from WAS and perennial ryegrass co-digestion. Error bars represent standard deviations of three different measurements.

According to **Figure 1a and 1b**, the optimum conditions for simultaneous enhancement of methane production and methane content in biogas were initial pH 12 and C/N 17/1 at the fermentation time of 30d. Most of the studies on the influence of pH and time on anaerobic digestion focused on either sludge or grass. It has been reported in our previous investigation that the methane generation from sludge anaerobic digestion can be enhanced after pretreated at pH 10 for 8d (Zhang et al., 2010); the alkaline pretreatment has also been taken as a useful pretreated method to enhance the hydrolysis process during the grass anaerobic digestion. Some studies have been carried out to investigate the suitable C/N ratio for anaerobic digestion and the suggested C/N ratio was 20/1-30/1 (Hossain Molla et al., 2004), because the feedstock and organic loading rate used were different. Our

previous study showed that the maximal short-chain fatty acids production was at C/N ratio 20/1 and fermentation time 12d when WAS and perennial ryegrass were co-digested (Jia et al., 2013); however, the effects of pH and C/N ratio on methane production and methane content in biogas have never been documented. Furthermore, it is difficult to figure out the effects of pH and C/N ratio on microorganism involved in methane production and methane content from WAS and perennial ryegrass co-digestion as various species of microbial community are in anaerobic digestion system. Although the optimum pH and C/N range are still debatable in the literature, it seems that the adjustment of pH and C/N ratio of sludge and perennial ryegrass, would be more suitable for microorganisms and benefit for the simultaneous enhancement of methane production and methane content in biogas, which will be investigated in detail in the following text to understand the mechanisms of pH and C/N ratio affecting methane generation and methane content in anaerobic co-digestion process.

3.2. Effects of initial pH and C/N ratio on the consumptions of fermentation substrates

Protein and carbohydrate are the two predominant organic compounds in WAS, while cellulose, hemicellulose and lignin are the three main types of polymers that constitute perennial ryegrass. During the anaerobic digestion process, these organic matters can be converted to methane, so the consumptions of substrates were expressed by these organic matters in this study. The consumptions of protein and carbohydrate are shown in **Figure 2**. It can be seen that the consumptions of protein and carbohydrate under the optimum conditions were much higher than that of the sole WAS and sole perennial ryegrass, reaching 166 ± 6 and 666 ± 20 g-COD/kg-TS, respectively. The protein and carbohydrate consumptions in sole perennial ryegrass were higher than that in sole WAS, but the methane production of sole perennial ryegrass was much lower than sole WAS; the reason was that these organic matters in perennial ryegrass converted to volatile fatty acids (VFAs) leading to an extremely low pH of the anaerobic digestion system that inhibited the activities of methanogenic bacteria to convert the VFAs into methane (Liu et al., 2008). Further, the protein and carbohydrate of WAS were more easily biodegradable than the perennial ryegrass. Also, it is well-known that the maximum possible methane yield is 350 mL/g COD during anaerobic

treatment of wastes at standard temperature and pressure, which is equal to 395 mL/g COD at 35°C and standard pressure, so the organic compounds consumed from sole WAS can produce the relatively high methane.

The consumptions of cellulose, hemicellulose and lignin of the optimum conditions and sole perennial ryegrass are shown in **Table S4** (Supplementary Data). In this study, the optimum conditions (initial pH 12 and C/N ratio 17/1) provided an alkaline condition that promoted the hydrolysis of cellulose, hemicellulose and lignin. The consumptions of cellulose, hemicellulose and lignin under the optimum conditions were 280 ± 13 , 227 ± 12 and 75 ± 6 g-COD/kg-TS, which were much greater than that of the sole perennial ryegrass. Obviously, the cellulose and hemicellulose were easily digested during the anaerobic digestion process, and the lignin was hard to be disrupted, because the cellulose and hemicellulose are macromolecules consisted of the same or different carbohydrate units and the lignin is an aromatic polymer made up of phenyl propanoid precursors (Sanchez, 2009), in which the strong inter-linkage of these polymers gives a stable shape and structure to the grass. In order to facilitate the optimum methane production, different treatment methods were used to overcome the limitation of hydrolysis of grass silage, such as steam explosion, diluted acid hydrolysis and alkaline delignification (Njoku et al., 2012; Rafique et al., 2010), but energy consumptions of these pretreatment methods, during which the electric energy, thermal energy and chemical energy were consumed, has been scarcely considered. Based on **Figure 2** and **Table S4** (Supplementary Data), this result showed a good conformity with the fact that the methane production was the highest under the optimum conditions.

Fig. 2 - Effects of C/N ratio and initial pH on the consumptions of fermentation substrates. Error bars represent standard deviations of three different measurements.

3.3. Synergistic effect of main organic compounds in WAS and perennial ryegrass on methane production

In an anaerobic co-digestion system of WAS and perennial ryegrass, the main organic compounds are protein, carbohydrate, cellulose and hemicellulose. These organic matters will be finally converted into methane. In order to study the impacts of these organic matters on methane generation in an anaerobic co-digestion system, a series

of synthetic wastewater batch experiments were conducted. **Figure 3** illustrates the effects of these organic matters on methane generation in an anaerobic digestion system. It can be seen that the methane production of sole BSA (a model protein compound, Fig. 3-B) and sole dextran (a model polysaccharide compound, Fig.3-D) were 134 ± 7 and 106 ± 6 mL/g-COD, respectively, while the methane production of BSA and dextran co-digestion (Fig. 3-B+D) was 150 ± 9 mL/g-COD, which was higher than that of sole BSA or sole dextran, indicating that the nutrients balance was beneficial to methane production in an anaerobic digestion. Nevertheless, the methane production of cellulose and hemicellulose co-digestion (Fig. 3-C+H) was 72 ± 5 mL/g-COD, but the methane production of BSA, dextran, cellulose and hemicellulose co-digestion (Fig. 3-All) was not improved significantly (92 ± 5.6 mL/g-COD), resulted in a negative effect of co-digestion of these organic substrates at C/N 17/1. However, when the initial pH of the co-digestion system of these organic substrates was adjusted to 12 (Fig. 3-All+pH), the methane production was enhanced greatly to 226 ± 10 mL/g-COD, which was approximately 2.5-fold of the control without pH adjustment (Fig. 3-All). This result was in good conformity with the results in **Figure 1**. The pH of WAS and perennial ryegrass (WAS) co-digestion was 6.7 ± 0.5 , which was close to pH 7.0. As shown in **Figure 1**, the methane production of WAS and perennial ryegrass co-digestion (C/N 17/1, pH 7.0) was 102 mL/gVS_{add}, while the methane production of the optimum conditions (C/N 20/1, pH 12.0) was 310 mL/gVS_{add}. Approximately 3.0-fold of methane production was obtained when pH was adjusted to 12 compared with control without pH adjustment. It can be concluded that the synergetic effects of initial pH and C/N ratio had positive interactions under optimal conditions; the co-digestion system can improve the methane production compared to the sole substrate.

Fig. 3 - Synergistic effect of main organic compounds in WAS and perennial ryegrass on methane production at anaerobic digestion time of 30 d. B: BSA; D: Dextran; C: Cellulose; H: Hemicellulose; All: BSA + Dextran + Cellulose + Hemicellulose; pH: pH=12. Error bars represent standard deviations of three different measurements.

3.4. Effects of initial pH and C/N ratio on variations of process pH and total alkalinity

Figure 4a illustrates the effects of initial pH and C/N ratio on variations of pH during the 30-d anaerobic digestion time. It revealed that in the anaerobic digestion system of sole perennial ryegrass, the pH dropped from the second day to the twelfth day, and then kept steady around 4.78 ± 0.22 ; however, in the anaerobic digestion system of sole WAS or optimum conditions, the pH variations had the similar trend that the pHs decreased first and then increased to a relatively stable pH value at 7.10 ± 0.20 and 7.22 ± 0.14 , respectively. It is well known that hydrolysis, acidogenesis, acetogenesis and methanogenesis are usually involved in an anaerobic digestion system (Zhang et al., 2011), so we can easily understand that the pHs decreased first due to the hydrolysis and acidification process, and then increased to a relatively stable value for the methanogenesis process. With the low buffer capacity, the pH of sole perennial ryegrass decreased dramatically to around 4.78 ± 0.22 , which inhibited the activity of methanogenic bacteria (Liu et al., 2008).

Fig. 4 - Effects of initial pH and C/N ratio on pH (a) and total alkalinity (b) variation during the 30-day anaerobic digestion time. Error bars represent standard deviations of three different measurements.

Alkalinity is an important parameter of the anaerobic digestion system. Carbonate alkalinity refers to the solutions where bicarbonate and carbonate are the only species considered, whereas total alkalinity also involves other species endowed with acid-base properties. The variations of total alkalinity in the anaerobic digestion system are shown in **Figure 4b**, which was in correspondence with the variations of pH. It can be seen that in the anaerobic digestion system of sole perennial ryegrass, the total alkalinity maintained almost low and stable, while in the anaerobic digestion system of sole WAS, the total alkalinity increased first and then kept stable during the 30-d anaerobic digestion process. In the optimum conditions, the total alkalinity increased first and then decreased during the initial 16-d anaerobic digestion time, followed by it increased and kept stable. Obviously, the total alkalinity of the optimum conditions was much higher than that of the sole WAS, meanwhile the pHs were relatively the same between the sole WAS and the optimum conditions. The ammonia can also consume total alkalinity besides carbonate alkalinity in the anaerobic digestion system, and the concentrations of ammonia in the optimum conditions

reactor and the sole sludge reactor were similarly reaching 547.3 ± 17.2 and 567.9 ± 16.3 mg/L, respectively, which implied that the carbonate alkalinity of the optimum conditions was much higher than the sole WAS. Thus, it can be concluded that more carbon dioxide was dissolved into the liquid under the optimum conditions during the anaerobic digestion process. The similar phenomenon was also found in previous publication that during the anaerobic digestion process without pH control the pH value increased to around 8.0 and reached up to 8.3, meanwhile the methane content in biogas was enhanced in a positive way for more carbon dioxide was dissolved in the medium and as a result, the methane content in gas phase increased (Jung et al., 2000). It can be explained that the methane content produced from the WAS and perennial ryegrass co-digestion under the optimum conditions was much higher than that of the sole WAS or sole perennial ryegrass.

3.5. Effects of C/N ratio and initial pH on the activities of key enzymes in the anaerobic digestion system

The key enzymes involved in the WAS and perennial ryegrass co-digestion system are shown in **Figure S2** (Supplementary Data). It can be seen that protease, α -glucosidase and cellulases play important roles in the hydrolysis process. Polysaccharide in sludge and polysaccharide released from ryegrass are firstly hydrolyzed into monosaccharide, meanwhile protein are hydrolyzed into amino acids, and then they are both converted into pyruvate. In the proposed metabolic pathway, the pyruvic acid and amino acids can be converted into acetyl-CoA which will eventually be converted to acetic acid by PTA along with AK. POR is the enzyme that catalyzes TPP (thiamine pyrophosphate) - dependent oxidative decarboxylation of pyruvate to form acetyl-CoA, carbon dioxide and hydrogen (Yakunin and Hallenbeck, 1998).

As shown in **Table S5** (Supplementary Data), the activities of protease and α -glucosidase in the optimum conditions were the highest compared with the sole WAS and sole perennial ryegrass. The activity of cellulase in the optimum conditions was much higher than that of the sole WAS, but the activity of cellulase was lower than that of the sole perennial ryegrass. In general, the activities of protease, α -glucosidase and cellulase in the optimum conditions were enhanced greatly, indicating that the hydrolysis process of the WAS and perennial

ryegrass co-digestion system became more active in the optimum conditions. PTA and AK in optimum conditions were also more active than those in the sole WAS or sole perennial ryegrass, which explained that the methane production was improved significantly comparing with others. POR enzyme plays an important role in hydrogen production during the anaerobic digestion. POR activity became more active in the optimum conditions than that of the sole WAS or sole perennial ryegrass, indicating that more hydrogen was produced in the anaerobic digestion system and more methane was produced via the interspecies H_2 /formate transfer pathway.

3.6. Effects of C/N ratio and initial pH on the activities of microbial community in the anaerobic digestion

Figure S3 (Supplementary Data) shows the FISH (fluorescent in situ hybridization) analysis of the key functional *Bacterial* and *Archaeal* groups in three semi-continuous reactors fed respectively with the sole WAS, sole perennial ryegrass and the mixture of WAS and perennial ryegrass, and the results are analyzed with the image analysis system (Image-Pro Plus, V6.0). It was observed that the relative abundance of *Bacteria* and *Archaea* were both higher in the optimum conditions than sole WAS or sole perennial ryegrass, indicating that the microbial activity of WAS and perennial ryegrass co-digestion under the optimum conditions was much greater. The activities of *Methanosarcinaceae* and *Methanosaetaceae*, mainly utilizing acetic acid for methane production, were also higher in the optimum conditions, which showed a good conformity with that of the observed methane production and methane content. *Acetogenic bacteria*, synthesizing acetate, formic acid, hydrogen and so on by its metabolic pathway, plays an important role in an anaerobic digestion process. The abundance of *Acetogenic bacteria* in the optimum conditions was much higher compared with others, indicating that more methane was produced via acetic, formic acid or hydrogen metabolic.

The structures of microbial community were studied by 454 pyrosequencing. To estimate the microbial structures of the *Bacterial* and *Archaea* communities in the anaerobic reactors, the trimmed sequences were grouped into operational taxonomic units (OTUs) using a 97% identity threshold. **Table S6** (Supplementary Data) shows the sequence diversity and library coverage estimates of the samples. The sufficient coverage found in each

sample suggested that the 454 pyrosequencing method had captured the most frequently occurring organisms. As it is shown in **Figure 5 (a, b, c)**, the bacterial sequences generated from the anaerobic digestion system were distributed among four major phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Spirochaetes*. In the sample of sole perennial ryegrass digestion system, the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Spirochaetes* accounted for 61.1%, 30.6%, 3.69% and 0%, respectively, of all the *Bacterial* sequences found. In the sample of sole WAS, these values were 35.59%, 8.03%, 5.22% and 1.94%, respectively. However, in the sample of the optimum conditions, these values were 50.88%, 19.41%, 8.34% and 6.53%. It is obviously that in the optimum conditions, the relative abundance of *Bacteroidetes* was much higher than that of sole WAS. The sole perennial ryegrass in the anaerobic digestion system showed the highest relative abundance of *Bacteroidetes* compared with the optimum conditions and the sole WAS. For the phylum *Bacteroidetes* species can attack the 1,4a-glycosidic bonds of plant polysaccharides (Kindaichi et al., 2004), it is possible to metabolize carbohydrates to produce VFAs. *Firmicutes* are well-known as fermenters and syntrophic bacteria that can biodegrade VFAs, such as butyrate and its analogs (Garcia-Pena et al., 2011). The predominance of *Firmicutes* is an indication that these products are readily available for anaerobic digestion. *Proteobacteria* and *Spirochaetes* are also important bacteria involved in the hydrolysis and acidification of anaerobic digestion system. In the optimum conditions, the total portions of the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Spirochaetes* in *Bacterial* accounted for 85.16%, while in the sole WAS and sole perennial ryegrass, the total portions accounted for 50.78% and 95.39%. It seems that in the co-digestion system of the optimum conditions the bacterial diversity and relative abundance (from Figures 5 and 6) were improved compared with the sole WAS.

The relative abundances of *Archaea* populations are also shown in **Figure 5 (d, e, f)**. It can be seen that in the sample of sole perennial ryegrass anaerobic digestion system, the hydrogenotrophic methanogenic geus *Methanobacterium* accounted for 92.21% of all the *Bacterial* sequences found. In the sole perennial ryegrass anaerobic digestion system, the pH value was very low, reaching around 4.78 ± 0.22 when the system was stable.

Under such circumstances, *Methanobacterium* was the dominate archaea that utilized hydrogen and carbon dioxide to generate methane (Smith et al., 2013). Although the portion of *Methanobacterium* was higher in the sole perennial ryegrass digestion, the quantity (or population) of *Methanobacterium* was fewer because of the inhibitory effect on methanogens via a rarely low pH. In the sample of sole WAS and the optimum conditions anaerobic digestion system, the *Archaea* sequences generated from the anaerobic digestion system were distributed among three major geuns: *Methanosaeta*, *Methanosarcina* and *Methanobacterium*. Nevertheless, in the sample of sole WAS anaerobic digestion system, the *Methanosaeta*, *Methanosarcina* and *Methanobacterium* accounted for 43.44%, 16.21% and 27.94%; in the sample of the optimum conditions, these values were 48.02%, 3.74% and 33.48%. *Methanosaeta* and *Methanosarcina* belong to the order *Methanosarcinales*, which are aceticlastic methanogens utilizing acetate as a substrate for methane production, while *Methanobacterium* is hydrogenotrophic methanogens. From **Figure 5**, it can be concluded that the relative abundance of *Methanobacterium* in the optimum conditions was much higher than that of the sole WAS, while the relative abundance of *Methanosaeta* and *Methanosarcina* were very close between the sole WAS and the optimum conditions, indicating that more methane was produced via the interspecies H₂/formate transfer pathway, which required validation. It may be a reason to explain the higher methane content in the optimum conditions compared with others.

Fig. 5 - Phylum-level distributions of *Bacterial* populations (a-c) and genus-level distributions of *Archaea* populations (d-f) in the anaerobic digestion system of sole perennial ryegrass (a and d), the optimum conditions (b and e) and the sole WAS (c and f).

4. Conclusions

The above studies investigated a new method of simultaneous enhancement of methane production and methane content in biogas by controlling the important anaerobic digestion parameters in WAS and perennial ryegrass co-digestion system. The optimum conditions for simultaneously enhancement of methane production and

methane content from WAS and perennial ryegrass were initial pH 12 and C/N ratio 17/1, and the maximal methane production and methane content were 310 mL/gVS_{add} and 74% at anaerobic digestion time of 30d. The microbial community analysis showed more hydrogen and methane were produced via the interspecies H₂/formate transfer pathway, leading to higher methane content in biogas.

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Appendix. supplementary information

This file contains additional methods details, Tables S1-S6, and Figures S1-S5.

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Figure 1

Fig. 1 - Effects of C/N ratio and initial pH on methane production (a) and methane content (b) produced from WAS and perennial ryegrass co-digestion. Error bars represent standard deviations of three different measurements.

Figure 2

Fig. 2 - Effects of C/N ratio and initial pH on the consumptions of fermentation substrates. Error bars represent standard deviations of three different measurements.

Figure 3

Fig. 3 - Synergistic effect of main organic compounds in WAS and perennial ryegrass on methane production at anaerobic digestion time of 30 d. B: BSA; D: Dextran; C: Cellulose; H: Hemicellulose; All: BSA + Dextran + Cellulose + Hemicellulose; pH: pH=12. Error bars represent standard deviations of three different measurements.

Figure 4

Fig. 4 - Effects of initial pH and C/N ratio on pH and cumulative biogas production (a) and total alkalinity (b) variation during the 30-day anaerobic digestion time. Error bars represent standard deviations of three different measurements.

Figure 5

Fig. 5 - Phylum-level distributions of *Bacterial* populations (a-c) and genus-level distributions of *Archaea* populations (d-f) in the anaerobic digestion system of sole perennial ryegrass (a and d), the optimum conditions (b and e) and the sole WAS (c and f).