An efficient method to improve the production of methane from anaerobic digestion of waste activated sludge

Xiaolan Li, Xueqin Xu, Shansong Huang, Yun Zhou and Haijiang Jia

ABSTRACT

Methane production from waste activated sludge (WAS) anaerobic digestion is always low due to slow hydrolysis rate and inappropriate ratio of carbon to nitrogen (C/N). In this work, a novel approach, i.e., co-digestion of WAS and tobacco waste (TW) using ozone pretreatment, to greatly enhance the production of methane is reported. Experimental results showed the optimal C/N and ozone dosage for methane production was 24:1 and 90 mg/g suspended solids, and the corresponding methane production was 203.6 mL/g volatile suspended solids, which was 1.3-fold that in mono-WAS digestion. Further investigation showed the co-digestion of WAS and TW was beneficial to the consumptions of protein and cellulose; also, the presence of ozone enhanced the disruption of organic substrates and production of short chain fatty acids, which provided sufficient digestion substrates for methane generation. Analysis of microbial community structure suggested that members of the phyla Bacteroidetes and Firmicutes were the dominant species when ozone pretreatment was applied. The findings obtained in this work might be of great importance for the treatment of WAS and TW.

Key words | anaerobic co-digestion, methane, ozone, tobacco waste, waste activated sludge

INTRODUCTION

Waste activated sludge (WAS) is generated from wastewater treatment plants (WWTPs) daily with great amounts; it is reported that around 34 million metric tonnes of WAS (~20% of solids) were produced in China in 2015 (Feng et al. 2018). WAS contains a large number of organic matter and pathogens, and it could cause secondary pollution to the environment if disposed improperly (Zhao et al. 2018a). Meanwhile, lots of organic compounds such as protein and polysaccharide involved in sludge make it a cheap energy recovery resource (Zhao et al. 2018b; Wang et al. 2019; Wu et al. 2016). Anaerobic digestion is a promising technology for organic solid waste treatment and energy recovery (Zhao et al. 2015b, 2016a).

Generally, sludge digestion has a quite low production of methane and long digestion time due to the low rate of hydrolysis and inapposite ratio of carbon to nitrogen (C/N). Wang et al. (2015) reported that the highest production of methane was 168.0 mL/g volatile suspended solids (VSS) when sludge was digested without pretreatment (Wang et al. 2015). Yan et al. (2015) found the production of methane from sludge was only 67.7 mL/g VSS with thermal pretreatment. Co-digestion with other C-rich organic matter is an effective method to enhance methane production (Wang et al. 2014; Dai et al. 2016).

Tobacco, a very important economic crop, has played a major role in the Chinese national economy. It was documented that the cultivation and production of China ranked first among the word. The annual yield of tobacco is 450–500 million tons, and around 25% of tobacco leaf is abandoned, which might pose great threat to the environment. Usually, tobacco waste (TW) is transferred to an incinerator for waste mass reduction. However, incineration can cause waste of organic matter and production of foul gas, which is not environmentally friendly. Because TW contains lots of organic matter and wood cellulose, anaerobic co-digestion of WAS and TW might be an efficient approach to improve methane production because co-digestion can overcome the drawbacks of WAS mono-digestion (Mata-Alvarez et al. 2000; Dai et al. 2016).

Pretreatments are applied to enhance the biodegradability of digested substrates. In previous studies, biological,
physical (mechanical) and chemical pretreatments were extensively employed to improve the decomposition of sludge (Zhou et al. 2013, Zhao et al. 2015c, 2016b; Lee et al. 2014). Among those pretreatments, ozone pretreatment has drawn much attention for its high efficiency to increase the biodegradability of sludge or lignocellulosic materials and no generation of by-product (Sawatdeenarunat et al. 2015). Ozone pretreatment can effectively disintegrate the sludge flocs and release the intracellular substances. Zhang et al. reported that the presence of ozone enhanced the solubilization of sludge and when the dosage of ozone was 50 mg/g dry sludge, the content of soluble chemical oxygen demand (SCOD) and protein increased by 699% and 602%, respectively (Zhang et al. 2009). Xu and colleagues noted that the combination of ozone and ultrasound posed a synergistic effect on release of organic matter (Xu et al. 2010). However, the applications of ozone in those studies were mainly focused on sludge; the use of ozone to enhance the hydrolysis of a mixture of WAS and TW has never been reported before now. Therefore, it is necessary to assess the effect of ozone pretreatment on the production of methane from the anaerobic co-digestion of WAS and TW.

The aims of this work are first to test the feasibility of co-digestion of WAS and TW, and determine the optimal ratio of C/N and ozone conditions for methane production. Finally, the mechanisms of C/N and ozone pretreatment for methane improvement were revealed by analysing the consumption of digested substrates.

MATERIALS AND METHODS

Source of WAS, TW, and inoculated sludge

The WAS employed in this work was obtained from the secondary tank of a municipal WWTP in Nanning, China. After the WAS was sent back to the laboratory, WAS was stored in a 4°C refrigerator for 24 h, and then the supernatant was removed. The main characteristics of WAS are shown in Table 1.

TW was taken from Guangxi China Tobacco Industry Co., Ltd. The TW was first cut into the size of 1 cm long before starting experiments, and then mixed with water. The main characteristics of TW are also shown in Table 1.

Inoculated sludge applied in this work was collected from anaerobic reactor for WAS treatment; the main characteristics of the inoculated sludge are also displayed in Table 1.

<table>
<thead>
<tr>
<th>WAS</th>
<th>TW</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.9 ± 0.1</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>12,200 ± 123</td>
<td>21,300 ± 256</td>
</tr>
<tr>
<td>VSS (mg/L)</td>
<td>8,560 ± 59</td>
<td>18,500 ± 210</td>
</tr>
<tr>
<td>SCOD (mg/L)</td>
<td>126 ± 8</td>
<td>213 ± 12</td>
</tr>
<tr>
<td>Protein (mg/L)</td>
<td>6,541 ± 310</td>
<td>2,650 ± 186</td>
</tr>
<tr>
<td>Carbohydrate (mg/L)</td>
<td>2,390 ± 185</td>
<td>4,410 ± 260</td>
</tr>
<tr>
<td>Cellulose (mg/L)</td>
<td>851 ± 95</td>
<td>5,230 ± 2,130</td>
</tr>
<tr>
<td>Total COD (mg/L)</td>
<td>13,600 ± 180</td>
<td>23,650 ± 260</td>
</tr>
<tr>
<td>VFA (mg/L)</td>
<td>52 ± 2.1</td>
<td>65 ± 3.9</td>
</tr>
<tr>
<td>Total nitrogen (mg/g)</td>
<td>29.6 ± 1.5</td>
<td>16.5 ± 0.9</td>
</tr>
<tr>
<td>NH₄⁺-N (mg/L)</td>
<td>17.2 ± 2.3</td>
<td>10.2 ± 0.5</td>
</tr>
</tbody>
</table>

*Standard deviations of triplicate measurements.

Comparison of different ratios of C/N affecting methane production during co-digestion of WAS and TW

The mixing ratio of WAS and TW was essentially the C/N ratio of the fermentation matrix, so it is more practical to explore the effect of C/N ratio on digestive matrices. A batch test was set up to assess the effect of different ratios of C/N on the production of methane from co-digestion of WAS and TW. First, four replicate anaerobic reactors with working volume of 1.0 L each received 0.6 L of a mixture of WAS and TW. Then, 0.2 L of inoculated sludge, abovementioned, was added into those anaerobic reactors. The ratio of C/N in those reactors was controlled at 12:1, 18:1, 24:1, and 30:1. As a comparison, another two reactors were also operated to assess the mono-digestion of WAS and TW. The initial organic load was maintained at 10.0 g/L. The pH in those reactors was maintained at 7.0 ± 0.1 by adding 2.0 M hydrochloric acid or 2.0 M sodium hydroxide (Appels et al. 2008; Wang et al. 2015). The addition of hydrochloric acid or sodium hydroxide also occupies a certain amount of reaction space; thus 0.2 L of reactor space was left for hydrochloric acid or sodium hydroxide. Afterwards, those reactors were flushed with nitrogen for 5 min, then sealed, and placed in an air-bath shaker (120 rpm) at 35 ± 1°C for 30 d. All the experiments were repeated three times. TW does contain a variety of toxic ingredients, but their levels are quite low; the main TW components are carbohydrates, protein, and cellulose (Table 1). Nicotine is a toxic substance in TW, and co-digestion of WAS and TW is bound to introduce nicotine; so it is necessary to consider the effect of nicotine on the production of methane from sludge and tobacco. However, the content of nicotine in tobacco is low, and the concentration of nicotine after
mixing with the sludge is further diluted. In this study, the maximum content of nicotine was less than 0.001 mg/L, and such content did not cause negative impact on the activity of microorganisms (Hartmann et al. 1980). Therefore, this study did not consider the effect of nicotine on the performance of sludge and tobacco co-digestion.

**Effect of dosage of ozone on methane production under the optimal C/N ratio**

Five replicate serum bottles with working volume of 0.6 L each were used to evaluate the effect of ozone dosage on methane production under the optimal C/N ratio. First, each bottle was fed with 0.1 L of inoculum and 0.5 L of WAS and TW mixture. The ratio of C/N was controlled at 24:1 by controlling the volume of WAS to TW. The dosage of ozone applied in this work was controlled at 10, 30, 60, 90, and 120 mg/L suspended solids (SS), respectively. Initial pH in all bottles was also controlled at 7.0 ± 0.1 by adding 2.0 M hydrochloric acid or 2.0 M sodium hydroxide. Other experimental conditions are the same as given in the previous section.

**Analytical methods**

VSS and total suspended solids (TSS) were determined by gravimetric method. Chemical oxygen demand (COD) was determined by potassium dichromate method (Wang et al. 2013). Methane was determined by a gas chromatograph equipped with a thermal conductivity detector, and the detailed determination methods are in the literature (Mu & Chen 2011). The contents of protein and carbohydrate were measured using the Lowry-Folin method with bovine serum albumin as the standard and the phenol-sulfuric method with glucose as the standard, respectively (Lowry et al. 1951; Herbert et al. 1971). Key enzymes including hydro-lase (protease, α-glucosidase, cellulase), acid-producing enzymes, i.e., phosphotransacetylase (PTA) and acetate kinase (AK), and methane-producing enzyme (F420) were measured, and the detailed determination methods are in the literature (Goel et al. 1998). The activity of protease was determined by mixing 3 mL fermentation mixture withdrawn from the anaerobic reactors with 1 mL 0.5% azocasein and incubating at 37 °C for 90 min. The reaction was terminated by 2 mL 10% trichloroacetic acid. And then the mixture was centrifuged at 4,000 rpm for 30 min, and 2 mL supernatant was mixed with 2 mL aqueous NaOH (2 M) before being monitored at 440 nm. The activity of α-amylase was measured by mixing 1 mL fermentation mixture withdrawn from anaerobic reactors with 1 mL 0.1% nitrobenzene-α-D-pyran glycosidase and 2 mL 0.2 M Tris-HCl (pH 7.6, buffer) and incubating at 37 °C for 90 min. The reaction was terminated in boiling water for 3 min. An elemental analyzer (Elemental Analyzer NA 2500) was employed to analyze the carbon and nitrogen elemental composition of the digestion substrates. The methane was calculated as mL CH4/g VSS added.

**454 high-throughput pyrosequencing**

Before microbial community analysis, the fermentation mixture which was withdrawn from semi-continuous-flow reactors was centrifuged at 5 min at 10,000 rpm, and the total genomic DNA was extracted using the method described by Feng et al. (2009). Then, the DNA was amplified by polymerase chain reaction (PCR) using the primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (5'-TTACCCGGCCTGCTGGCAC-3') for the V1-V3 region of the 16S rRNA gene. To achieve the sample multiplexing during pyrosequencing, barcodes were incorporated between the 454 adaptor and forward primer. The 20.0 mL PCR mixture contained 4 mL of 5× FastPfu buffer, 2.0 mL of 2.5 mM dNTPs, 0.4 mL of each primer, 10.0 ng template of DNA and 0.4 mL FastPfu polymerase (TransStart FastPfu DNA Polymerase, TransGen). PCR chimeras were filtered out using Chimera Slayer. The reads flagged as chimeras were extracted out, and the non-chimera reads formed the database of ‘effective’ reads for each sample.

**Statistical analysis**

All experiments were conducted in triplicate. Analysis of variance was used to assess the significance of results obtained in this work and p < 0.05 was statistically significant.

**RESULTS AND DISCUSSION**

**Comparison of different ratios of C/N affecting methane production**

Methane production from organic solid waste anaerobic digestion is a series of continuous biological reactions, in which the ratio of C/N is a very important parameter. Figure 1 displays the effect of C/N ratio on the production of methane from co-digestion of WAS and TW. The production of methane in all reactors presented a rising trend with digestion time. The highest production of methane in mono-WAS and -TW reactors was respectively 159.2 and
172.3 mL/g VSS, which were similar to that reported in previous literature (Wang et al. 2015). However, when the WAS and TW were combined, the maximal yield of methane was significantly improved. When the C/N ratio was 12:1, 18:1, 24:1, and 30:1, the highest production of methane was 186.3, 198.2, 203.6 and 181.9 mL/g VSS, respectively. Those data suggested that the ratio of C/N can affect the production of methane, and the optimal C/N ratio was 24:1. It was also reported that the suitable C/N ratio for the activity of anaerobic microorganisms was 20:1–30:1 (Molla et al. 2004), and the optimal value obtained in this work was just in the suggested range. Obviously, under the optimal C/N ratio, the production of methane was 1.3 and 1.2-fold that in mono-WAS and -TW digestion, respectively.

### Effect of ozone pretreatment on the production of methane under the optimal C/N

Although suitable C/N ratio was conducive to the production of methane, extracellular polymeric substance bound with sludge and cellulose in TW seriously slowed the process of hydrolysis. And protein consumption in WAS was around 30% (Table 2), which means lots of refractory protein was still present in sludge. Figure 2 shows the effect of ozone dosage on the production of methane from the co-digestion of WAS and TW. It was found that the highest yield of methane increased from 203.6 to 281.3 mL/g VSS when the dosage of ozone increased from 0 to 90 mg/g SS, which suggested the increase of ozone promoted the yield of methane from co-digestion of WAS and TW. However, further increase of ozone to 120 mg/g SS had insignificant effect on methane generation, as compared with that in the presence of 90 mg/g SS ozone ($p > 0.05$). The above experimental result further indicated that the optimal dosage of ozone for methane production was 90 mg/g SS, and the corresponding yield of methane was

### Comparison of consumptions of main organic compounds during co-digestion of WAS and TW

The main substrates in WAS are protein and polysaccharide, and the main substrate in TW is cellulose; thus, their consumptions are close to the production of methane. Table 2 gives the consumptions of those main substrates with digestion time. From Table 2, it can be seen that a synergistic effect on the consumptions of those substrates was observed when WAS and TW were co-digested. For instance, the consumption of protein and cellulose in sole WAS anaerobic digestion was $2,341 \pm 124$ and $126 \pm 5$ mg/L, respectively; however, the consumption of protein and cellulose increased from $2,598 \pm 256$ and $2,014 \pm 142$ mg/L to $3,965 \pm 302$ and $2,856 \pm 158$ mg/L, respectively, when the ratio of C/N increased from 12:1 to 24:1, which indicated suitable increase of C/N promoted the consumptions of protein and cellulose. However, with further increase of C/N ratio to 30:1, a negative impact on the consumptions of protein and cellulose was observed. More consumption of main substrate gave rise to more substrates for methane production; thus it is easy to understand that when the C/N was 24:1 the highest production of methane was obtained. Co-digestion of WAS and TW contributes to the consumption of organic matter, which can achieve the reduction of WAS and TW and help the final disposal.

### Table 2 | Comparison of consumptions of protein and cellulose at different ratios of C/N after 20 d*

<table>
<thead>
<tr>
<th>C/N Ratio</th>
<th>WAS</th>
<th>TW</th>
<th>12:1</th>
<th>18:1</th>
<th>24:1</th>
<th>30:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein consumption (mg COD/L)</td>
<td>$2,341 \pm 124$</td>
<td>$856 \pm 36$</td>
<td>$2,598 \pm 256$</td>
<td>$3,240 \pm 278$</td>
<td>$3,965 \pm 302$</td>
<td>$2,443 \pm 216$</td>
</tr>
<tr>
<td>Cellulose consumption (mg COD/L)</td>
<td>$126 \pm 5$</td>
<td>$1,596 \pm 102$</td>
<td>$2,014 \pm 142$</td>
<td>$2,351 \pm 147$</td>
<td>$2,856 \pm 158$</td>
<td>$1,985 \pm 125$</td>
</tr>
</tbody>
</table>

*± standard deviations of triplicate measurements.
281.3 mL/g VSS, which was almost 1.4-fold that in the control. It was reported that the presence of ozone could oxidize and decompose certain recalcitrant organic compounds, such as protein and cellulose (Kindaichi et al. 2003; Kurniawan et al. 2009). The amount of methane produced can also be expressed as the millilitres of CH₄ produced per gram of COD removal. Wang et al. (2015) noted that the production was 246.1 mL/g VSS from WAS with high level of poly-hydroxyalkanoate. Obviously, the yield of methane was higher than that reported in the literature. The decomposition of protein and cellulose provided sufficient organic matter for production of methane. Previous studies have shown that ozone pretreatment of sludge to promote the production of methane is economical (Goel et al. 2003; Chu et al. 2009); although the ozone dosage in this study was similar to that reported in the literature (Goel et al. 2003), the yield of methane from WAS and TW in this study was significantly higher than that (203.6 vs 140 mL/g) reported in the literature (Goel et al. 2003). Therefore, application of ozone pretreatment to promote the production of methane from WAS and TW has economic benefits.

Effect of ozone dosage on the disruption of WAS and the solubilization of TW

Figure 3 shows the profile of SCOD during the co-digestion of WAS and TW with different dosages of ozone addition. From Figure 3, it was found that the content of SCOD first increased and then decreased with digestion time, and the highest content of SCOD occurred at the fermentation time of 6 d. The concentration of SCOD increased with the dosage of ozone at each given time, and the highest SCOD increased from 1,259 to 2,356 mg/L when the dosage of ozone increased from 0 to 90 mg/g SS, but further increase to 120 mg/g SS caused insignificant improvement of SCOD (p > 0.05). Those observations suggested that higher dosage of ozone led to higher content of SCOD,
providing more substrates for methane production. In addition, the results of this study showed that the addition of ozone can accelerate the solubilization of WAS and TW, the solubilization of organic matter can provide a digestive matrix for acidogenic bacteria and methanogens, and the residual matter in the residue after digestion was less, which was beneficial to the subsequent disposal (e.g., landfill) of sludge and TW.

Effect of ozone dosage on the variations of SCFA during co-digestion of WAS and TW

Short-chain fatty acids (SCFA), an intermediate product of anaerobic digestion, can be further consumed for methane generation. Also, they can accumulate in high amounts, which could cause the decrease of pH, and then pose a negative impact on methanogens. Figure 4 displays the effect of ozone dosage on the variations of SCFA during co-digestion of WAS and TW. It can be seen that the profiles of SCFA with different dosages of ozone first increased and then decreased with digestion time. The highest production of SCFA increased from 755.4 to 1,295.8 mg/L when the dosage of ozone increased from 0 to 90 mg/g SS, but further increase of ozone dosage to 120 mg/g SS promoted insignificant production, as compared with the presence of 90 mg/g SS ozone. Those observations indicated the presence of ozone improved the yield of SCFA. The accumulated SCFA can be further consumed for methane production. The change of pH during the whole process was also monitored and is shown in Figure 5. As shown in Figure 5, the pH in all reactors first decreased and then increased to the initial pH. For example, when the ozone dosage was 90 mg/g SS, pH decreased from the initial 7.0 to 6.2 at the fermentation time of 9 d, and then slowly rose to 7.0 by 25 d, and maintained stability during the rest of the time. The lowest pH in blank was 6.4 at the fermentation time of 15 d, which was similar to that in the presence of ozone. The similar data of pH in the presence of different ozone dosages suggested that pH change was not the reason for methane decrease.

The composition of SCFA is very important for their subsequent utilization. Figure 6 shows the effect of ozone dosage on the composition of SCFA under their optimal conditions. It can be seen that the main components in all reactors were acetic acid and propionate, which accounted for around 65–70% of the total SCFA. Nevertheless, valerate acid had the lowest content of acids in all reactors, with a percentage of 2.9–4.2%. Further analysis indicated that the general order of SCFA under their optimal conditions were in the sequence: acetic acid > propionic acid > isovalerate > butyric acid > iso-butyric acid > valerate. Those data suggested that the presence of ozone had no obvious effect on the composition of SCFA.

Figure 4 | Effect of ozone dosage on the variations of SCFA during co-digestion of WAS and TW. Error bars represent standard deviations of triplicate measurements.
Figure 5 | Effect of ozone dosage on the variations of pH during co-digestion of WAS and TW. Error bars represent standard deviations of triplicate measurements.

Figure 6 | Effect of ozone dosage on the composition of SCFA during anaerobic co-digestion of WAS and TW. Error bars represent standard deviations of triplicate measurements.
Effect of ozone dosage on the activities of key enzymes responsible for methane production

Methane production from organic waste is catalyzed by related enzymes; thus the activities of key enzymes are measured to reflect the effect of ozone dosage on the production of methane. Protease and α-glucosidase are the key enzymes responsible for the hydrolysis of protein and polysaccharide, respectively. Cellulases play an important role in the degradation of cellulose. Because acetate is the primary acid in SCFA, the key enzymes (i.e., PTA and AK) responsible for acetate production are also detected. Co-enzyme F420 is the main enzyme for methane production; thus co-enzyme F420 was selected to be measured. As shown in Table 3, the activities of protease, α-glucosidase and cellulases increased with ozone dosage, which indicated that the hydrolysis was greatly enhanced in the presence of ozone. The activities of PTA and AK are also increased with ozone dosage. However, the activities of F420 were similar in all reactors.

Effect of ozone dosage on the microbial communities during anaerobic co-digestion of WAS and TW

Analysis of microbial community structure is an alternative method to reflect the underlying mechanism of how ozone affects methane production. In this study, 454 pyrosequencing was applied to determine the structures of the microbial community with different dosages of ozone during anaerobic co-digestion of WAS and TW. Figure 7 shows the distributions of bacterial sequences from the aspect of phyla. It can be seen

Table 3 | Effects of ozone dosage on specific activities of key enzymes involved in the methane production

<table>
<thead>
<tr>
<th>Ozone dosage (mg/g SS)</th>
<th>Protease</th>
<th>α-Glucosidase</th>
<th>Cellulase</th>
<th>PTA</th>
<th>AK</th>
<th>F420</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0085 ± 0.0005</td>
<td>0.049 ± 0.008</td>
<td>9.26 ± 0.5</td>
<td>0.325 ± 0.004</td>
<td>5.16 ± 0.2</td>
<td>0.38 ± 0.5</td>
</tr>
<tr>
<td>10</td>
<td>0.0095 ± 0.0004</td>
<td>0.051 ± 0.007</td>
<td>10.65 ± 0.7</td>
<td>0.395 ± 0.008</td>
<td>5.65 ± 0.3</td>
<td>0.39 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.0125 ± 0.0006</td>
<td>0.059 ± 0.009</td>
<td>12.64 ± 0.8</td>
<td>0.415 ± 0.009</td>
<td>5.92 ± 0.2</td>
<td>0.38 ± 0.3</td>
</tr>
<tr>
<td>60</td>
<td>0.0165 ± 0.0005</td>
<td>0.068 ± 0.011</td>
<td>16.54 ± 0.7</td>
<td>0.426 ± 0.011</td>
<td>5.98 ± 0.2</td>
<td>0.40 ± 0.4</td>
</tr>
<tr>
<td>90</td>
<td>0.0185 ± 0.0004</td>
<td>0.075 ± 0.019</td>
<td>17.69 ± 0.9</td>
<td>0.458 ± 0.016</td>
<td>6.23 ± 0.1</td>
<td>0.41 ± 0.5</td>
</tr>
<tr>
<td>120</td>
<td>0.0189 ± 0.0008</td>
<td>0.076 ± 0.008</td>
<td>17.85 ± 0.8</td>
<td>0.463 ± 0.019</td>
<td>6.34 ± 0.5</td>
<td>0.40 ± 0.5</td>
</tr>
</tbody>
</table>

The data are the averages and their standard deviations in duplicate tests. The unit is U/mg VS.

Figure 7 | Distribution of bacterial community at phylum level.
that four major phyla, Bacteroidetes, Firmicutes, Proteobacteria and Spirochaetes, were observed in the co-digestion of WAS and TW with different dosages of ozone. When the ozone dosage was absent, the phyla Bacteroidetes, Firmicutes, Proteobacteria and Spirochaetes accounted for 29.3%, 20.6%, 16.5% and 13.9% of the total bacterial sequences detected, respectively. When the dosage of ozone increased to 90 mg/g SS, the abundance of Bacteroidetes and Firmicutes also increased to 51.2% and 23.9%, respectively. However, the abundance of Proteobacteria and Spirochaetes remained unchanged (p > 0.05). It is well known that the species of Bacteroidetes can break down the 1,4α-glycosidic bonds of plant polysaccharides (Zhao et al. 2015a). The increase of Bacteroidetes with higher ozone dosage enhanced the degradation of TW during the co-digestion. Firmicutes are well-known fermenters that can decompose SCFA to methane (Zhao et al. 2015b), the enrichment of Firmicutes is one reason for the improvement of methane production with ozone addition. The predominance of Firmicutes is an indication that these products are readily available for anaerobic digestion. Ozone disrupted the refractory organic matter in WAS and TW, and provided easily degradable materials for the utilization of microorganisms, and then those microorganisms gradually evolved into dominant strains.

CONCLUSION

This study reported an efficient approach (i.e., co-digestion of WAS and TW with ozone pretreatment) to significantly improve methane production. Experimental results showed the optimal C/N ratio for methane production was 24:1, and the corresponding production of methane was 203.6 mL/g VSS, which was 1.3-fold that in sole WAS digestion. Further, ozone pretreatment was applied to improve the production of methane, and the optimal concentration of ozone was 90 mg/g SS. Mechanism studies revealed that ozone improved the disruption of digestion substrates and enhanced the production of SCFA, providing sufficient substrates for methane generation. Analysis of the microbial community showed that Bacteroidetes and Firmicutes were the main anaerobic microorganisms with the presence of the ozone, which was in accordance with the enhancement of methane.

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