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Co-fermentation of waste activated sludge and agricultural waste for hydrogen production: effect of the carbon-to-nitrogen mass ratio

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ABSTRACT

H_2 production under anaerobic conditions was greatly affected by the C/N ratio of the substrate. Co-fermentation of waste activated sludge (WAS) and agricultural waste was used to modulate the C/N ratio for H_2 production. In this study, the effects of different C/N ratios, ranging from 6:1 to 55:1, on H_2 production from the anaerobic digestion of a mixture of WAS and wheat straw were studied. It was observed that the mixture system with a C/N ratio of 45:1 provided optimal conditions for H_2 production. H_2 production in the mixture system was 5.2- and 1.2-fold greater than that in the sole sludge (C/N 6:1) and sole straw (C/N 55:1) systems, respectively. Under the optimal C/N ratio, soluble chemical oxygen demand, volatile fatty acids and activities of key enzymes were improved. Firmicutes and Bacteroidetes were the dominant microorganisms in the mixture and sole straw systems that were beneficial to organic matter degradation, utilization, and H_2 production. Clostridia played an important role in H_2 production. A balanced C/N ratio favors the co-fermentation process.

Keywords: Co-fermentation; Hydrogen production; Waste activated sludge; Agricultural waste; C/N ratio

1. Introduction

With the development of wastewater treatment plants (WWTPs), large amounts of waste activated sludge (WAS) are produced annually [1,2]. WAS must be treated to reduce its volume, improve its stability, and reduce associated health problems. The treatment of municipal sludge is now a major challenge for environmental protection [3]. Due to secondary pollution, capital intensive costs, and more stringent regulatory pressures, conventional methods such as landfilling, incineration, and beneficial use are less preferable. Anaerobic digestion not only provides sludge stabilization and volume reduction treatment but also reduces the potential for pathogens and odors. In addition, anaerobic digestion does not require oxygen and does produce biogas, thereby reducing energy consumption [4–6]. However, anaerobic digestion of WAS as the sole substrate has some disadvantages, such as low organic conversion efficiency, long retention time, and low methane yield. A balanced carbon-to-nitrogen (C/N) ratio favors co-fermentation, as well as macroscopic and micro-nutrients, pH, inhibitors/toxic compounds that will affect hydrolysis, acidogenesis, acetogenesis, and methanogenesis [7,8]. The C/N ratio is an important indicator of anaerobic digestion. WAS is characterized by a relatively low C/N ratio and a high buffering capacity, and thus can tolerate high C/N ratios and low alkalinity values for co-substrates [9].

Studies have shown that agricultural waste with a high C/N ratio is characterized by a low pH substrate, poor buffering capacity, poor nutrient content, and the possibility of high volatile fatty acid (VFA) accumulation during digestion [8,10]. Crop straw is the main byproduct of agriculture

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production after an agricultural crop harvest. Agricultural waste is considered to be the most abundant renewable resource. It is necessary and important for the sustainable development of bio-fuel production in the long run [11]. As the largest agricultural country, rich in biomass resources, China experiences serious haze pollution every year due to the incineration of straw. It is therefore important to properly treat this agricultural waste. Biogasification as a key technology shows great promise for sustainable utilization of wheat straw as a renewable energy source [12].

Several studies are using straw and sludge for co-digestion. Traditionally, methane recovery has been the key focus of anaerobic digestion. In recent years, for several reasons, the focus has shifted to H_2 production, and H_2 is a promising alternative energy source. This emerging sustainable energy source is clean, efficient, renewable, and does not produce toxic byproducts. Kim et al. [13] studied the production of H_2 and methane from untreated rice straw and raw sludge under thermophilic anaerobic conditions. During H_2 fermentation, they obtained a high H_2 yield (21 mL/g-VS) and stable H_2 content (60.9%) [13]. In another rice straw and sewage sludge co-digestion experiment, it was found that biogas and H_2 production in all raw sludge samples increased faster than the heat-treated sample and showed longer and higher H_2 production [14]. Alemahdi et al. [15] studied the effects of thermal pretreatment, pH, and substrate size on biohydrogen production from the co-digestion of rice straw and activated sludge. Abudi et al. [16] investigated the influence of thickened WAS and rice straw pretreatment on the organic fraction of municipal solid waste, thickened WAS, and rice straw co-digestion. All these studies involved additional high-energy input treatment or lacked microbial community analysis.

In this study, we explored H_2 production using sludge and wheat straw for co-digestion under mesophilic conditions. The first objective of this study was to reveal the effect of different C/N ratios on H_2 production from the co-fermentation of WAS and wheat straw. Second, soluble organic matter conversion and enzyme activities in different substrates with different C/N ratios were studied. Third, shifts in the microbial community under different anaerobic co-digestion systems were investigated.

2. Materials and methods

2.1. Characteristics of WAS and wheat straw

The WAS used for anaerobic fermentation was obtained from the secondary sedimentation tank of a municipal WWTP in Shanghai, China, which was operated with a traditional activated sludge process. After settling at 4°C for 24 h, the sludge was concentrated. The wheat straw was harvested in 2015 from Zhengzhou, China. The wheat straw was dried and then cut into 1 cm lengths. Samples were stored in sealed bottles at 4°C before being fed into the reactors. The main characteristics of the WAS and wheat straw are shown in Table 1.

Table 1
Primary characteristics of waste activated sludge (after settling) and wheat straw[a]

Parameter	WAS	Wheat straw
pH	6.8 ± 0.1	/
TS (total solids), %	1.96 ± 0.06	85 ± 2
VS (volatile solids), %	73.4 ± 1.2	93.4 ± 3
Total carbohydrate, g COD/kg-TS	102 ± 4	134 ± 8
Total protein, g COD/kg-TS	492 ± 30	66.6 ± 5
Soluble carbohydrate, g COD/kg-TS	0.38 ± 0.02	
Soluble protein, g COD/kg-TS	3.25 ± 0.05	
Carbon to nitrogen ratio	6 ± 1	55 ± 2
Cellulose, g/kg	/	321 ± 15
Hemicellulose, g/kg	/	253 ± 13
Lignin, g/kg	/	187 ± 15

[a]The data presented are averages and standard deviations based on duplicate tests.

2.2. Batch experiments for comparison among different carbon to C/N ratios affecting H_2 production

All reactors, maintained at 35°C ± 1°C, were made of Plexiglas and stirred at 120 rpm to mix the contents. The effective volume of each reactor was approximately 1 L. All experiments were conducted in triplicate and one-way analysis of variance at the 0.05 level was used to analyze the data. The anaerobic digestion experiments to study the impacts of initial C/N ratio on H_2 production were conducted in 13 reactors. These reactors maintained a range of 13 C/N ratios (6:1, 8:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 22:1, 25:1, 35:1, 45:1, and 55:1). The C/N ratio of WAS is 6:1, and the C/N ratio of sole wheat straw is 55:1. According to the total solid (TS) of WAS and TS of straw, when the C/N ratio is 8:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 22:1, 25:1, 35:1, and 45:1, the amount of WAS and straw added can be calculated. The total volatile solids (VS) of each reactor were maintained at 3.0 g/L, and the liquid volume of each reactor was approximately 300 mL. Throughout the experiment, pH was kept at 10.0; pH was controlled by the automatic addition of 5 M NaOH or 4 M HCl with an automatic titrator. These reactors were sealed with rubber stoppers and then fermented for 9 d. Every 24 h, H_2 biogas production was monitored. The gas produced from these anaerobic reactors was collected using the water displacement method, and the composition of the gas was investigated using gas chromatography (Agilent 6890N, USA) with a thermal conductivity detector; nitrogen was used as the carrier gas. VFA concentrations (acetic acid, propionate, iso-butyrate, *n*-butyrate, iso-valerate, and n-valerate) were monitored after anaerobic digestion. To compare soluble organic matter transformation in the reaction process, soluble COD, proteins, and polysaccharides were determined at the beginning and after 2 d of digestion. Quantities of hemicelluloses, cellulose, lignin, protein, and polysaccharide were measured at the beginning and following digestion to calculate inversion quantities.

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

Three semi-continuous-flow reactors with working volumes of 2 L were operated to investigate enzyme

activities related to the decomposition of organic compounds. Reactors 1 and 2 were set as blank tests: one was filled with only sludge and the other was filled with only wheat straw. Reactor 3 was conducted with the optimum mixture of sludge and wheat straw (C/N 45:1). The temperature was maintained at 35°C±1°C, and the agitator speed was set at 120 rpm. Every day, a certain amount of fermentation substrate was manually poured out from each reactor, and the same amount of new substrate was added, in order to control the sludge retention time at 9 d. After 3 months of stable operation, enzyme activities and the microbial community were tested.

2.4. Analytical methods

The methods used to determine pH, TS, VS, protein, and carbohydrate were the same as described in previous publications [17,18]. H_2 content of the biogas was measured using a gas chromatograph (GC) (Agilent Technologies 6890 N, CA, USA) with a thermal conductivity detector equipped with Hayesep Q mesh and Molsieve 5A columns. To analyze VFAs, the sludge samples from the reactors were centrifuged at 10,000 rpm for 10 min. Then the supernatant was passed through a microfiber filter (0.45 μm) and the filtrate was acidified using formic acid to adjust the pH to approximately 2.0 before VFA was analyzed using a GC (Agilent Technologies 6890N, CA, USA) with a flame ionization detector. The C/N ratio of the material was measured using an organic element analyzer (Vario EL III, Elementar, Germany) after freeze drying. The contents of hemicelluloses, cellulose, and lignin in wheat straw were determined using the detergent extraction method [19].

Pyruvate:ferredoxin oxidoreductase is an important enzyme that is involved in pyruvate decomposition and acetyl coznzyme A (CoA) production. The reduction of a proton by Fe (red) produces hydrogen through hydrogenase activity. The activity of hydrogenase, an iron-containing enzyme, is one of the most important factors in the overall hydrogen fermentation. Hydrogenase and pyruvate:-ferredoxin oxidoreductase activities were tested according to the method provided in previous publications [20,21]. Oxaloacetate transcarboxylase (OAATC) is an important enzyme for propionic acid synthesis, catalyzing pyruvate to oxaloacetate and methylmalonyl CoA to propionyl CoA. The detection of OAATC was performed according to the method of Wood et al. [22].

To determine the activities of protease and cellulase, the fermentation substrates (25 mL) were sampled from the different anaerobic digestion reactors and then washed and suspended in a 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 enzyme activity assays. The activity of protease was measured according to Miron et al. [23]. The activity of cellulase was determined using the filter paper assay method of Ghose [24]. One unit of enzyme activity was defined as the amount of enzyme necessary to catalyze 1 μmol of substrate per minute. The specific enzyme activity was defined as the unit of enzyme activity per gram of VS.

Genomic DNA was extracted and purified from the biomass samples using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions (Figs. 1 and 2). The nucleotide concentration was examined by a Nanodrop spectrophotometer (Nanodrop Technologies Inc., USA). The purified genomic DNA (20 ng/μL) was sent to the Majorbio company for 16S rRNA gene-based amplicon sequencing using a MiSeq desktop sequencer (Illumina, USA). Polymerase chain reaction (PCR) of bacteria 16S rRNA genes with 7F (5′-CAGAGTTTGATCCTGGCT-3′) and 1540R (5′-AGGAGGTGATCCAGCCGCA-3′) as bacterial primers was carried out [25]. The amplification reaction was performed with 2.5 μL of 5× PCR buffer with Mg^{2+}, 1 μL of 2.5 mM deoxynucleoside triphosphate solution, 0.5 μL of each primer (10 μM), 0.5 μL template, and 0.2 μL DNA polymerase (Dream Taq-TM, MBI, Lithuania) in a 25 μL PCR stock solution and diluted with deionized water to 25 μL. PCR conditions were as follows: initial reaction mixtures were held at 94°C for 4 min followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min per cycle. Finally, an extension step of 10 min at 72°C was performed. Purified product sizes and concentrations were checked by electrophoresis on a 1% (w/v) agarose gel with UV emission. Correctly sized PCR products were randomly selected. The sequenced 16S rDNA gene was analyzed using the NCBI search tool to identify the closest matching sequence. The sequences were compared with the GenBank databases using the basic local alignment search tool algorithm to determine the approximate phylogenetic affiliations. Representatives from the different clone types were submitted to the ribosome database.

3. Results and discussion

3.1. Effect of different C/N ratios on H_2 production from co-fermentation of sludge and wheat straw

Carbon and nitrogen are important nutrients for microorganisms. The substrate C/N ratio affects bacterial activity, which affects the anaerobic digestion process. In the anaerobic digestion process, microorganisms use carbon 25–30

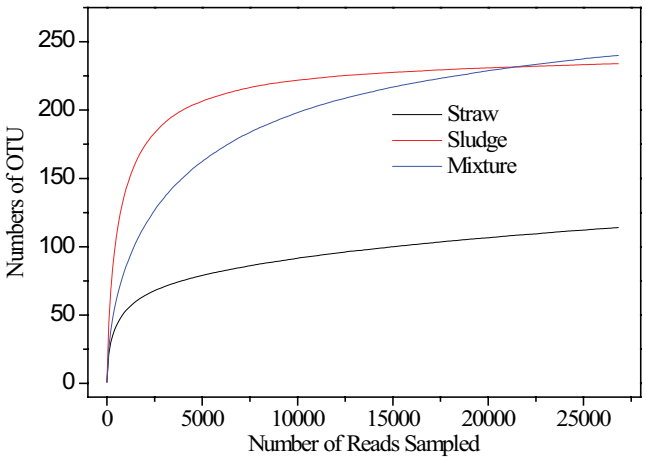

Fig. 1. Rarefaction curves of bacterial sequences from the fermentation reactors of straw (solid black), sludge (solid red), and mixture (solid blue). The OTUs were defined by clustering sequences at dissimilarity levels of 3%.

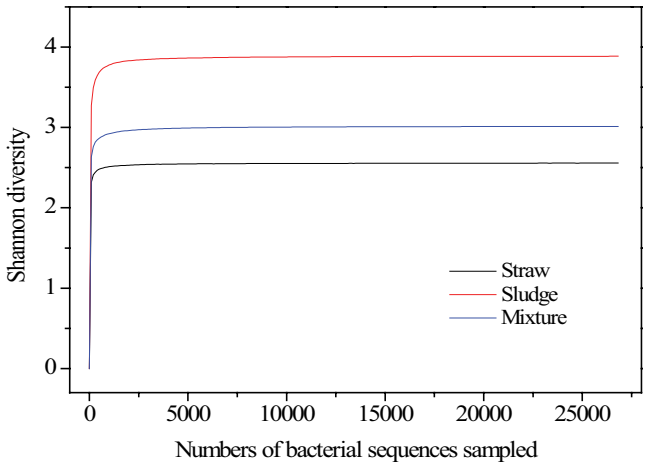

Fig. 2. Shannon diversity curves of bacterial sequences from the fermentation reactors of Straw (solid black), Mixture (solid blue), and Sludge (solid red). The OTUs were defined by clustering sequences at dissimilarity levels of 3%.

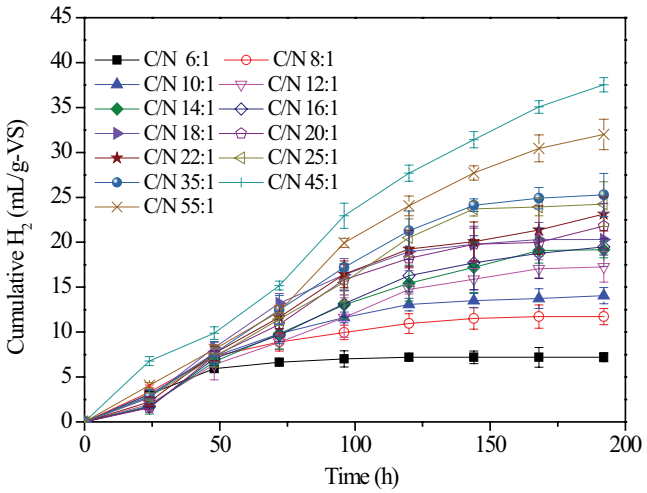

Fig. 3. Effect of different C/N ratios on H_2 production from the co-fermentation of WAS and wheat straw.

times faster than they use nitrogen [26,27]. Inadequate carbon content may lead to the accumulation of NH_4^+ and then slow down the microbial growth rate and the conversion reactions of substrate to biogas [28]. Anaerobic digestion of excess C/N ratios leads to VFA accumulation and a lower pH, leading to process inhibition. It can be seen from Fig. 3 that as the C/N increased from 6:1 to 45:1, H_2 production increased daily, and the cumulative production increased from 7.2 to 37.5 mL/g-VS in 9 d. Then the H_2 production decreased to 32.0 mL/g-VS as C/N further increased to 55:1. Thus, the best C/N ratio for the system was determined to be 45:1. In Kim et al.'s [14] study, it was found that a C/N ratio of 25:1 was the best choice for H_2 production when rice straw and sewage sludge were used as substrates. Alemahdi et al. [15] reported that when using rice straw and activated sewage sludge, a C/N ratio of 30:1 was suitable for H_2 production. There have been many studies on C/N ratios in anaerobic co-digestion systems, and different results have been obtained under different conditions, such as temperature, substance, and pretreatment. It can be speculated that the optimal C/N ratio required for the production of hydrogen from different co-digestion substrates is different. This might be attributed to the fact that different co-digestion substrates produce different microbial communities, which is considered to be an important factor influencing hydrogen production. In the following experiment, only C/N ratios of 6:1 (sludge), 45:1 (mixture of sludge and straw), 55:1 (straw) were tested.

3.2. Soluble organic matter conversion in different substrates with different C/N ratios

Soluble organic matter (recorded as COD) in sole sludge (C/N 6:1), sole straw (C/N 55:1), and a mixture system (C/N 45:1) was determined in order to evaluate organic matter conversion in the reaction process. Soluble COD, protein, and polysaccharide in the mixture, straw, and sludge systems on the first day (before hydrolysis) and the third day (after hydrolysis) are shown in Fig. 4a. After 2 d of fermentation, the soluble COD, protein, and polysaccharide were increased in these three systems compared with the initial stage (on first day). The soluble COD increased from 4.38 g/kg-TS to 63.0 g/kg-TS in the mixed system; protein and polysaccharide increased to 32.43 and 24.38 g COD/kg-TS, respectively. In the sole sludge system, the protein concentration was higher than the polysaccharide concentration, as protein accounts for a large portion of the sludge. In a sole straw system, the polysaccharide concentration was much higher than the protein concentration, because carbohydrates are the primary component of the straw. The results showed that the appropriate C/N greatly enhanced the conversion of organic matter from a solid phase to liquid phase and then affected H_2 production.

When the fermentation was complete, the carbohydrates and proteins consumed in the three systems were measured and compared. As shown in Fig. 4b, the mixture system (C/N 45:1) was more favorable for polysaccharide degradation than the sole straw system (C/N 55:1). These results indicate that the addition of nitrogen from the sludge enhanced the degradation of the polysaccharides. Increased protein consumption was observed in the sole sludge system (C/N 6:1) because the protein concentration was greater than that in the other two systems. An additional experiment was conducted to analyze the consumptions of cellulose, hemicellulose, and lignin. Because no cellulose, hemicellulose, and lignin were detected in the sole sludge system (C/N 6:1), these substrates were not consumed. Although the sole straw system (C/N 55:1) contained the largest amounts of cellulose, hemicellulose, and lignin, this system consumed less than the mixture system (C/N 45:1). These results indicate that the balanced C/N of the mixture system was beneficial to microbial growth and substrate consumption. It can also be noted that cellulose and hemicellulose are more biodegradable than lignin.

Complex organic compounds such as proteins, carbohydrates, and lipids are converted into simple soluble products such as amino acids, sugars, and long-chain fatty acids, and then the soluble products of the first step are hydrolyzed into

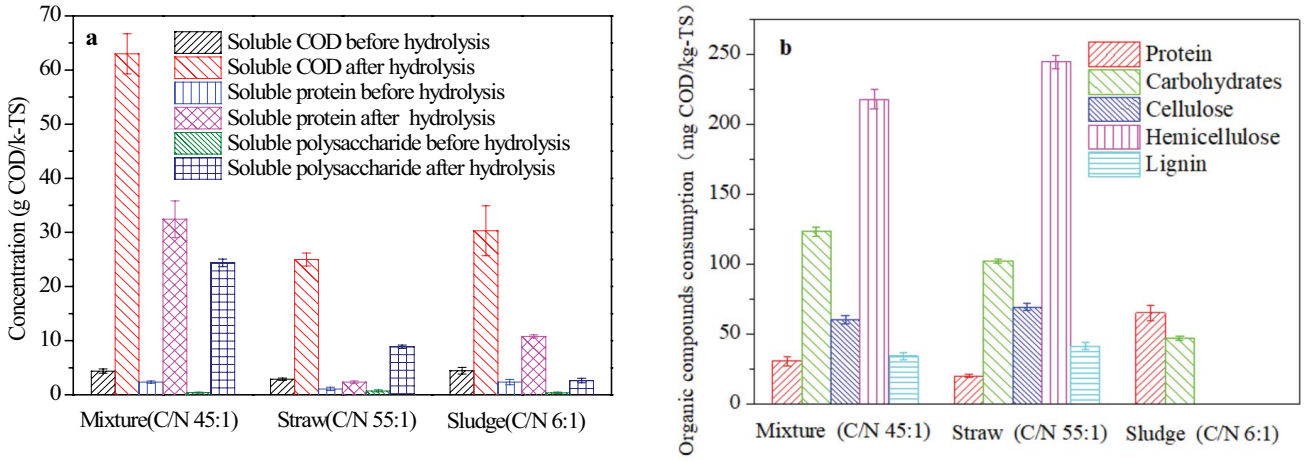

Fig. 4. (a) Soluble COD, protein, and polysaccharide in different substrates (mixture, sole straw, and sole sludge) on the first day (before hydrolysis) and the third day (after hydrolysis); (b) consumptions of protein, carbohydrates, cellulose, hemicellulose, and lignin in different substrates (mixture, sole straw, and sole sludge) after anaerobic digestion.

a mixture of organic acids, H_2, and CO_2 by fermentative bacteria. Acidogenesis is the generation of VFAs (C > 2), such as propionic and butyric acid. These VFAs, along with ethanol, are converted to acetic acid, H_2, and CO_2 by another group of bacteria known as hydrogen-producing acetogenic bacteria. Of the hydrogen-producing microorganisms, Clostridia and enteric bacteria have been extensively studied. The H_2 production pathway through dark fermentation is affected by microbial types. Acetate and butyrate are produced by Clostridia via fermentation to produce H_2. Using glucose as a model substrate, H_2 production with either acetate or butyrate formation can be expressed as:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

Then, a maximum of 4 mol of H_2 can be obtained by the acetate type fermentation, but only 2 mol of H_2 can be produced in the butyrate type fermentation. Higher H_2 production means a higher acetate concentration. VFAs in all three systems measured on Day 9 can be seen in Fig. 5. In each of the three systems, acetate was the main product. The amount of acetate was highest in the mixture system (C/N 45:1), which was twice that measured in the sole sludge system (C/N 6:1). Acetate in the sole straw system (C/N 55:1) was also higher than that of the sole sludge system (C/N 6:1), suggesting that the sole straw system was most favorable for acetic acid production. The concentration of acetic acid in the three systems was positively related to H_2 production.

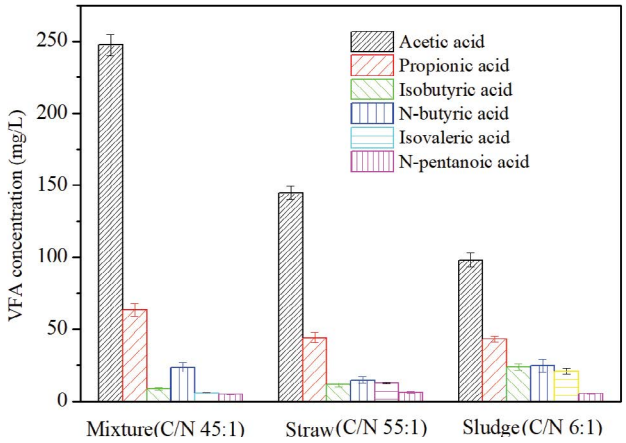

Fig. 5. VFA concentrations in different substrates (mixture, sole straw, and sole sludge) with different C/N ratios after anaerobic digestion.

3.3. *Enzyme activities in different substrates with different C/N ratios*

Pyruvate:ferredoxin oxidoreductase and hydrogenase are important enzymes associated with H_2 production. Cellulase and protease are used to characterize hydrolysis. Propionic acid production is associated with OAATC activity: increased OAATC activity leads to higher propionic acid production. All five enzyme activities were then tested to evaluate microbial activity (Fig. 6). It was assumed that the enzymes activities in the sludge system were 100%. The enzyme activities in the mixture and the straw systems were compared with the enzyme activities in the sludge system. With an increase of C/N, the cellulase activity increased with an increase of cellulose. Protease activity was lowest in the sole straw (C/N 55:1) because the amount of protein in that system was relatively low, but the protease activity in the mixture system (C/N 45:1) was approximately 1.5 times as high as that of the sludge system (C/N 6:1), indicating that the improved C/N ratio increased the protease activity and more protein was degraded. The activities of pyruvate:-ferredoxin oxidoreductase and hydrogenase were lowest in the sole sludge system (C/N 6:1), but they were highest in the mixture system (C/N 45:1). The results were positively correlated with H_2 production. It can be concluded that the mixture of sludge and straw at C/N 45:1 was beneficial to

microbial activity, thus increasing H_2 production. It can be seen that OAATC activity decreased with an increase of straw.

3.4. Microorganism communities in different substrates with different C/N ratios

High-throughput sequencing of the 16S rRNA genes was used to reveal the microbial community composition in the reactors with different substrates. To estimate the microbial structures of the bacterial communities in the anaerobic reactors, trimmed sequences were grouped into operational taxonomic units (OTUs) using a 97% identity threshold. Table 2 shows the sequence diversity and library coverage estimates of the samples. The sufficient coverage found in each sample suggested that the 16S rRNA method captured the most frequently occurring organisms. Microbial community structure at the phylum level can be seen in Fig. 7a. Microbial diversity in the sole sludge system (C/N 6:1) was higher than those in the mixture and sole straw systems. The diversity index of the mixture, sole sludge, and sole straw systems is presented in Table 2. This may be because the sludge composition was more complex. Firmicutes, Proteobacteria, Actinobacteria, Chlorobi, and Saccharibacteria were the dominant bacterial phyla. Actinobacteria, Chloroflexi, Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria have also been frequently found in other studies [29,30]. In the sole straw and mixture systems, Firmicutes and Bacteroidetes were enriched, accounting for approximately 90% of the microbial community. Firmicutes can biodegrade VFAs, such as butyrate and its analogs. The predominance of Firmicutes is an indication that these products are readily available for anaerobic digestion. Species within the phylum Bacteroidetes can attack the 1,4a-glycosidic bonds of plant polysaccharides [31], such that these carbohydrates can be metabolized to produce VFAs. Proteobacteria is also an important bacterium

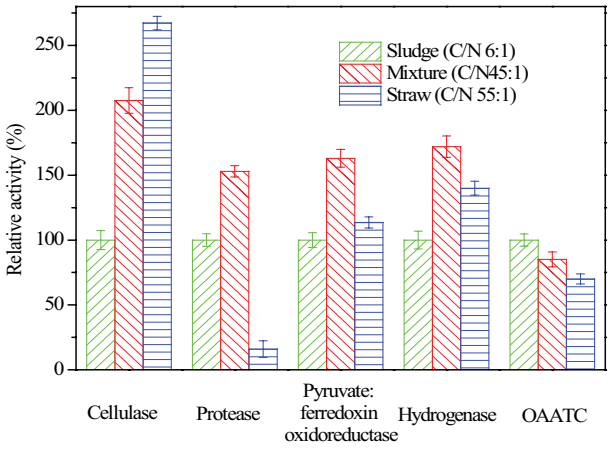

Fig. 6. Enzyme activities in different substrates (mixture, straw, and sludge) with different C/N ratios.

Table 2
Diversity index in mixture, sludge, and straw systems

Sample	Reads	0.97					
		OTU	Ace	Chao	Coverage	Shannon	Simpson
Mixture	26,810	240	261	269	0.998657	3.01	0.0972
Sludge	26,810	234	238	245	0.99959	3.89	0.0567
Straw	26,810	114	143	141	0.999068	2.56	0.1579

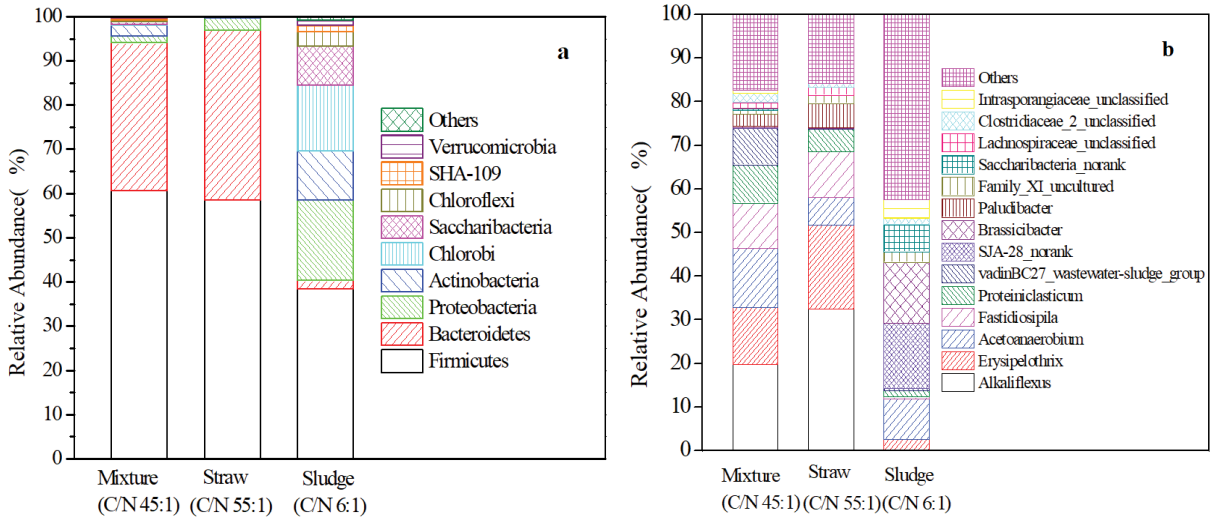

Fig. 7. Microbial community structure at the phylum level (a) and genus level (b).

involved in the hydrolysis and acidification of anaerobic digestion system. At the genus level, bacteria were much more diversified in the sludge system than in the mixture and straw systems (Fig. 7b). *Alkaliflexus*, *Erysipelothrix*, *Acetoanaerobium*, *Fastidiosipila*, and *Proteiniclasticum* were the major genera found in the mixture and straw systems. As shown in Fig. 7b, with an increase of straw, some bacteria that played an important role on organic degradation and H_2 production were enriched.

4. Conclusion

C/N ratio has a great influence on H_2 production under anaerobic conditions. It was observed that the system with a mixture of sludge and straw (C/N 45:1) was the optimal system for H_2 production. Under the optimal conditions, H_2 production was 5.2- and 1.2-fold higher than those measured in the sole sludge and the sole straw systems. Under the optimal conditions, soluble COD, VFAs, and key enzyme activities were improved. Firmicutes and Bacteroidetes, which were in relatively high abundance, were the dominant microorganisms beneficial to the degradation and utilization of organic matter. Clostridia was the major class of bacteria involved in H_2 production.

Acknowledgments

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