Journal of Bioscience and Bioengineering
VOL. 127 No. 1, 99–106, 2019

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Unravelling the influence of sulfate loading on enhancing anaerobic co-digestion of corn stover and bio-kerosene production wastewater

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Received 15 November 2017; accepted 10 July 2018
Available online 23 September 2018

This study was conducted to investigate the effect of sulfate loading on methane production and organic matter degradation during the mesophilic anaerobic co-digestion of corn stover and bio-kerosene production wastewater (BKPW). The highest methane production of 192.04 mL/gVS was obtained at a sulfate concentration of 86 mg/L. This was 46.80% higher than that achieved by a sulfate concentration of 113 mg/L. Additional degradation of organic matter was obtained at a sulfate concentration of 113 mg/L because organic matter in the corn stover and BKPW was oxidized by sulfate-reducing bacteria (SRB). The concentration of sulfate declined by approximately 23% after 29 days of anaerobic co-digestion, and this reduction in sulfate was enhanced when the soluble chemical oxygen demand (SCOD)/sulfate ratio was less than 15. The results of a mass balance analysis showed that 34.87% of C element and 10.04% of S element in substrate, respectively, were converted to biogas during anaerobic co-digestion of corn stover and BKPW at a sulfate concentration of 86 mg/L. The microbial community was analysed using 16S rDNA sequencing technology, and the results showed that the relative abundance of *Synergistes* (related to methane production with acetic acid) at a sulfate concentration of 86 mg/L had obviously increased and was approximately 287% higher than the abundance achieved at a sulfate concentration of 113 mg/L.

[**Key words:** Sulfate loading; Corn stover; Bio-kerosene production wastewater; Anaerobic co-digestion; Methane production; Microbial community]

It has been estimated that approximately 216 million tons of corn stover are generated in China each year (1). Because it is more cost-effective and environmentally friendly than other methods, anaerobic digestion is widely utilized in residual corn stover treatment (2). However, the complex structure and chemical properties of corn stover make its biodegradation difficult and likely to result in low biogas yields when corn stover is directly used as a substrate. Pre-treatment of corn stover is considered an effective method for improving the digestive efficiency of corn stover and biogas production (3). However, it cannot be applied in practical situations because of its high costs and risk of secondary contamination. Consequently, some researchers have searched for alternative methods to improve methane production resulting from the anaerobic digestion of corn stover.

In recent years, the production of bio-kerosene using lignocellulose substances (such as corn stover) as raw materials has aroused great attention from researchers and government officials around the world. During production, large amounts of sulfuric acid are added to the reactor as a catalyst to hydrolyse cellulose into furfural and levulinic acid. Bio-kerosene was obtained by catalytic hydrodeoxygenation of platform chemicals, which were prepared from furfural and levulinic acid in a sodium hydroxide solution. According to production practice, a ton of bio-kerosene produces approximately 20–30 tonnes of sulfate-containing wastewater. Because of its high temperature and volatile fatty acid (VFA) concentration, bio-kerosene production wastewater (BKPW) can be used as a substrate for methane production. However, BKPW contains a high concentration of sulfate because large amounts of sulfuric acid and sodium hydroxide are added to the reactor as catalysts. Therefore, the inhibitory effect of sulfate on methane production should be considered.

Reports have indicated that a high concentration of sulfate in anaerobic fermentation liquor can cause significant problems resulting from sulfate reduction (4). During the anaerobic digestion of high-sulfate wastewater, sulfate-reducing bacteria (SRB) and methanogenic bacteria (MA) often compete for the same substrates and produce poisonous sulfides during sulfate reduction (5), leading to decreased methane production (6). In addition, unnecessary SRB has been shown to result in a loss of methane generation when the chemical oxygen demand (COD) is oxidized directly into CO_2 in the presence of sulfate. However, Qatibi et al. (7) also found that propionate oxidation was strongly accelerated by the presence of sulfate. Adding sulfate also improved methane fermentation performance and propionate degradation (8) because propionate oxidation reactions mediated by SRB are thermodynamically more favourable than those mediated by acetogens (9). Currently, lowering the concentrations of sulfate and sulfide is considered an effective method for enhancing anaerobic treatment performance (10,11). Therefore, it is believed that high methane production and

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https://doi.org/10.1016/j.jbiosc.2018.07.010

organic matter degradation may be achieved by anaerobic co-digestion of corn stover and BKPW.

The main objective of this study was to delineate the effects of sulfate loading on enhancing anaerobic co-digestion of corn stover and BKPW. Pre-experiments demonstrated that methanogenic activity is not inhibited at sulfate concentrations of 95 mg/L (SCOD/sulfate ratio of 65). This study investigated the influence of sulfate loading on methane production and organic matter degradation when sulfate concentrations near 95 mg/L. At the end of the anaerobic co-digestion of corn stover and BKPW, a mass balance analysis of C and S elements was carried out. The microbial community of the fermentation liquor after anaerobic co-digestion was analysed by 16S rDNA sequencing, and its role in methane production is discussed.

MATERIALS AND METHODS

Experimental design The BKPW investigated in this study was obtained from the pilot plant of the Liaoning Institute of Energy Conversion in Yingkou City. The wastewater sample was filtered through a 0.45 μm polyethersulfone membrane before analysis. The corn stover used in this study was obtained from a farm at Northeast Agriculture University in Harbin City and dried prior to use. The dried corn stover was crushed in a shredder. The crushed corn stover diameter was approximately 2 cm. The anaerobic sludge used as an inoculum was collected from an anaerobic digester (working volume: 2000 mL) that digested cattle manure at 35°C for 40 days. The characteristics of the BKPW, corn stover and inoculums used in this study are shown in Table 1.

The batch reactors consisted of 1000 mL glass flasks. The experiments were divided into 3 groups: control group 1, control group 2, and experimental group 3. In control group 1, 580 mL of anaerobic sludge and 22 g of corn stover were added to obtain an inoculum-to-substrate ratio of 1.25 (based on total solids), which is optimal for methane production. The sulfate concentration in control group 1 was small enough to be safely disregarded. To obtain different sulfate concentrations in the fermentation liquor, 580 mL of anaerobic sludge and 22, 25, 27, 30 or 33 mL of BKPW were added to group 2. Finally, in experimental group 3, 580 mL of anaerobic sludge and 22 g of corn stover were added, and sulfate concentrations of 77, 86, 95, 104, or 113 mg/L were achieved in the fermentation liquor by adding 22, 25, 27, 30 or 33 mL of BKPW, respectively. Some distilled water was added if necessary to ensure that the quantities of fermentation liquor in the control and experimental groups remained consistent. The batch experiments were performed at a mesophilic level of 35 ± 1°C. All reactors were warmed in a Digital Biochemical incubator (Memmert IPP260, Memmert GmbH + Co. KG, Schwabach, Germany). Each sulfate concentration was tested in six flasks, including three for the gaseous phase (including biogas volume and biogas composition), and three for the liquid phase (including the SCOD, sulfate, pH and VFAs) (12). Gas and liquid samples with varying sulfate concentrations were analysed every day and then every 2 days after the initial incubation time, and the analysis interval was gradually increased over time. The total solid (TS), volatile solid (VS), hemi-cellulose, cellulose and lignin levels in the fermentation liquor were analysed at the end of anaerobic co-digestion of corn stover and BKPW. The reactors were monitored for 29 days. The batch experiment data were analysed using Origin 8.0 (OriginLab Corporation, Northampton, MA, USA).

Analytical methods TS, VS and pH were measured according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). SCOD and sodium ions were analysed using the rapid-digestion method (HH-6, Jiangsu Jiangfen Electroanalytical Instrument Co., Ltd., Taizhou, China) and flame photometry (F-100, Shanghai Metash Instruments Co., Ltd., Shanghai, China), respectively. Hemi-cellulose, cellulose and lignin levels were measured using semi-automatic cellulose analyser (ANKOM 200i, ANKOM Technology, New York, USA). Biogas volumes were measured via a water displacement method. The biogas composition (hydrogen, methane, nitrogen, hydrogen sulfide and carbon dioxide) was determined via gas chromatography (GC-6890N, Agilent Inc., Santa Clara, CA, USA) using a thermal conductivity detector (TCD). VFAs (acetic, propionic, butyric and total VFAs) and solvents (methanol and ethanol) were also analysed via gas chromatography (GC-6890N, Agilent Inc.) using a flame ionization detector (FID). The sulfate and nitrate concentrations were determined with a Skalar flow analyser (SA-5000, Skalar Analytical B.V., Breda, Netherlands). Carbon and nitrogen levels were determined using an elemental analyser (EA 3000, Leeman Technologies Co., Ltd., Beijing, China). All measurements were conducted in triplicate, and the averaged data are presented.

The microbial community was analysed using 16S rDNA sequencing technology. The fermentation liquor was collected from the reactor (control group 1, sulfate concentrations of 86 mg/L and 113 mg/L) at the end of the experiment for DNA extraction. A universal primer set (515F/806R, 5′-GTGCCAGCMGCCGCGGTAA -3′/5′-GGACTACHVGGGTWTCTAAT-3′) was used to amplify the V4 region of the 16S rDNA gene. To minimize variations, polymerase chain reaction (PCR) assays were performed in triplicate for each sample. The thermal cycling consisted of one initial denaturation cycle at 94°C for 120 s; 25 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The sample was then stored at 4°C. The PCR products were examined via agarose gel electrophoresis and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then, 16S rDNA sequencing was performed on an Illumina MiSeq Benchtop Sequencer by the Centre for Genetic & Genomic Analysis, Genesky Biotechnologies, Inc. (Shanghai, China).

TABLE 1. Characteristic of the BKPW, corn stover and inoculum.

Parameter	BKPW	Corn stover	Inoculum
TS (%)	4.66 ± 0.01	94.07 ± 0.50	4.46 ± 0.03
VS (%)	1.32 ± 0.00	88.95 ± 0.91	3.12 ± 0.01
pH	5.66 ± 0.02	–	6.94 ± 0.01
SCOD (g/L)	15.03 ± 0.21	–	3.30 ± 0.23
Sulfate (g/L)	1.94 ± 0.15	–	0.01 ± 0.00
Nitrate (mg/L)	4.12 ± 0.02	–	1.24 ± 0.01
Na^+ (g/L)	13.53 ± 0.34	–	–
Methanol (g/L)	0.14 ± 0.00	–	–
Acetic acid (g/L)	1.88 ± 0.01	–	–
Propionic acid (g/L)	0.15 ± 0.00	–	–
Butyric acid (g/L)	0.40 ± 0.00	–	–
C (%)	10.10 ± 0.07	38.63 ± 0.02	30.04 ± 0.04
N (%)	0.01 ± 0.00	0.78 ± 0.03	2.55 ± 0.10
Cellulose (%)	–	34.10 ± 0.11	11.59 ± 0.06
Hemi-cellulose (%)	–	28.36 ± 0.08	24.99 ± 0.00
Lignin (%)	–	5.57 ± 0.03	25.04 ± 0.01

% of dry matter weight; –, not determined. Values are expressed as means ± standard deviations (n = 3).

RESULTS AND DISCUSSION

Methane production Fig. 1 illustrates the cumulative amount of methane produced by anaerobic co-digestion of corn stover and BKPW over the digestion time in experimental group 3 (sulfate concentrations of 77, 86, 95, 104, and 113 mg/L). The results indicated that all sulfate concentrations were able to ferment normally, and the cumulative methane production rapidly increased over 3–12 days, possibly because of the characteristics of the inoculum. The inoculum was acclimated using cattle manure for 40 days, and methanogens were predominant. This assumption may also be supported by the microbial characteristics of the fermentation liquor in control group 1, which contained high relative abundances of

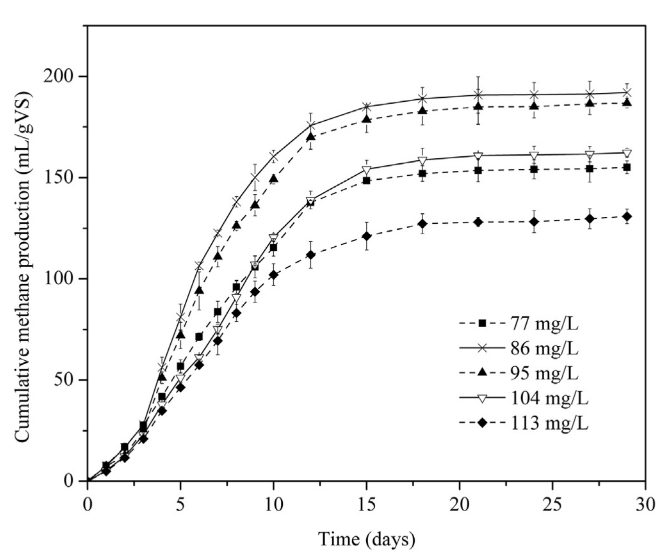

FIG. 1. Cumulative methane production at various sulfate concentrations throughout the entire experiment during the anaerobic co-digestion of corn stover and BKPW. Symbols: squares, 77 mg/L; crosses, 86 mg/L; up triangles, 95 mg/L; down triangles, 104 mg/L; diamonds, 113 mg/L. Values are expressed as the means ± standard deviations (n = 3).

fermentative bacteria, such as *Clostridium*, *Synergistetes* and *Methanosarcina*. In addition, the methane production rapidly increased over 3–12 days may have been related to a discrepancy in the substrate affinity characteristics of SRB and MA for acetate (9). The saturation constant (K_S) for acetate of MA may be 15-fold higher than that of acetate of SRB (13); therefore, MA predominates in carbon source utilization under higher organic matter concentrations during the initial incubation period. Ninety percent of the total methane yield was produced by the 12th day for various sulfate concentrations; however, for sulfate concentrations of 104 mg/L and 113 mg/L, approximately 90% of the total methane was produced by day 15. The cumulative methane production had almost ceased for all sulfate concentrations after 15 days of digestion, whereas sulfate reduction was apparently enhanced (Fig. 2). This may be because SRB usually effectively competes with MA for acetate and H_2 at low substrate levels, as shown in the study by Isa et al. (14). However, Zhao et al. (15) found that SRB was not dominant when the effluent of this upflow anaerobic sludge blanket (UASB) contained more than 350 mg/L of COD.

Cumulative methane production was 155.11, 192.04, 186.82, 162.29 and 130.81 mL/gVS for sulfate concentrations of 77, 86, 95, 104, and 113 mg/L (experimental group 3), respectively. The highest level of methane production was obtained with a sulfate concentration of 86 mg/L, at which production was 34.18% and 46.80% higher than that in the control group 1 (143.10 mL/gVS) and for a sulfate concentration of 113 mg/L, respectively. The cumulative methane production for control group 2, which contained only anaerobic sludge and 22, 25, 27, 30 and 33 mL of BKPW, were 130.88, 145.61, 136.65, 132.56 and 125.53 mL/gVS, respectively. The results of a C element mass balance analysis showed that the C content in BKPW was only 0.74% of the total C element in the fermentation liquor. Therefore, the sulfate load was the main factor affecting the anaerobic co-digestion of corn stover and BKPW. Except for sulfate concentrations of 113 mg/L, the cumulative amount of methane produced by anaerobic co-digestion of corn stover and BKPW was higher than the summed amount of methane produced by the separate anaerobic digestion of corn stover and BKPW. The reason for this increase in methane production may be that low concentrations of sulfate can promote anaerobic co-digestion of corn stover and BKPW. It was reported that sulfide produced by sulfate reduction can be used as an important sulfur source for the growth of methanogens and maintains a low oxidation-reduction potential in the anaerobic digestion system (16). The cumulative methane production with sulfate concentrations of 113 mg/L was obviously lower, indicating that the sulfate loading threshold for anaerobic digestion of corn stover was 113 mg/L. In addition, previous studies of the influence of sulfate-containing wastewater on anaerobic digestion have mostly focused on competition between SRB and MA (17,18). However, SOB also plays an important role in anaerobic digestion (19), and the role of SOB in anaerobic fermentation will be discussed in the next section.

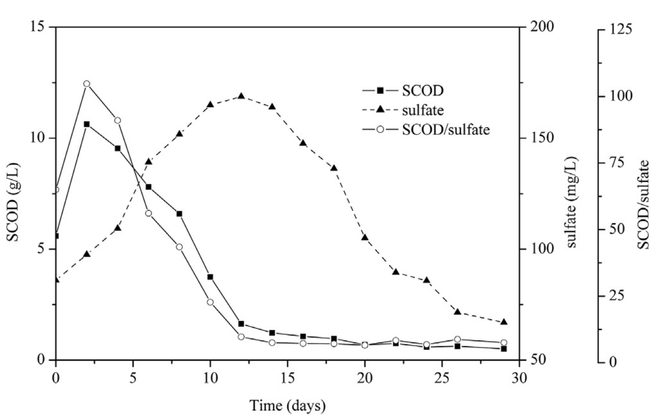

FIG. 2. Variations in SCOD and sulfate concentration and the SCOD/sulfate ratio at a sulfate concentration of 86 mg/L throughout the entire experiment performed to evaluate anaerobic co-digestion of corn stover and BKPW. Symbols: squares, SCOD concentration; up triangles, sulfate concentration; circles, SCOD/sulfate ratio. Values are expressed as the means (n = 3).

Except for sulfate concentrations of 113 mg/L, the methane content of biogas produced using different sulfate concentrations always exceeded 60% after day 4 (data not shown), suggesting that specific compounds in the BKPW have a limited inhibitory influence on methane production during anaerobic co-digestion of corn stover and BKPW. The high biogas methane content indicated that anaerobic co-digestion of corn stover and BKPW is an effective method to improve methane fermentation performance.

Degradation of organic matter and removal of sulfate The impact of sulfate loading on the degradation of organic matter was evaluated in terms of TS and VS reduction and lignocellulosic biomass reduction. As shown in Table 3, an increase in sulfate loading resulted in an increase in TS and VS removal. The highest TS and VS removals, 27.19% and 48.54%, respectively, were obtained at a sulfate concentration of 113 mg/L, and these were approximately 20.31% and 37.08% higher, respectively, than the sulfate concentrations achieved by 77 mg/L (Table 3). The degradation of cellulose, hemi-cellulose and lignin increased with increasing sulfate concentrations. When the sulfate concentrations increased from 77 mg/L to 113 mg/L, cellulose, hemi-cellulose and lignin degradation significantly increased from 23.16%, 22.64% and 14.32%, respectively, to 37.31%, 32.27% and 21.15%, respectively, consistent with the results reported by Liu et al. (1). In this study, the highest cumulative methane production was obtained with a sulfate concentration of 86 mg/L, whereas more degradation of organic matter was reached at a sulfate concentration of 113 mg/L, possibly because of the oxidation of organic matter by SRB during anaerobic co-digestion of corn stover and BKPW. This result was consistent with the results of 16S rDNA sequencing, which showed that the relative abundance of *Desulfovibrio* (a type of SRB) increased as the sulfate concentration increased.

As shown in Fig. 2, for different sulfate concentrations, SCOD increased rapidly and reached a maximum of approximately 10 g/L on day 2. Then, the SCOD concentration rapidly dropped to approximately 1 g/L on day 12 before descending more slowly to 0.50–0.80 mg/L after 29 days of anaerobic co-digestion of corn stover and BKPW. The rapid decline in the SCOD concentration might be related to the utilization of organic matter, which is primarily processed by MA, as evidenced by the high methane production observed during this period. Acetoclastic sulfidogenesis (Table 2, reaction 4) may have greatly contributed to the SCOD

TABLE 2. Reactions involved in anaerobic co-degradation of corn stover and BKPW respective Gibbs free energies.

	Reaction	$\Delta G'^0$(kJ mol^{-1})	Reference
1	$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O$	−743.9	20
2	$S^{2-} + 0.4NO_3^- + 2.4H^+ \rightarrow S^0 + 0.2N_2 + 1.2H_2O$	−191.0	21
3	$S^{2-} + NO_3^- + 2H^+ \rightarrow S^0 + 0.8NO_2^- + H_2O$	−130.4	21
4	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−47.3	22
5	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2$	+48.4	23
6	$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + 3H_2 + CO_2$	+76.1	23
7	$2CH_3CH_2CH_2COO^- + SO_4^{2-} \rightarrow HS^- + 4CH_3COO^- + H^+$	−55.5	22
8	$4CH_3CH_2COO^- + 3SO_4^{2-} \rightarrow 4HCO_3^- + 3HS^- + 4CH_3COO^- + H^+$	−150.6	22

TABLE 3. Degradation of organic matter after 29 days of anaerobic co-digestion of corn stover and BKPW.

Sulfate concentration (mg/L)	Degradation (%)				
	TS	VS	Cellulose	Hemi-celluloses	Lignin
77	22.60 ± 0.10	35.41 ± 0.12	23.16 ± 0.07	22.64 ± 0.00	14.32 ± 0.06
86	23.01 ± 0.08	38.73 ± 0.09	26.91 ± 0.02	25.95 ± 0.12	16.87 ± 0.04
95	25.92 ± 0.00	39.95 ± 0.11	30.37 ± 0.14	28.19 ± 0.01	18.17 ± 0.28
104	26.39 ± 0.41	45.14 ± 0.35	32.21 ± 0.05	30.64 ± 0.08	20.53 ± 0.07
113	27.19 ± 0.30	48.54 ± 0.12	37.31 ± 0.13	32.27 ± 0.00	21.15 ± 0.19

Values are expressed as means ± standard deviations (n = 3).

removal observed at all sulfate concentrations beginning at 12 days after digestion and lasting until the end of the experiment. This is mainly due to the thermodynamic advantages of acetoclastic SRB over acetoclastic methanogenic bacteria at low acetate concentrations. The SCOD values presented here were lower than those found by Liu et al. (1) for COD concentrations in microbial-pre-treated corn stover, and the value was 3 g/L at the end of digestion. In this study, SCOD removal was only slightly affected, although cumulative methane production was significantly impacted by sulfate concentrations. The variation observed in SCOD concentrations supported the previously discussed results for methane production as well as the results for sulfate production and consumption.

The variation in sulfate concentration temporal profiles is shown in Fig. 2. For all sulfate concentrations, the sulfate concentration increased with time during the initial incubation period and reached a peak at approximately 12 days. The obvious increases in sulfate concentrations may be attributed to SOB, which utilizes nitrate as an electron acceptor for the oxidation of sulfide into sulfate (Table 2, reaction 1), because of the relatively high concentration of nitrate found in the fermentation liquor during the initial incubation period. Additionally, nitrite produced by nitrate reduction is a strong inhibitor of SRB (19) and might therefore lead to acetate, and the H_2 released by fermentative bacteria was primarily utilized by MA and not SRB at the initial incubation time. A decrease in the sulfate concentration was observed after peak values were reached because of the sulfate reduction promoted by the high sulfate concentration, which was produced by SOB. Previous studies reported thermodynamic advantages for SRB over MA at low substrate levels, and the maximum growth rate of SRB at low acetate concentrations may be up to 15-fold higher than that of MA (24). All of the sulfate concentrations declined by approximately 23% after 29 days of anaerobic co-digestion of corn stover and BKPW, indicating that more sulfate was consumed by SRB than produced by SOB. Similar results were reported by Jing et al. (2), who found that the removal rate of sulfate was 28.2–42.5% when the organic loading rate (OLR) ranged from 6.1 to 37.8 g COD/L/d.

In this study, the SCOD/sulfate ratio ranged from 7 to 125 during anaerobic co-digestion of corn stover and BKPW. Cumulative methane production nearly ceased, and SRB governed organic matter consumption for SCOD/sulfate ratios less than 15 (Fig. 2). This result is consistent with the results of Kiyuna et al. (12), who observed that methane production was enhanced only for COD/sulfate ratios higher than approximately 25–30 and that sulfate reduction apparently ceased simultaneously at these levels. Barrera et al. (25) reported that both MA and SRB were inhibited by sulfide at COD/sulfate ratios from 10.0 to 5.0 in an up-flow anaerobic sludge blanket reactor continuously fed with sugarcane vinasse supplemented with Na_2SO_4.

VFA production and consumption Fig. 3 demonstrates the temporal profiles of VFA (mainly acetic acid, propionic acid, and butyric acid) concentrations at different sulfate concentrations. The variations in acetic acid and propionic acid concentrations derived from sulfate concentrations of 77 mg/L and 86 mg/L presented a similar pattern. A rapid increase in acetic acid concentration was observed at over 48 h of digestion. This was followed by a marked decrease and a subsequently continuous drop to a concentrations less than 0.13 g/L (Fig. 3A, B). The above-described increase in acetic acid was possibly caused by acetogen metabolism, whereas the later decrease was caused by the consumption of acetic acid, primarily by MA. For sulfate concentrations of 77 mg/L and 86 mg/L, propionic acid concentrations slightly increased during the initial incubation period before reaching peak values of 1.61 and 1.73 g/L, respectively, on the 6th day of incubation. After the acetic acid and butyric acid levels reached values less than 0.56 and 0.11 g/L (Fig. 3A, B), a decline in propionic acid concentration was observed, which was due to the Gibbs free energy of the oxidation of propionic acid into acetic acid by acetogen is 76.1 kJ/mol was thermodynamically higher than that of butyric acid (48.4 kJ/mol) (Table 2, reactions 5 and 6). The concentration of propionic acid dropped to 0.05–0.07 g/L on day 12 because propionic acid was converted to acetic acid and then rapidly consumed by MA. This result is consistent with previous results showing that cumulative methane production was nearly at peak on day 12 for sulfate concentrations of 77 mg/L and 86 mg/L (Fig. 1). The concentration of butyric acid in sulfate concentrations of 77 mg/L and 86 mg/L reached peak values on days 2 and 4, respectively. Regardless of the sulfate concentration, the concentration of butyric acid rapidly decreased to less than 0.08 g/L after day 8, while the concentration of propionic acid dropped to near its lowest level on day 12. These results may be attributed to the fact that the thermodynamic characteristics of propionic acid are less favourable than those of butyric acid during degradation to acetic acid, the latter of which requires less energy (23).

For sulfate concentrations of 95 mg/L, 104 mg/L and 113 mg/L, a rapid increase in the acetic acid concentration may occur during the initial period of incubation, and peak values of 4.80, 4.15 and 3.74 g/L, respectively, were reached on day 2. Thereafter, the acetic acid concentration decreased rapidly and was followed by a transient increase and subsequent decline in concentration to less than 0.22 g/L (Fig. 3C–E). The transient increase was most likely caused by the oxidation of butyric acid to acetic acid by SRB under higher sulfate concentrations, and this increased sharply as the butyric acid concentration decreased supporting our hypothesis during this period. This pattern did not appear in the other two experimental groups because of the low sulfate concentration. The Gibbs free energy for the oxidation of butyric acid into acetic acid (Table 2, reactions 5 and 7) indicates that less energy is required for butyric acid oxidation by SRB than for butyric acid oxidation by acetogens. A similar pattern was observed for the production and consumption of propionic acid at all sulfate concentrations, and a low propionic acid concentration was observed because propionate oxidation was enhanced by the presence of sulfate (Table 2, reaction 8) after anaerobic co-digestion of corn stover and BKPW. The concentration of butyric acid produced from sulfate concentrations of 95 mg/L, 104 mg/L and 113 mg/L reached peak values of 3.24, 3.79 and 2.83 g/L, respectively, on day 4 before rapidly decreasing to 0.07, 0.06 and 0.06 g/L, respectively, on day 8. For all sulfate concentrations, negligible variations in VFA concentrations were observed after the 18th day of incubation (Fig. 3A–E).

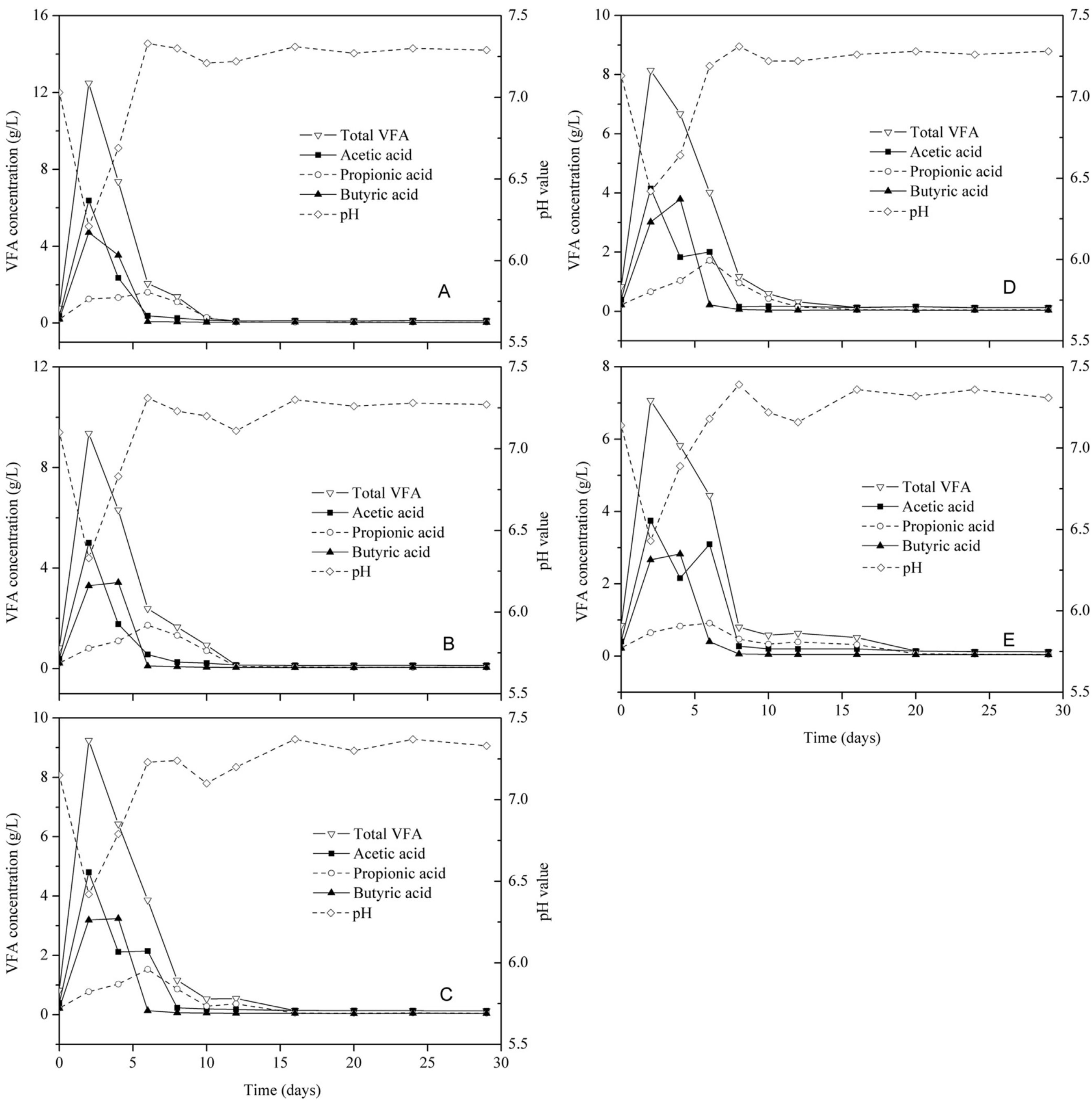

FIG. 3. Variations in VFA concentrations and pH at different sulfate concentrations throughout the entire experiment performed to evaluate anaerobic co-digestion of corn stover and BKPW: (A) 77 mg/L, (B) 86 mg/L, (C) 95 mg/L, (D) 104 mg/L, and (E) 113 mg/L. Symbols: down triangles, total VFA; squares, acetic acid; circles, propionic acid; up triangles, butyric acid; diamonds, pH. Values are expressed as the means (n = 3).

pH is an important parameter for anaerobic co-digestion of corn stover and BKPW, and it greatly depends on VFAs. pH decreased as VFA production increased. The lowest pH (approximately pH 6.3) was obtained on day 2 for all sulfate concentrations, and after day 6, pH increased to between 7.25 and 7.35 as the VFA concentration decreased because of the consumption of VFAs by methanogens. In addition, the pH increased as the sulfate concentration increased during the early stage of digestion (Fig. 3), and this effect was partially attributed to hydrogen ions produced by fermentation bacteria that were converted to water by SOB (Table 2, reactions 1–3). During the anaerobic co-digestion of corn stover and BKPW, SOB and SRB form a symbiotic relationship to promote VFA consumption and organic matter degradation (26). Fig. 3 shows that total VFAs (mainly acetic acid, propionic acid, and butyric acid) also decreased as sulfate concentrations increased. Fortunately, this decrease did not lead to decreases in methane production because degradation of organic matter is promoted due to the presence of sulfate. The results of this study indicate that anaerobic co-digestion of corn stover and BKPW could reduce the risk of acidification, increase methane production and organic matter degradation. It was also known that stable fermentation performance is difficult to achieve under a high OLR because of VFA accumulation, and acidification frequently occurs under anaerobic conditions, especially when using easily degradable substrates. Therefore, the anaerobic co-digestion of easily degradable substrates (corn stover) and BKPW (sulfate-containing wastewater) might be an effective method for overcoming such acidification problems.

Mass balance analysis of C and S elements The mass balance analysis data for C and S elements of anaerobic co-digestion of corn stover and BKPW process are shown in Table 4. The volumes of methane, carbon dioxide and hydrogen sulfide were calculated from the total volume and its percentage content. The mass balances for C and S are shown in Fig. 4. First, the C content of 17.81% in corn stover, BKPW and inoculum were converted to biogas and found to be 12.72% methane and 5.09% carbon dioxide. The remaining 82.21% of the C element flowed into the biogas residue. The reason why the carbon conversion efficiency is low is that the carbon in the inoculum accounts for almost 50% of the total carbon content, and this part of carbon can hardly be used by microorganisms. If the carbon from the inoculum were excluded, the conversion efficiency of carbon in the substrate (corn straw and BKPW) was 34.87% during anaerobic co-digestion of corn stover and BKPW, which was slightly higher than that reported by Niu et al. (27), 30.8% of C element was converted into biogas during anaerobic digestion of rice hulls. Second, S elements mainly flowed into the biogas residue. Among these, 74.54% existed as sulfates, while 15.42% existed as sulfur, sulfites, sulfides and so on. About 10.04% of S elements were converted to biogas.

TABLE 4. Mass balance analysis data for C and S elements at a sulfate concentration of 86 mg/L.

Item	Material	Total mass (g)	Element content (%)		Element flow (g)	
			C	S	C	S
Import flow	Inoculum	580	1.34	0.33	7.77	0.0019
	Corn stover	22	36.34	0	7.99	0
	BKPW	25[a]	0.47	0.0647	0.12	0.0162
Export flow	Biogas CH_4	2.69[b]	0.7500		2.02	
	CO_2	2.96[b]	0.2727		0.81	
	H_2S	0.0019[b]		0.9412		0.0018
	Biogas residue	605	0.0216	0.0022[c]	13.05	0.0133
				0.0005[d]		0.0030

Values are expressed as means ± standard deviations (n = 3).
[a] Units in mL.
[b] $TM_{biogas} = TV_{biogas} \times \rho_{biogas}$.
[c] Existed as sulfates.
[d] Existed as sulfur, sulfites, sulfides and so on.

Microbial community analysis The bacterial communities in control group 1, sulfate concentrations of 86 mg/L and 113 mg/L, were analysed by 16S rDNA sequencing after 29 days of anaerobic co-digestion of corn stover and BKPW, and the results demonstrated that sulfate loading had a real effect on the microbial communities. In this study, a total of more than 125,000 clean reads from each sample were obtained after sequences below a quality score of 20 and shorter than 100 bp in length were excluded. The dominant length distribution of the sequences obtained from the three samples was approximately 250–257 bp.

As shown in Fig. 5B, *Clostridium* and *Synergistetes* were the dominant groups in the fermentation liquor. *Clostridium* can utilize carbohydrates for H_2 production (28), and *Synergistes* is a recently recognized genus of anaerobic bacteria that is associated with methane production with acetic acid (29). The relative abundance of *Synergistetes* at a sulfate concentration of 86 mg/L was approximately 287% and 46% higher than the abundance achieved at a sulfate concentration of 113 mg/L and the control group 1, respectively, and this may be the main reason for increased methane production. The relative abundances of methanogens (such as *Methanosarcina*, *Methanobacterium* and *Methanosaeta*) (30) in control group 1 and at a sulfate concentration of 86 mg/L were no significantly different, indicating that sulfate at this concentration would not affect the specific methanogenic activities of the inoculum, consistent with the results reported by Wang et al. (31). The lowest relative abundance of methanogens was obtained at a sulfate concentration of 113 mg/L, consistent with the lowest methane production for this condition.

In addition, the relative abundance of SOB (such as *Sulfuricurvum*) increased as sulfate concentrations increased. The relative abundance of *Sulfuricurvum* increased from 1.01% to 3.35% when the sulfate concentration increased from 86 mg/L to 113 mg/L, and this might explain why the concentration of sulfate increased with time from the initial period of incubation. The relative increase in

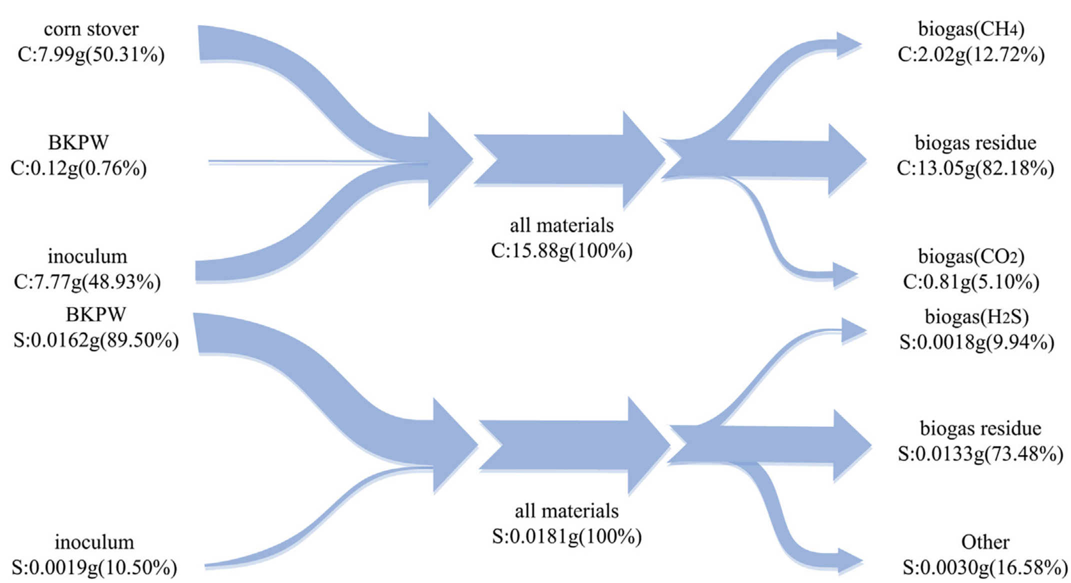

FIG. 4. Mass balance of C and S elements at a sulfate concentration of 86 mg/L.

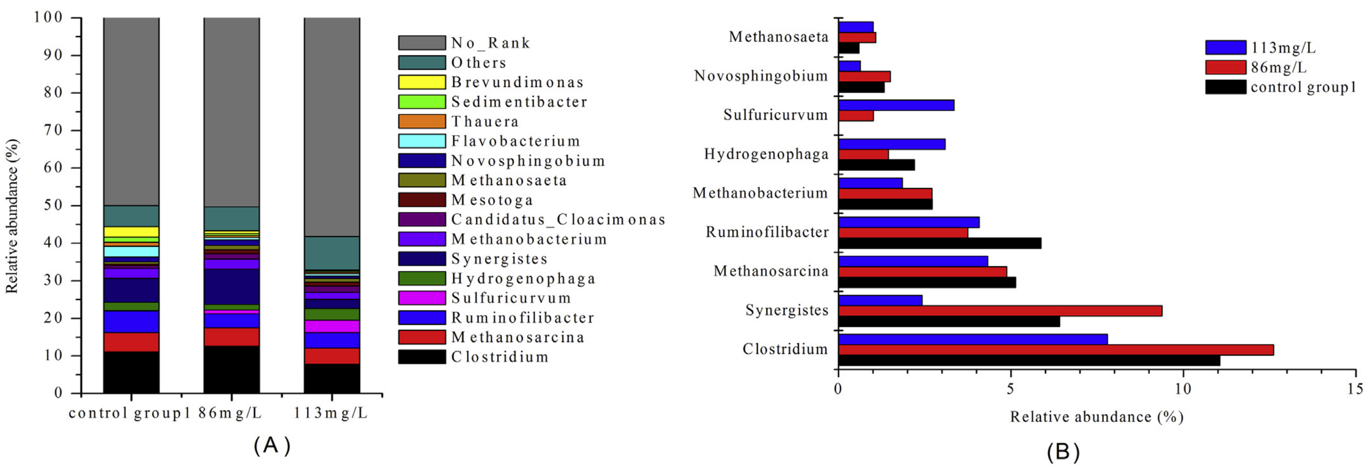

FIG. 5. Microbial communities determined by 16S rDNA sequencing technology. (A) The bacterial communities in control group 1 for sulfate concentrations of 86 mg/L and 113 mg/L. (B) The relative abundance of dominant bacterial communities (with relative abundance greater than 1%) for control group 1, sulfate concentrations of 86 mg/L and 113 mg/L in the fermentation liquor after 29 days of anaerobic co-digestion. In order to more clearly show the relative abundance difference of the dominant bacterial communities among control group 1, sulfate concentrations of 86 mg/L and 113 mg/L, the relative abundance of bacterial communities less than 1% in three groups are not listed.

the abundance of SRB (*Desulfovibrio*) followed a similar pattern relative to SOB and increased as the sulfate concentration increased.

ACKNOWLEDGMENTS

This work was supported by the Central Specialized Fund for the Development of Local Science and Technology (No. ZY17C05) and the Science and Technology Service Platform Project of Heilongjiang Province (No. PA17J201). This study was also partially supported by the Scientific and Technical Cooperation Program of Heilongjiang Province, the Chinese Academy of Sciences and Chinese Academy of Engineering (No. YS17A01) and the "Young Talents" Project of Northeast Agricultural University (No. 16QC16).

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