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Effect of organic loading rate on thermophilic methane fermentation of stillage eluted from ethanol fermentation of waste paper and kitchen waste

Li Tan,[1,2] Hiroto Nishimura,[3] Yan-Fang Wang,[4] Zhao-Yong Sun,[4] Yue-Qin Tang,[4] Kenji Kida,[4] and Shigeru Morimura[3,*]

Key Laboratory of Environmental and Applied Microbiology, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China,[1] Environmental Microbiology Key Laboratory of Sichuan Province, Chengdu 610041, China,[2] Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan,[3] and College of Architecture and Environment, Sichuan University, Chengdu 610065, China[4]

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Thermophilic methane fermentation was a valid approach for handling the stillage eluted from ethanol fermentation of waste paper and kitchen waste. The wide organic loading rate (OLR) range (2–14 g VTS/(L d)) for stable performance and relatively high energy recovery efficiency (79.0%) were achieved, and OLR of 8 g VTS/(L d) was optimum for achievement of highest biogas evolution and VTS removal efficiency. Microbial community analysis revealed that hydrolysis of cellulose was the critical step for methane production from the stillage.

[**Key words:** Organic loading rate; Anaerobic digestion; Microbial community; Waste paper; Kitchen waste]

Waste paper and kitchen waste collected as combustible waste are incinerated generally. However, incineration of this water-rich waste is energy-consuming and generates dioxins and dust when the incineration temperature is not high enough (1,2). Since both waste paper and kitchen waste are rich in carbohydrates such as cellulose and starch, a process was developed to convert the mixture of waste paper and kitchen waste to ethanol in our previous study (3). After distillation, the stillage contains organic compounds like residual sugars, protein, and organic acids. Our previous studies demonstrated that methane fermentation is an excellent strategy to handle the stillage, maximize energy recovery, and reduce emission of organic compounds (4–6).

Organic loading rate (OLR) is a critical operational parameter for methane fermentation. Methane fermentation is improved with increase of OLR at an appropriate range. However, an extreme high OLR will lead to accumulation of volatile fatty acids, decrease of biogas yield, and eventually irreversible failure (6–9). The optimum OLR of methane fermentation depends on the material supplied, process, and conditions. Generally, an appropriate high OLR is preferred due to reduction of the capacity requirements of reactor, save of energy cost for heating, and improved biogas evolution (10). Moreover, OLR is potentially used as bioengineering management tool to improve the stability and control the efficiency of methane fermentation (11,12).

Thermophilic methane fermentation is a potential strategy to reach a relatively high OLR, due to a strong capacity for metabolism indicated by the increased activity of microorganisms. As reported by Liu et al., the optimum volatile total solid (VTS) loading rate for methane fermentation of food waste under thermophilic condition (55°C) was 2.5 g/(L d), while it was 1.5 g/(L d) under mesophilic condition (37°C) (13). Besides, thermophilic methane fermentation offers several benefits over mesophilic digestion, including high degree of waste stabilization through destruction of viral and bacterial pathogens, a decreased retention time, a small reactor capacity, and a high biogas production (13,14). At present, there are plenty reports on performance of methane fermentation with increase in OLR under mesophilic condition (10–12,15,16), but the effect of OLR on performance of thermophilic methane fermentation is studied rarely (13).

Understanding the microbial community and function with regard to the operational parameters is benefit for optimization of operational conditions and maximize energy recovery of methane fermentation (17). Bacteria and archaea were able to adapt to the OLR disturbances during anaerobic treatment of tequila vinasses under mesophilic condition, favoring the interactions between syntrophic bacteria and *Methanosaeta* at high OLRs (16). Sun et al. (10) reported that the relative abundance of *Firmicutes*, *Bacteroidetes*, *Methanomicrobiales*, and *Methanobacteriales* increased dramatically as a response of increase in OLR during mesophilic methane fermentation of macroalgae. Compared with these mesophilic methane fermentations, there are few reports concerning effect of OLR on microbial community of thermophilic methane fermentation.

In this study, the stillage eluted from ethanol fermentation of waste paper and kitchen waste (hereafter called stillage) was supplied to thermophilic methane fermentation, to develop the process for maximizing energy recovery from waste paper and kitchen waste and reducing emission of organic compounds. The effect of OLR on physicochemical parameters and microbial community characteristics was studied to determine the optimum OLR.

* Corresponding author. Tel./fax: +81 96 342 3669.
E-mail address: morimura@kumamoto-u.ac.jp (S. Morimura).

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MATERIALS AND METHODS

Stillage and seed sludge Ethanol fermentation of waste paper and kitchen waste was carried out as described previously (3). Briefly, kitchen waste was liquefied at 90°C for 1 h and the mixture of waste paper and kitchen waste was presaccharified at 50°C for 16 h. After presaccharification, the mixture was cooled to 35°C, and yeast inoculum was added to the mixture to initiate simultaneous saccharification and fermentation (SSF). SSF was carried out at 150 rpm for 48 h. The fermented mash was subjected to vacuum distillation using an evaporator (N-11, EYELA, Tokyo, Japan) at 45°C and 65 mmHg. Distillation was terminated until 40% of the fermented mash was distilled. Stillage was prepared by adding tap water (the same amount of distillate) to the distillation residue.

The seed sludge was obtained from a local municipal sewage treatment plant. The total solids (TS), soluble total organic carbon (STOC), and pH were 8.3% (w/w), 5.7 g/kg, and 7.9, respectively. The seed sludge also contained 22.4 mg/L of SO_4^{2-}, 771.0 mg/L of NH_4^+, and 102.6 mg/L of total volatile fatty acids (VFAs). The seed sludge was acclimatized by feeding the stillage at a OLR of 2 g VTS/(L d) till steady volume of biogas was evolved.

Operation of thermophilic methane fermentation Thermophilic methane fermentation was carried out in a 10-L completely stirred-tank reactor (jar fermentor, MDL-10L; B.E. Marubishi, Chiba, Japan) with working volume of 8 L, as shown in Fig. 1. The stillage was fed once a day by the draw-and-fill method. A certain amount of the sludge was removed and replaced with the same amount of the stillage. The headspace of the reactor was replaced with nitrogen gas for 5 min after the feed every day. The OLR was increased stepwise from 2 g VTS/(L d) to 16 g VTS/(L d) with interval of 2 g VTS/(L d). The operation period for each OLR was 1–2 weeks according to our previous study (18). Ni^{2+}, Co^{2+}, and Fe^{2+} were added at concentrations of 0.05 mg/g VTS, 0.05 mg/g VTS and 0.65 mg/g VTS, respectively, to accelerate the methane fermentation rate (19). Methane fermentation was carried out at 53°C and 85 rpm. The reactor was micro-aerated by continuous supply of air at 3% amount of biogas produced from the atmosphere to reduce H_2S content in the biogas. The flow rate of air was controlled by a mass flow meter (KOFLOC 3200, Kojima Instruments Inc., Kyoto, Japan) connected to a control panel, which can adjust the air flow rate based on the real-time biogas production rate. After the evolved biogas was cooled and dried, the amount of biogas produced was recorded using an electromagnetic flow meter.

Microbial community analysis The sludge used for microbial community analysis was directly sampled from the reactor, before feeding stillage. Metagenomic DNA was extracted from approximately 0.4 g of each sludge sample using Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instruction. DNA quality was confirmed using agarose gel (1.0 % w/v) electrophoresis. The quality-confirmed DNA samples were sent to Sango Biotech (Shanghai) Co., Ltd. (Shanghai, China) for sequencing, and the V3 and V4 hypervariable regions of 16S rRNA genes were sequenced. The sequencing approach and data analysis followed the methods as described in our previous study (6). We used Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences (20). It does not contain the identities. Instead, a Confidence Threshold is available using the RDP classifier. The Confidence Threshold was 80% in our analysis. The Confidence Threshold of 80% is appropriate for our analysis due to the length of our sequences are approximately 400 bp. The sequencing data discussed in this study are available from the NCBI Sequence Read Archive (SRA) database under the accession number SRP148421.

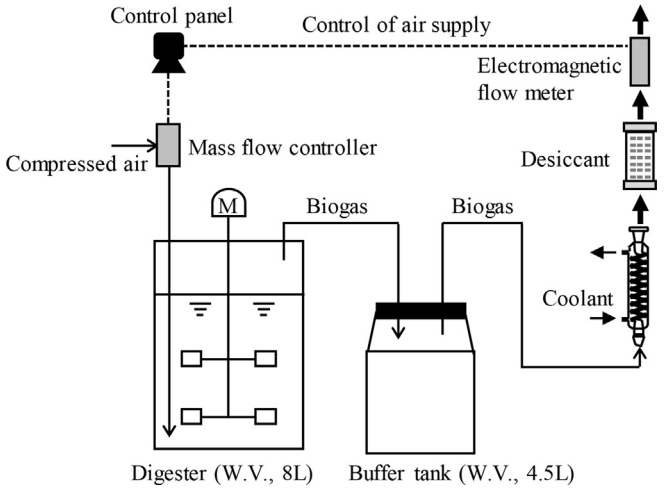

FIG. 1. Schematic diagram of the thermophilic methane fermentation system.

Analytical methods TS and VTS were analyzed in accordance to standard methods (21). The elements, including C, H, N and S, were measured with an elementary analyzer (Elementar, Germany). Element O was calculated as follow: O= 1−C−H−N−S−Ash. Ethanol was assayed by gas chromatography (GC353B; GL Sciences, Tokyo, Japan) using an internal standard (isopropanol). VFAs were analyzed using a post-label method by a high performance liquid chromatograph (HPLC) system equipped with a UV detector (at 450 nm) and Shimpack SCR-101H (Shimadzu, Kyoto, Japan) column, as previously described (22). STOC was measured using a TOC analyzer (TOC-V CSH, Shimadzu), and the pH was measured using a pH meter (HM-25G, TOA-DKK, Tokyo, Japan). The concentration of NH_4^+ was analyzed with the ion chromatography system (ICS-1500, Dionex, CA, USA) equipped with a CS-12A column for the analysis and a CG-12A guard column. The concentration of SO_4^{2-} was analyzed with a different ion chromatography system (ICS-1100, Dionex) equipped with an AS-12A column for the analysis and an AG-12A guard column. The methane content of the biogas was measured by gas chromatography using a thermal conductivity detector (KOR-2G, GL Sciences) equipped with a packed column (Porapack Q, GL Sciences). The contents of H_2S in the biogas were measured using Kitagawa precision gas detector tubes (Komyo Rikagaku Kogyo, Kawasaki, Japan).

Calculations The theoretical gas production ($V_{gas\text{-}T}$, in mL/g VTS) was calculated from Eqs. 1 and 2 (23).

$$C_aH_bO_cN_d + \frac{4a-b-2c+3d}{4}H_2O \rightarrow \frac{4a+b-2c-3d}{8}CH_4 + \frac{4a-b+2c+3d}{8}CO_2 + dNH_3 \quad (1)$$

$$V_{gas-T} = \left(\frac{4a+b-2c-3d}{8} + \frac{4a-b+2c+3d}{8}\right) \times 22.4 \times \frac{273+25}{273} \times 1000/W \quad (2)$$

where 22.4 (in L/mol) is the volume of 1 mol gas at standard temperature and pressure; (273+25)/273 was used to convert the volume of gas at standard temperature and pressure to room temperature (25°C) and atmospheric pressure (101 kPa); W (in g/mol) is the molecular weight of feedstock, which equals 12a+b+16c+14d; 1000 converts liters to milliliters;

The biogas evolution efficiency was calculated from Eq. 3.

$$\text{Biogas evolution efficiency} = \frac{V_{gas-M}}{V_{gas-T}} \times 100\% \quad (3)$$

where $V_{gas\text{-}M}$ (in mL/g VTS) is the measured gas evolution.

Koch's equation (Eq. 4) was used to estimate VTS degradation efficiency, assuming that the mass of undegradable material (inorganic fraction) remained constant (24).

$$\text{VTS degradation efficiency} = \left[1 - \frac{VTS_{digestate} \times \left(100 - VTS_{feed}\right)}{VTS_{feed} \times \left(100 - VTS_{digestate}\right)}\right] \times 100\% \quad (4)$$

where $VTS_{digestate}$ is the VTS content (in %, dry base) after dry methane fermentation; VTS_{feed} (in %, dry base) is the VTS content of feedstock.

We have previously reported that the lower heating value (LHV) calculated from the elemental contents was in good agreement with the combustion energy measured with the calorimeter (4). Thus, the LHV of feedstock (HI, in kJ/kg VTS) was calculated from Eq. 5.

$$HI = [8100C + 2900 \times (H - O/8)] \times 4.184 \quad (5)$$

where C, H, and O are the contents of C, H, and O, respectively; one calorie is equal to 4.184 J.

RESULTS AND DISCUSSION

Composition of stillage Three batches of stillage were used for thermophilic methane fermentation. The three batches of stillage contained similar TS and VTS of approximately 11.5% (w/w) and 10% (w/w, on a wet weight basis), respectively. Few ethanol (2.6–3.8 g/L) remained in the stillage. Glucose was not detected due to completely consumption during ethanol fermentation, while xylose of 55.5–125.5 mg/L was detected because the yeast strain *Saccharomyces cerevisiae* KF-7 used for ethanol fermentation cannot utilize xylose. As per the ethanol yield of 70.2% after ethanol fermentation, it was predicted that cellulose existed in the solid fraction (3). Relatively low concentrations of propionic acid (12.1–17.5 mg/L) were detected. The concentrations of lactic

acid were significantly different among the three batches, and the concentration of acetic acid of batch 2 was higher than those of batch 1 and batch 3, causing the different concentration of STOC. The contents of elements (C, H, N, and S) were also similar among the three batches, giving similar C/N ratio of approximately 20, which was just in the range of the optimum C/N ratio for anaerobic digestion of 20–30 (25). Theoretically, biogas of 928–953 mL/g VTS could be produced by the stillage, in which contains CH_4 of 53.1%–55.4% and H_2S of 3.1–4.8 ppm. H_2S is harmful for methane fermentation even at a low content of few hundred milligram per liter. Although the theoretical H_2S content is low, the thermophilic methane fermentation of stillage was micro-aerated by supplying air continuously at 3% of the amount of biogas produced to reduce the inhibition by H_2S, as reported previously (19).

Performance of thermophilic methane fermentation Time course of physicochemical parameters during thermophilic methane fermentation of stillage is shown in Fig. 2, and the average values of some of the parameters at steady state are presented in Table 1. Biogas evolution had increased from 504.1 ± 45.5 mL/g VTS to 605.4 ± 52.7 mL/g VTS with mean ± SD with increase in OLR from 2 g VTS/(L d) to 8 g VTS/(L d), while it decreased to 518.3 ± 29.6 mL/g VTS with further increase in OLR to 14 g VTS/(L d), which was consistent with the published literature (6,7). Change trend of VTS removal efficiency was same with that of biogas evolution, with the highest removal efficiency of 61.6% at OLR of 8 g VTS/(L d). It was also reported that biogas evolution and organic removal efficiency were improved with increase in OLR at appropriate ranges (6–8). Methane content reached above 70% at OLRs of 2 g VTS/(L d) and 4 g VTS/(L d), and it decreased to approximately 60%–66% at OLRs of 6–14 g VTS/(L d). The highest methane evolution of 389.6 ± 44.0 mL/g VTS at OLR of 4 g VTS/(L d), followed by 388.1 ± 33.8 mL/g VTS at OLR of 8 g VTS/(L d) without statistical difference (Table 1). H_2S content of below 50 ppm was relatively low due to supply of air. At OLRs of 2–14 g VTS/(L d), concentrations of acetic acid and propionic acid were 0–82 mg/L and 0–330 mg/L, respectively. Lactic acid was not detected, although it was higher than 10,000 mg/L in the stillage (Fig. 2). The pH was 7.9 at OLR of 2–8 g VTS/(L d), and it decreased slightly to 7.6 at OLR of 14 g VTS/(L d) (Table 1). The concentration of STOC was kept at approximately 2200 mg/L. NH_4^+ fluctuated regularly and SO_4^{2-} was barely detected (Fig. 2). At OLR of 16 g VTS/(L d), thermophilic methane fermentation of stillage had a trend of failure, as reflected by decrease in biogas evolution and pH to 7.0–7.2, accumulation of propionic acid, and residue of SO_4^{2-} (Fig. 2). The effect of OLR on physicochemical parameters is summarized in Fig. 3. Biogas evolution (mL/(L d)) increased with increase of OLR from 2 g VTS/(L d) to 14 g VTS/(L d), however, it became decreased at 16 g VTS/(L d). Concentration of STOC increased at 16 g VTS/(L d) with the increase of propionic acid concentration. As reported in our previous study on

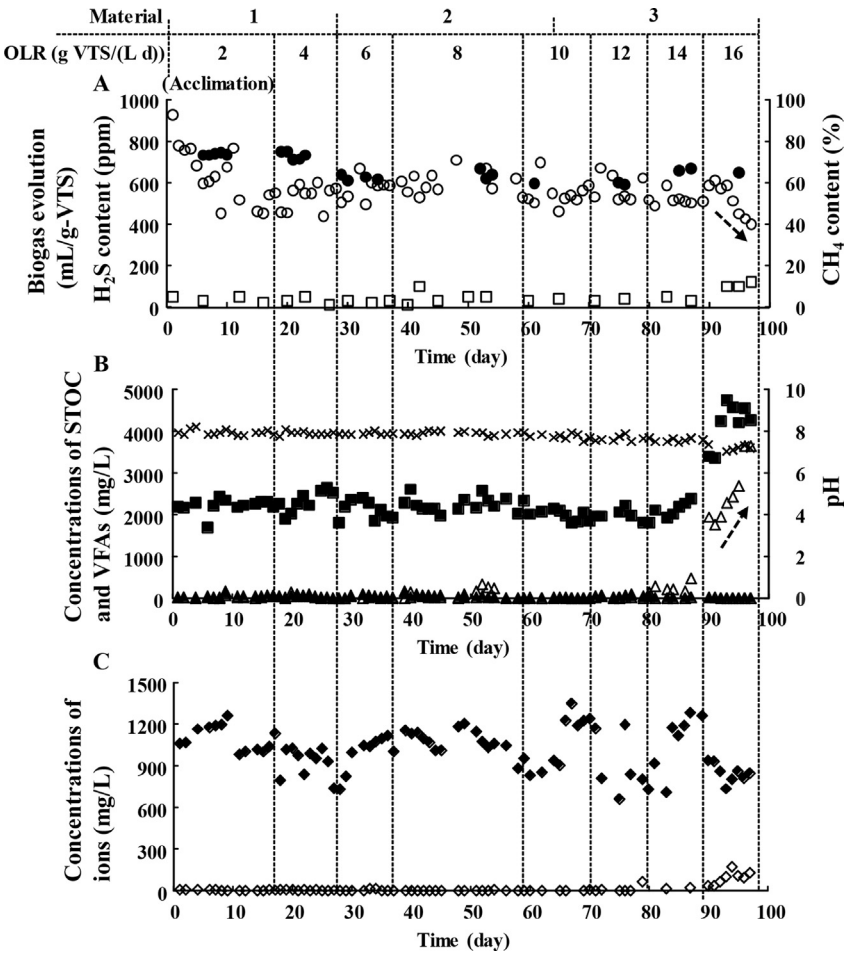

FIG. 2. Time course of physicochemical parameters during thermophilic methane fermentation of stillage. (A) Open circle, biogas evolution; closed circle, methane content; open square, H_2S content. (B) Closed square, soluble total organic carbon; open triangle, propionic acid; closed triangle, acetic acid; cross, pH. (C) Closed diamond, NH_4^+; open diamond, SO_4^{2-}.

TABLE 1. Changes of pH, methane content, gas evolution, solid content, and volatile total solid (VTS) removal efficiency on average with increase in organic loading rate (OLR) during thermophilic methane fermentation of stillage at steady state.

OLR (g VTS/(L d))	pH	Methane content (%)	Gas evolution (ml/g VTS)		VTS/TS[a] (%)	VTS removal efficiency (%)
			Biogas	Methane		
2	7.9 ± 0.1 [6][b]	73.6 ± 0.5 [5]	504.1 ± 45.5 [5]	370.9 ± 33.5 [5]	75.3 ± 4.3 [7]	53.0 ± 8.9 [7]
4	7.9 ± 0.1 [10]	73.0 ± 1.9 [5]	533.3 ± 60.2 [10]	389.6 ± 44.0 [10]	77.7 ± 2.7 [3]	54.7 ± 6.6 [3]
6	7.9 ± 0.1 [8]	62.2 ± 1.2 [4]	569.9 ± 56.8 [8]	354.6 ± 35.3 [8]	78.0 ± 1.8 [3]	54.1 ± 4.9 [3]
8	7.9 ± 0.1 [15]	64.1 ± 2.3 [3]	605.4 ± 52.7 [11]	388.1 ± 33.8 [11]	74.5 ± 3.5 [7]	61.6 ± 6.6 [7]
10	7.8 ± 0.1 [10]	59.6 ± 0.0 [1]	543.9 ± 59.9 [11]	323.9 ± 35.7 [11]	76.1 ± 0.9 [3]	59.1 ± 2.1 [3]
12	7.6 ± 0.1 [7]	59.5 ± 0.7 [2]	575.0 ± 63.8 [7]	342.1 ± 38.0 [7]	76.6 ± 0.4 [3]	58.0 ± 0.8 [3]
14	7.6 ± 0.1 [8]	66.2 ± 0.8 [2]	518.3 ± 29.6 [8]	343.1 ± 19.6 [8]	77.7 ± 1.2 [3]	55.0 ± 3.1 [3]

[a] TS, total solid.
[b] All data are shown as mean ± standard deviations. The numbers of sample used for calculation of means are in the brackets.

thermophilic dry methane fermentation of distillation residue eluted from ethanol fermentation of kitchen waste, accumulation of acetic acid followed by propionic acid went worse with increase in OLR (6). Therefore, it could be predicted that acetic acid would accumulate if thermophilic methane fermentation at OLR of 16 g VTS/(L d) was carried out for a longer time. Prior to complete failure of thermophilic methane fermentation of stillage, OLR was decreased to 10 g VTS/(L d). Thermophilic methane fermentation was recovered successfully, as reflected by the recovered biogas evolution and no more increase in accumulation of propionic acid. The OLR was subsequently rose to 14 g VTS/(L d), and steady biogas evolution was obtained and concentration of propionic acid had a decrease trend (data not shown). These results indicated that 14 g VTS/(L d) was the maximum OLR for stable performance of thermophilic methane fermentation of stillage, and OLR of 8 g VTS/(L d) was suitable for achievement of high biogas and methane evolution.

Microbial community analysis Microbial community was focused on the most abundance (relative abundance of >1%), while the rest are represented as minor phyla. The relative abundances of microbes (bacteria and archaea) for each OLR are shown in Fig. 4A and B. Totally, there were 13 genera of bacteria and 2 genera of archaea with relative abundance of >1%, accounting for 81.6%–90.2% and 80.0%–98.1% of the total bacterial and archeal reads, respectively.

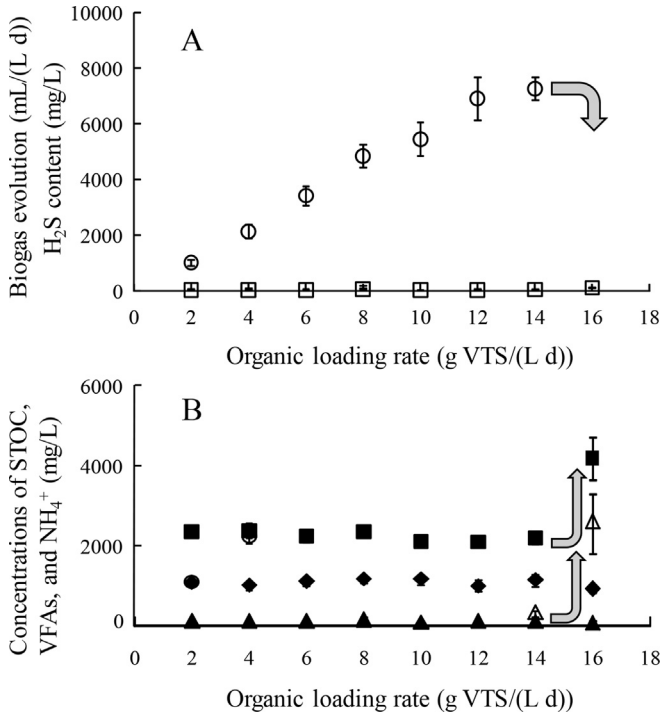

FIG. 3. Effect of organic loading rate on physicochemical parameters during thermophilic methane fermentation of stillage. (A) Open circle, biogas evolution; open square, H_2S content. (B) Closed square, soluble total organic carbon; open triangle, propionic acid; closed triangle, acetic acid; closed diamond, NH_4^+.

Genera 060F05-B-SD-P93 and *Thermosyntropha* were the core dominant bacteria, which accounted for 9.2%–49.5% and 16.6%–35.3% of total bacteria at steady state, respectively. The genus 060F05-B-SD-P93 could produce extracellular polymeric substances (EPS) which promote the formation of stable cellular aggregates and facilitate interspecies hydrogen transfer in anaerobic digestion reactor (26,27). This genus is close to *Defluviitoga tunisiensis* with 99% similarity of 16S rRNA gene in BLAST. *D. tunisiensis* grows at 37–65°C (optimum 55°C) with 0.2%–3% (w/v) NaCl (optimum 0.5%) (28). Since our thermophilic digestion (53°C) of stillage contained NaCl deriving from kitchen waste, it allowed a suitable habitat for the genus 060F05-B-SD-P93 and led this genus dominance reasonably. *Thermosyntropha lipolytica* could grow syntrophically with methanogens on lipids utilizing only the liberated fatty acid moieties but not the glycerol (29). Both dominant genera could accelerate methane production in a digester. Stillage contained residual cellulose, fatty acids, and protein as the main carbon source for methane fermentation. Actually, the known bacterial genera were clustered into 4 groups based on the main function of bacteria, namely, long-chain fatty acids-degrading bacteria (*Thermosyntropha* (29) and *Syntrophomonas* (30)), proteolytic bacteria (*Coprothermobacter* (31), *Proteiniphilum* (32), and *Caldicoprobacter* (33)), cellulolytic bacteria (*Ornatilinea* (34) and *Herbivorax* (35)), and acidogenic and acetogenic bacteria (060F05-B-SD-P93 (26,27), *Gelria* (36), *Defluviitalea* (37,38), *Anaerobaculum* (39), and *Lactobacillus* (27)). Proteolytic bacteria could also produce fatty acids by deamination of amino acids. The change of relative abundance of each group is shown in Fig. 4C. The relative abundance followed the order of acidogenic and acetogenic bacteria > long-chain fatty acids-degrading bacteria > proteolytic bacteria > cellulolytic bacteria at OLRs of 2–14 g VTS/(L d). The relative abundance of long-chain fatty acids-degrading bacteria increased from 24.9% to 37.8% with increase in OLR from 2 g VTS/(L d) to 6 g VTS/(L d), and then decreased to 21.0% at OLR of 8 g VTS/(L d) and kept relatively stable with further increase in OLR to 14 g VTS/(L d). The relative abundance of proteolytic bacteria increased from 5.2% to 14.2% with increase in OLR from 2 g VTS/(L d) to 8 g VTS/(L d) and kept relatively stable at OLRs of 8–14 g VTS/(L d). The relative abundance of cellulolytic bacteria had two peaks at OLRs of 8 g VTS/(L d) and 14 g VTS/(L d) with peak values of 9.7% and 8.4%, respectively. The relative abundance of acidogenic and acetogenic bacteria decreased from 56.0% to 37.4% with increase in OLR from 2 g VTS/(L d) to 6 g VTS/(L d) and increased to 45.6% when the OLR was increased to 10 g VTS/(L d). It re-decreased to 31.2% at OLR of 14 g VTS/(L d).

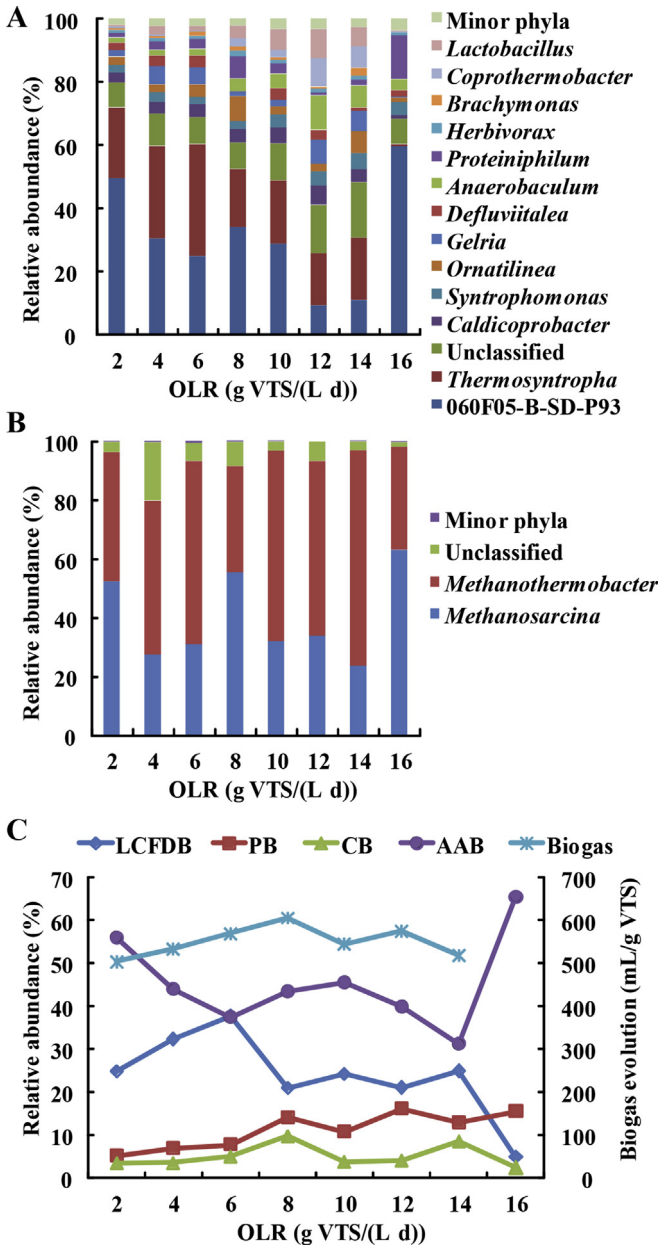

FIG. 4. Changes of relative abundance of (A) bacteria, (B) archaea, and (C) functional bacteria with increase in organic loading rate (OLR). LCFDB, long-chain fatty acids-degrading bacteria, consisting of *Thermosyntropha* and *Syntrophomonas*; PB, proteolytic bacteria, consisting of *Coprothermobacter*, *Proteiniphilum*, and *Caldicoprobacter*; CB, cellulolytic bacteria, consisting of *Ornatilinea* and *Herbivorax*; AAB, acidogenic and acetogenic bacteria, consisting of 060F05-B-SD-P93, *Gelria*, *Defluviitalea*, *Anaerobaculum*, and *Lactobacillus*; biogas, biogas evolution.

Judging from the inflection point of each curve in Fig. 4C, OLRs of 6 g VTS/(L d) and 8 g VTS/(L d) were the critical values for changes of relative abundance of bacteria. Integrating change of biogas evolution, it concluded that OLR of 8 g VTS/(L d) was the most critical during thermophilic methane fermentation of stillage. Meanwhile, changes of the relative abundance of cellulolytic bacteria were similar with change of biogas evolution, indicating that hydrolysis of cellulose was the critical step during thermophilic methane fermentation of stillage. When thermophilic methane fermentation of stillage went worse due to overloading (16 g VTS/(L d)), significant decrease in the relative abundances of long-chain fatty acids-degrading bacteria and cellulolytic bacteria were the primary reason for decrease in biogas evolution, and significant increase in the relative abundances acidogenic and acetogenic bacteria were the main reason for accumulation of propionic acid. Although the relative abundance of proteolytic bacteria changed insignificantly, the relative abundance of *Proteiniphilum* increased sharply from 1.9% to 14.0% (Fig. 4A). As reported by Li et al. (40), the relative abundance of *Proteiniphilum* increased significantly at the failure stage of methane fermentation of food waste. Also, as reported in our previous study on thermophilic dry methane fermentation of distillation residue eluted from ethanol fermentation of kitchen waste, the relative abundance of proteolytic bacteria was still high when overloading, resulting in significant accumulation of NH_4^+ (6).

Specifically, *Brachymonas* with relative abundance of 0.1%–2.4% was detected in this study. *Brachymonas* has a respiratory type of metabolism with oxygen or nitrate as the terminal acceptor, encodes methane monooxygenases, and does not produce acid from carbohydrates (41–44). In this study, air was supplied to reduce H_2S inhibition to methane fermentation, giving *Brachymonas* living condition. The existence of *Brachymonas* in steady state of thermophilic methane fermentation indicated that there was biological pathway to prevent methane fermentation from toxicity of oxygen.

Compositions of archaea were mainly *Methanosarcina* and *Methanothermobacter*, which had no notable changes. However, distinct difference was observed in the relative abundance of each genus with increase in OLR. The relative abundance of *Methanosarcina* increased from 27.4% to 55.5% with increase in OLR from 4 g VTS/(L d) to 8 g VTS/(L d), and it decreased to 23.7% at OLR of 14 g VTS/(L d). On the contrary, the relative abundance of *Methanothermobacter* decreased from 52.3% to 36.0% with increase in OLR from 4 g VTS/(L d) to 8 g VTS/(L d) and increased to 73.3% with further increase in OLR to 14 g VTS/(L d). This result also indicated that OLR at 8 g VTS/(L d) was critical for thermophilic methane fermentation of stillage, and the most dominance of *Methanosarcina* suggested that methane production from stillage preferred acetoclastic pathway at this critical value (45). With unsteady performance at OLR of 16 g VTS/(L d), the relative abundances of *Methanosarcina* and *Methanothermobacter* suddenly changed to 63.2% and 34.9%, respectively. The genus *Methanosarcina* has been reported to be more favorable in elevated VFA concentrations (46,47).

The results above indicated that the microbial community shifted gradually with increase in OLR, even though steady state was achieved. However, when thermophilic methane fermentation suffered from overloading, microbial community changed suddenly and significantly.

Mass and energy balance Based on the result of thermophilic methane fermentation of stillage at OLR of 8 g VTS/(L d), mass and energy balance was calculated integrating ethanol fermentation and methane fermentation. According to the previous study on ethanol fermentation of waste paper and kitchen waste at the pilot scale, waste paper of 3.11 kg (dry weight) and kitchen waste of 1.30 kg (dry weight) were used, which contained energy of 56,433.9 kJ and could produce ethanol of 1.43 kg theoretically (3). As shown in Fig. 5, after ethanol fermentation, ethanol of 1.00 kg containing energy of 28,319.3 kJ was produced, corresponding to energy recovery efficiency of 49%. After distillation, energy of 51% was recovered in the stillage. Thermophilic methane fermentation of the stillage produced methane of 0.48 m^3 containing energy of 17,324.5 kJ, corresponding to energy recovery efficiency of 59%. Totally, energy of 79% containing in waste paper and kitchen waste was recovered as ethanol and methane. It was indicated that methane fermentation following ethanol fermentation of waste paper and kitchen waste is a valid

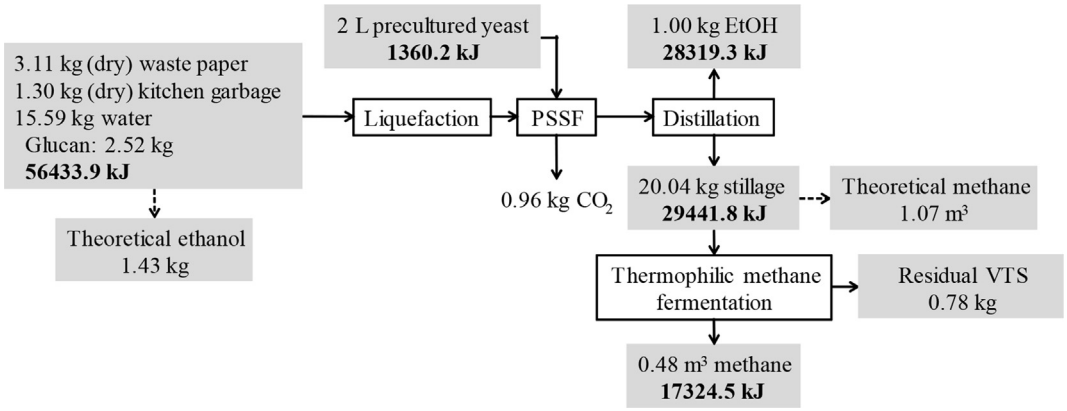

FIG. 5. Mass and energy balance integrating ethanol fermentation and methane fermentation of the mixture of waste paper and kitchen waste. PSSF, pre-saccharification and simultaneous saccharification and fermentation.

method to maximize the energy recovery and reduce the organic compounds.

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