Effect of substrate to inoculum ratio on biogas production and microbial community from hemi-solid state batch anaerobic co-digestion of rape straw and dairy manure

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Abstract: Substrate to inoculum (S/I) ratio is crucial for the fast start-up of SS-AD digesters. In this study, performances of methane production, digestion stability, and microbial community structure in co-digestion of rape straw (RS) and dairy manure (DM) at different S/I ratios (2:3, 1:1, 2:1, 3:1 and 4:1) in batch HSS-AD tests. The highest methane yield of 209.1 mL/g VSadded and the highest volumetric methane production of 0.4 L/(L·d) was achieved at S/I ratio of 2:3 and 2:1, respectively. Digesters at lower S/I ratios (1:2, 1:1, and 2:1) produced biogas steadily throughout the whole AD period, while digesters at higher S/I ratios (3:1 and 4:1) failed in producing biogas at the initial stage of AD as a consequence of excess VFAs accumulation and low pH. The 16S Illumina sequencing and qPCR quantification analyses indicated that significant differences in the bacterial and archaeal community resulting from the S/I ratios. In digesters at S/I ratio of 2:3 and 2:1, the predominant bacteria and archaea was the phyla Firmicutes and the genus Methanosaeta, respectively. Meanwhile, in digesters at S/I ratio of 4:1, the main dominant bacteria shifted to the phyla Bacteroidetes and the relative abundances of Methanobacterium increased from about 10% to 23%. The amounts of bacteria and archaea were significantly inhibited in acidic digesters. The findings of this study may provide useful information to enhance the efficient methane production and advance understanding of microbiome in HSS-AD of RS and DM at different S/I ratios.

Keywords: Substrate to inoculum ratio, hemi-solid state digestion, rape straw, methane production, microbial community

1. Introduction

Anaerobic digestion (AD) plays a key role converting organic wastes decomposition by a complex bacterial consortium in oxygen-free environment into methane-rich biogas as a renewable energy, either for solving the fossil energy shortage, or for reducing environmental pollution [1-2]. Based on the total solids(TS) content of feedstock, AD has been developed as wet (≤10% TS), hemi-solid (10-15%TS) and solid (>15% TS) state technologies [3]. Wet anaerobic digestion (W-AD) is typically applied for substrates with the high moisture content, including domestic and industrial wastewater [4]. However, it is not suitable for high solid content wastes, such as agricultural crop straws, livestock manures and municipal solid wastes, because of high consumption of water and large volume of digester for treating low moisture feedstocks. In general, compared to the conventional W-AD, solid state anaerobic digestion (SS-AD) has several advantages including higher volumetric methane productivity, smaller digester volume, less water for dilution and wastewater generation, no floating substrates, and positive energy balances [5]. In recent years, SS-AD has been given great attention recently practice in handling agricultural wastes [6]. This technology was adopted for more than 60% of recently built anaerobic digesters in European countries but no in others such as China [7]. The Chinese government has set the goal of production 8 billion m³ bio-methane to generate heat, electricity, and vehicle fuels as substitutes for 9.6 million fossil fuels every year by 2020 [8]. Currently, almost all medium and large-scale biogas plants are using W-AD technology with the low TS content (≤10% TS) animal manure as feedstock alone to produce biogas. However, a large amount of wastewater is generated and difficult to be treated causing secondary contamination in W-AD [9]. On the other hand, manure has limited availability and is hard to be as a stable feedstock supply for scaled biogas production in many places of China [10]. These shortages of AD technology have become major bottlenecks for realizing the goal. Therefore, alternative feedstocks such as crop residues and an efficient methane production process need to be developed urgently.

Agricultural crop straws are abundantly available feedstocks for SS-AD. In China, an estimated 30 million tons of rape straw (RS) is generated every year and over 50% of it as waste is dumped or burned in open field during harvest season, resulting in serious environmental pollution [11]. Hence, it is imperative to recycle RS in order to govern the pollution associated through AD. Many studies have been conducted for SS-AD of various crop straws, such as corn stover, wheat straw and rice straw, and confirmed the economic feasibility [12-14]. However, a few studies focused on the rape straw as a feedstock for biogas production from SS-AD. Rape straw as SS-AD feedstock in the agricultural area has attractive advantages such as easier harvest and transport, lower operating cost, because of its geographically concentrated cultivation, low sulphur content in favour of producing electricity and purifying methane from biogas to produce compressed bio-natural gas, and high cellulose content (>50%) with excellent biochemical methane potential (BMP) [15-16]. But for all this, RS as feedstock of SS-AD has a low methane production yield per unit mass and an instable process during the digestion because of hydrogenolysis rate limitation of its recalcitrant lignocellulose structure, imbalanced nutrients of its high carbon to nitrogen (C/N) ratio (>50) for microbial growth, and mass transfer limitation and start-up time extension at high TS(>20%) [17-18]. Although SS-AD with leachate recirculation seems an appropriate method for the valorization of RS at 20% TS content, which enhances mass transfer by redistributing substrates, nutrients, and microorganisms, the accumulation of ammonia, volatile fatty acids (VFAs), and other metabolism products is occurred in leachate with repeated recirculation resulting in inhibit microbial activities [18-19]. Meanwhile, the contradictory results of significant

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increase methane production have been obtained in some studies on leachate recirculation tests [6, 20]. Co-digestion is widely applied in commercial scale digesters to achieve higher methane yield and to increase more stable the feedstock supplying capacity than digesting individual feedstocks in SS-AD [7]. Many studies showed that co-digestion could balance the C/N ratio, dilute inhibitors, improve buffer capacity and achieve synergistic effect, which are beneficial to the SS-AD process [3, 21]. In consideration of the complementary nature of C/N ratio between RS and animal manures with low C/N ratio (<25), the mixing of both substrates in proper C/N ratio (25-35) is feasible for methane production. Nevertheless, co-digestion of them has not yet been received the attention using SS-AD system.

Generally, inoculation dose is considered to be one of the most important factors in batch SS-AD with lignocellulosic feedstocks to improve high methane yield and digester stability. Previous studies reported that inoculation dose affected not only microbial population, but also physical and chemical properties of fermentation during SS-AD [22-23]. Larger inoculation dose has been proved to short start-up time and increase specific methane production rate (SMPR) based on weight because of providing higher initial total VFAs, buffering capacity, and more methanogens in SS-AD [24-25]. However, excessive inoculum takes up more space and thus decreases volumetric methane production rate (VMPR). On the contrary, too low inoculum dose leads to the accumulation of VFAs and the drop in pH, which can inhibit microbial activities and induce the failed solid-state anaerobic digester [25-26]. To achieve the proper balance between SMPR and VMPR, the substrate to inoculum (S/I) ratio is often optimized for SS-AD lignocellulosic biomass. The optimal S/I ratio was reported to be in the range of 1-30 for SS-AD reactors with different agricultural crop straws including corn stover, rice straw, wheat straw at 15%-25% TS [4,12, 27-28]. A great difference of the preferred S/I ratio values above mentioned was found because the performance reactor also varied with other factors, such as lignocellulosic characteristics, TS content of substrate, and inoculum source except S/I ratio in batch SS-AD. Motte et al.[28] reported that S/I ratio has only an effect on the start-up phase, TS content is the main parameter governing the methane production during the growing phase of the SS-AD, and methane production rate of wheat straw at 15% TS is clearly higher than that of feedstock at 20% and 25% TS. The highest MVPR from co-digestion of corn stover and dairy manure (DM) is reached at 15% TS in a continuous reactor [29]. The conclusion is consistent with other studies [30-31]. Furthermore, the continuous and intensive mixing at 10%-15% TS of feedstock is easier to operate compared to 20-25% TS in large-scale digesters [32]. These results imply that hemi-solid state AD (HSS-AD) could be a threshold for efficient methane production from lignocellulosic feedstocks. To our knowledge, there is no investigation so far focusing on the evaluation of methane production, digestion stability, and microbial community structure in co-digestion of RS and DM at different S/I ratios in HSS-AD system.

Based on that, the objective of the present study was to: (1) obtain the proper ratios of S/I to achieve high SMPR and VMPR from co-digestion of rape straw and dairy manure in batch tests under HSS-AD (15%TS) condition, (2) evaluate the digestion stability at different S/I ratios of digesters from dynamical changes of biogas production, pH value and VFAs concentration during AD, and (3) monitor the microbial communities including bacteria and archaea in representative digesters by using quantitative real-time PCR (qPCR) and Illumina sequencing to understand the correlations of microbes and digester performance.

2. Methods

2.1. Feedstock and inoculum

Rape stalk (RS) was collected in July 2016 from a crop straw processing station (Jingyan Country, Leshan City, Sichuan, China), then was oven-dried at 60°C for 24 h in a Circulation Oven (Model DHG-9075A, Qixin, China) to obtain a dry mass content of more than 90%, smashed with a hammer mill (Model BAFS-20C, Baozheng, China) to particle size of 1-mm, and stored in zip-lock bags. Dairy manure (DM) was obtained in August 2016 from a dairy farm (Suqi Town, Leshan City, Sichuan,China), and conserved at -4°C in a refrigerator (Model HYC-198, Haier, China) to prevent biological decomposition. DM was unfroze at 4°C before using. Inoculum was collected from a 55-L well-run anaerobic digester under mesophilic operation, which was fed with rape stalk and dairy manure in our laboratory. Prior to use, the inoculum was acclimated and degassed at 37°C for 30 days to minimized the background biogas production, and then concentrated to achieve a total solid content (TS) of more than 15% by a refrigerated centrifuge (Model ST 16R, Thermo Fisher Scientific, USA). The characteristics of RS, DM and inoculum are shown in Table 1.

2.2. Batch anaerobic digestion tests

Batch tests were carried out in triplicate using 1 L home-made glass bottles with sample outlet at the bottom, and the working volume of each reactor was about 0.7 L. The substrate to inoculum (S/I) ratios were 2:3, 1:1, 2:1, 3:1 and 4:1, on a volatile solid content (VS) basis. Three reactors without any substrate addition and with the same amount of inoculum and water were used as controls. The initial total solid content (TS) of digestion system was 15% and the mixture of RS to DM ratio was adjusted to 34:1 (C/N) as the optimized result in our previous study [33]. All tests were purged with N₂ for 5 min to remove the oxygen and sealed using silicone stoppers, and then incubated in a biochemical incubator (Model LRH-250, Yiheng, China) for 30-60 days at 37±1°C. The biogas produced by each reactor was collected in a 2-L gas aluminium foil bag. The composition and volume of were measured every 1-3 days and all reactors were manually shaken twice a day for about 1 min to complete mixing. About 6-7 g of digestion substrate was sampled from the reactors and frozen in −20°C for further analysis. The sampling time depended on the daily biogas production of each digester. Before analysis, all samples were unfrozen at room temperature and divided two portions. One portion was measure contents of TS and VS to estimate organics the removal rates of substrate. Another portion was
centrifuged at 10,000 for 10 min, the supernatant was obtained to measure pH value and VFAs concentration, and the sediment was gained to extract total genomic DNA and monitor VS content, which was used to analyze the amount of biomass for DNA extraction.

2.3. Analytical methods

Biogas volume was measured using the water displacement method at ambient temperature, and corrected according to the standard temperature (0°C) and pressure (101.325 kPa). Methane and carbon dioxide contents of biogas were analyzed by a biogas analyzer (Model Biogas 5000, Geotech, Britain). Specific methane production rate (SMPR) and volumetric methane production rate (VMPR) was respectively expressed as shown in formulas (1) and (2) [3, 8]:

$$SMPR \left( \text{mL} \cdot \text{g VS}_{\text{added}}^{-1} \right) = \frac{V_1}{W}$$

$$VMPR \left( \text{mL} \cdot \text{mL}^{-1} \cdot \text{d}^{-1} \right) = \frac{V_1}{(V_2 \times T_{80})}$$

where $V_1$ means the cumulative methane volume during the whole digestion period (mL), $W$ is the weight of substrate VS added to digester (g VS$_{\text{added}}$), $V_2$ means represents the volume of reactor (mL), $T_{80}$ stands for the shortest technical digestion time (d), which is calculated as the time of cumulative methane volume to achieve 80% of $V_1$.

TS, VS, total nitrogen, total carbon and alkalinity of samples were measured according to the APHA methods [34]. The pH value was determined using a portable pH meter (Model SX-610, Sanxin, Shanghai, China). The lignocellulosic compositions (soluble substance, cellulose, hemicellulose, and lignin) of feedstocks and inoculum were analyzed by a fiber analyzer (Model 2000, ANKOM, USA) as the same in the study Li et al. [3]. The VFAs concentrations including formic acid (HFO), acetic acid (HAc), propionic acid (HPr), lactic acid (HLa), and butyric acid (HBu) of samples were estimated using a high-performance liquid chromatograph (Model LC-20A, Shimadzu, Japan) with a Aminex HPX-87H column (300mm×7.8mm, Bio-Rad, USA) and a refractive index detector (RID). A dilute H$_2$SO$_4$ with concentration of 0.005 M (pH=2.2) was used as the mobile phase at a flow rate of 0.6 mL/min, and temperatures of column oven and injection were maintained at 40 and 90 °C, respectively. Column pressure and injection volume was set 1300 kPa and 20 μL, respectively. Gradient multiple dilutions of the corresponding VFAs were monitored for manufacturing standard curves ($R^2$$>$0.99) according to quantify the compounds and confirm the peak position. Before analysis of VFAs with HPLC, the checking samples were obtained from extracts liquid of digestion substrate and mixed 1:1 with acetonitrile, filtered through a hydrophobic 0.22 μm filter by an injector [35].

2.4. Kinetic model fitting

Methane production can be expressed as a function of microbial growth during AD in batch test. For complex lignocellulosic materials, a modified Gompertz model (shown in Eq. (3)) is considered to be used for fitting the cumulative methane production [3, 35]. The model can improve the accuracy of prediction the potential methane production, the maximum methane production rate, and the lag period of digestion from specific feedstock as follows Eq. (3):

$$M = P \times \exp \left\{ -\exp \left[ \frac{R_m \times e}{P} (\lambda - t) + 1 \right] \right\}$$

where $M$ represents the cumulative methane yield (mL·g VS$_{\text{added}}^{-1}$), $P$ is the ultimate methane yield (mL·g VS$_{\text{added}}^{-1}$), $R_m$ refers to the maximum methane production rate (mL·g VS$_{\text{added}}^{-1} \cdot \text{d}^{-1}$), $\lambda$ stands for the delay period of digestion (d), $e$ is the natural constant (2.718), and $t$ means the digestion time (d).

2.5. Microbiological analysis

Inoculum and samples from the batch tests with different S/I ratios (2:3, 2:1 and 4:1) on day 12 were collected and extracted total genomic DNA, because the three digesters showed differences in digestion process stability and methane production at this time point. A DNeasy PowerSoil Kit (Model 12888-50, Qiangen, Germany) was used to extract the total genomic DNA. The DNA quality was detected using 1% agarose gel electrophoresis, and DNA contents were measured by a NanoDrop spectrophotometer (Model1000, Thermo Fisher Scientific, USA).

2.5.1 16S Illumina sequencing analysis

The community structures of bacteria and archaea of the chosen samples were analysed using sequencing the V3-V4 hypervariable region of 16S rRNA gene. The V3-V4 region was amplified with the universal primer sets of 343F (5’-TACGGRAGGCAGCAG-3’) and 798R (5’-AGGGTATCTAATCCT-3’), and 344F (5’-TGYCAGCCGCCGCGGTAA-3’) and 915R (5’-YCCGGCGTTGAVTCCAATT-3’) targeting for bacteria and archaea, respectively. The two sets of primer included the Illumina barcode sequences for each sample for multiplexing. The amplicon libraries from all samples were sequenced by the Illumina Miseq system using the pair-ended 2×250bp and 2×300bp protocol for bacteria and archaea, respectively [35-36]. Raw data was first purified using Trimmomatic (v.0.35), and then fragment assembly was performed using FLASH (v.1.2.11). Sequences shorter than 200 bp were filtered out. After demultiplexing, the valid reads were assigned species-equivalent operational taxonomic units (OTUs) at 97% sequence similarity using VSEARCH (v.2.4.2). Taxonomic classification of the remaining OTUs and calculation of alpha diversity metric were carried out by using Mothur (v.1.30.1), and then the levels of phylum and genus were denominated according to the Silva database (v.123) (http://www.arb-silva.de).

2.5.2 Quantitative PCR analysis

Quantitative real-time PCR (qPCR) reactions were carried out by an ABI 7500 system (Life Technologies, Singapore). For the qPCR total bacterial content of samples, the primer pairs of 63F (5’-GCAGGCTAAACACATGCAAGT-3’) and 335R (5’-CTGCTGTCCCTCCCAGGTAGGT-3’) were used (Castillo et al., 2006). Component in 20 μL qPCR reaction system (Takara...
Biotechnology, China) and the amplification protocol were performed using the previously reported method by Zheng et al. [8]. Standard curves were set up as described in Hua et al. [37].

For the four species qPCR of archaeal contents, the specific primer sets and 5 ’nuclease probes (TaqMan) were used as designed by Yu et al. [38]: MBT (Methanobacteriales; MBT857F, MBT929F, MBT1196R; amplicon length: 343 bp), MMB (Methanomicrobiales; MMB282F, MMB749F, MMB832R; amplicon length: 506 bp), Msc (Methanosarcinaeae; Msc380F, Msc492F, Msc828R; amplicon length: 408 bp), and Mst (Methanosaetaceae; Mst702F, Mst753F, Mst862R; amplicon length: 164 bp). The TaqMan probes were labelled with fluorescent dyes FAM (reporter) and BHQ-1 (quencher). The mixture of 20 μL qPCR reaction system (Takara Biotechnology, China), the two-phase amplification protocol and standard curves were conducted as presented by Ren et al. [39].

All DNA samples from reactor were estimated with each primer/probe set in triplicate. The DNA volume-based concentration (copies/μL) was converted to the digestion substrate biomass-based concentration (copies/ g VS) according to the following formula Eq. (4):
\[ C = C' \times \frac{V_s}{V_d} \] (4)
where \( C \) means the bacterial/archaeal concentration in the digestion substrate (copies/g VS), \( C' \) represents the bacterial/archaeal concentration in extracted DNA (copies/μL), \( V_s \) stands for the extracted DNA volume (μL), and \( V_d \) is the biomass of digestion substrate used to extract DNA (g VS).

2.6. Statistical analysis
The primary data was standardized by using the Excel 2010 software (Microsoft, USA), the figures of statistical data were plotted and the kinetic model of modified Gompertz equation was fitted by using Sigmaplot version 10.0 (Systat, USA), and the significant differences of each tested parameter was analyzed by using SPSS version 17.0 (IBM, USA).

3. Results and discussion
3.1. Characteristics of substrates and inoculum
The characteristics of substrates and inoculum are shown in Table 1. On a total weight basis, the total solid (TS) and volatile solid contents (VS) of rape straw (RS) were higher than that of dairy manure (DM). Compared with DM, RS has higher carbon content and lower nitrogen content. The C/N ratio of RS and DM was achieved to be 70.7 and 23.8. In consideration of the balanced nutrients of anaerobic microbes, solid-sate co-digestion of RS and DM has the potential to obtain a proper C/N ratio (20-35) and to improve methane yield [8, 13]. RS contained higher structural carbohydrates including cellulose, hemicellulose and lignin than CM. Similar results were also found by Tian et al. [18]. However, the content of soluble matters such as free sugars, oligomers, and organic acids in CM was higher than in RS. In general, the non-structural carbohydrates are easily degradable and can potentially contribute to improve biogas production rate [8]. Another parameter that is worth mentioning is the total alkalinity, which could increase the buffering capacity and maintain stable pH in digesters to prevent VFAs excess accumulation causing the failed AD occurrence [4]. The alkalinites of CM and inoculum were significantly higher (15.3 and 16.2 g CaCO₃ kg⁻¹, respectively) than that of RS (p < 0.01). This result suggested that the optimal CM addition amount and inoculation size could improve methane yield through increasing organic loading capacity of hemi-solid state co-digestion.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rape straw</th>
<th>Dairy manure</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (%) (^{a})</td>
<td>96.2 ± 0.0</td>
<td>12.8 ± 0.1</td>
<td>14.2 ± 0.1</td>
</tr>
<tr>
<td>Volatile solids (%) (^{b})</td>
<td>91.6 ± 0.1</td>
<td>11.4 ± 0.2</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Total carbon (%) (^{c})</td>
<td>52.3 ± 0.3</td>
<td>47.2 ± 0.4</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>Total nitrogen (%) (^{d})</td>
<td>0.7 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>C/N</td>
<td>70.7 ± 2.5</td>
<td>23.8 ± 2.1</td>
<td>12.2 ± 1.4</td>
</tr>
<tr>
<td>Soluble matters (%) (^{e})</td>
<td>15.1±1.1</td>
<td>40.3±2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Cellulose (%) (^{f})</td>
<td>45.7±1.9</td>
<td>22.8±3.1</td>
<td>ND</td>
</tr>
<tr>
<td>Hemicellulose (%) (^{g})</td>
<td>25.9±1.2</td>
<td>24.1±2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Lignin (%) (^{h})</td>
<td>12.7±0.5</td>
<td>6.7±1.0</td>
<td>ND</td>
</tr>
<tr>
<td>pH</td>
<td>ND</td>
<td>8.2±0.1</td>
<td>7.8±0.1</td>
</tr>
<tr>
<td>VFAs content (HAc g·kg(^{-1})) (^{i})</td>
<td>ND</td>
<td>2.8±0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Total alkalinity (CaCO₃ g·kg(^{-1})) (^{j})</td>
<td>7.2±1.8</td>
<td>15.3±1.5</td>
<td>16.2±1.6</td>
</tr>
</tbody>
</table>

Note: The letter \( a \) and \( b \) represents the value based on total weight of sample and TS of sample respectively, ND means not determined.

3.2. Performances of digesters
3.2.1 Daily methane production and methane content
The daily methane productions and methane contents from digesters with RS and DM at different S/I ratios are shown in Fig. 1. As shown in Fig.1A, different trends were obtained for all digesters. The digesters with S/I ratios of 2:3, 1:1, and 2:1 produced methane yield steadily in the entire digestion period of AD, while the digesters with S/I ratios of 3:1 and 4:1 ceased methane production at around day 7, and then recovered after 8 and 11 days, respectively. The similar phenomenon was found in previous study [8], which might be due to digesters with the strong buffering capacity and maintaining a balance of acidification and methanation at low S/I ratio. Yang et al. [26] reported that the failed solid-stated anaerobic digesters could be recovered efficiently by addition of suitable inoculum. Meanwhile, the peak time and the duration of methane production were delayed, and the highest daily methane production was decreased with increasing S/I ratios from 2:3 to 4:1. At S/I ratios from 2:3 to 4:1, the peak time of methane production was 3 to 40 day, and the duration of AD was 32 to 60 day, respectively. It suggested that S/I ratio might be play an important role in the start-up phase and methane production speed of anaerobic digester with solid state feedstocks [28].
The highest daily methane production of digester was 19.7 mL/gVS<sub>added</sub> at S/I ratio of 2:3 and only 5.7 mL/gVS<sub>added</sub> at S/I ratio of 4:1. The value obtained was in the range of yields (0.9-23.7 mL/g VS<sub>added</sub>) in the previous study using different mixtures of corn stalk and chicken manure in HSS-AD (10.1-11.2% TS) at S/I ratio of 1:2 [4]. It was worth mentioning that two successive peaks of methane production were observed in the digesters with S/I ratios 1:1 and 2:1 during AD. The possible reason that the easily digestible components of feedstocks such as soluble matters were converted into methane sooner than the structural carbohydrates including cellulose, hemicellulose and lignin in digesters with certain S/I ratios [8, 28].

![Fig. 1. Daily methane production (A) and methane contents of biogas (B) from digesters with RS and DM at different S/I ratios.](image)

As seen from Fig.1B, the variation trends of methane contents from digesters with different S/I ratios were similar with the daily methane productions (Fig.1A). At S/I ratios of 2:3, 1:1 and 2:1, methane contents of biogas in digesters sharply raised after the first 2 days in the initial phase of AD, and then kept in a stable range, suggesting that these digesters achieved a fast start-up. Whereas, at S/I ratios of 3:1 and 4:1, methane contents of biogas dramatically fallen after the first 3 days from the beginning of AD, and then significantly increased from stopped producing methane for the interval of about 10 days and maintained in a stabilization phase until around day 20 and 26, respectively. The average methane contents were respectively 57.4%, 58.2%, and 54.8% from digesters at S/I ratios of 2:3, 1:1, and 2:1 in stable range, which had no significant difference (<i>p</i> < 0.05) with 54.9% and 54.4% from digesters at S/I ratios of 3:1 and 4:1. It indicated that digester with a high S/I ratio had more substances and low inoculum in the start-up phase. These organic substrates were quickly decomposed to various VFAs and carbon dioxide by the fermentation bacteria with faster growth in the initial period of AD. At the time, the activity of methanogenic consortia with slower growth and its degradation capacity of initial hydrolysis products might be inhibited, probably because of the excessive accumulation of VFAs and the poor buffering capacity in digester under the low inoculum condition. Therefore, the low methane content and the suspended producing methane afterwards in digester might be caused by an imbalance between acidification and methanogenesis. To avoid the problem, it is necessary to increase the inoculum dose in adaptation phase in order to improve buffering capacity and shorten start-up time in batch SS-AD [28, 40].

### 3.2.2 Methane production rate

The special methane production rate (SMPR) and volumetric methane production rate (VMPR) of digesters at different S/I ratios can be found in Fig. 2. As shown in Fig. 2A, the highest SMPR of 209.1 mL/g VS<sub>added</sub> was obtained at S/I ratio of 2:3, which was significantly higher (<i>p</i> < 0.05) than that of 195.8, 177.5, 121.7, and 103.6 mL/g VS<sub>added</sub> at S/I ratio of 1:1, 2:1, 3:1, and 4:1, respectively. Previous result showed that the methane yields for rape straws ranged from 188 to 243 mL/g VS<sub>added</sub> in BMP tests at S/I ratios of 1 [18], which were close to the present study. It is noted that MPR decreased with increasing S/I ratios from 2:3 to 4:1 in this study. This phenomenon was also seen from the batch solid state anaerobic co-digestion of food yard waste and food waste where the methane yield declined with an increasing substrate to inoculum ratio [41]. The higher methane yields in batch SS-AD at lower S/I ratios were obtained because of the additional moisture content and the more methanogens provided by increasing inoculum, which contributes to the mass transfer and the efficient conversion from VFAs to methane [6, 22]. As can be seen from Fig. 2B, the comparisons of VMPR are quite other than those of SMPR among digesters with different S/I ratios. The highest VMPR of 0.42 mL/(mL·d) was obtained in the digester at S/I ratio of 2:1, which was 1.3-, 1.2-, 2.2-, and 2.6- fold (<i>p</i> < 0.05) higher compared with the value of digesters with S/I ratio of 2:3, 1:1, 3:1, and 4:1, respectively. There was no significant difference between S/I ratios of 2:3 and 1:1. Similar to SMPR, VMPRs were also much lower for the digesters at S/I ratios of 3:1 and 4:1. It can be seen that the S/I ratio of the highest SMPR may not always that of the highest VMPR, because the overlarge inoculum size could take up more volume and decrease organic loading of digester. Therefore, it could be an effective way to improve methane production of SS-AD from lignocellulosic substrates by optimizing the S/I ratio of operational parameters.
Although an extensive range of S/I ratios has been studied in SS-AD of lignocellulosic feedstocks in previous studies, the results are greatly differ from each other. The highest methane yield of corn stover was obtained with S/I ratio of 2.4 [27], while the highest production of yard trimmings was found to be 1 (based on VS) in mesophilic SS-AD [41]. Under the addition of buffers condition, the higher S/I ratios of 28-47 were shown to be effective for SS-AD of wheat straw with varying of TS and particle size of feedstock, and the AD duration (around 300 days) was about 5-10 times longer than that of digesters with low S/I ratios [28]. The differences might be due to the heterogeneity of feedstocks, but also the activity of inoculum.

3.3. Evaluation of AD process stability

Many studies of lignocellulosic feedstocks in batch SS-AD have conducted improving methane production and assessment of the AD process stability, but only for the initial and final phase [3, 7, 41]. To our knowledge, few studies have focused on the SS-AD stability including the intermediate stage. Based on the process stability of methane production in the entire AD, analysis of the dynamic performances of digesters is recognized to be an effective approach for understanding metabolic mechanisms of microbes, predicting the performances of reactor, and optimizing the parameters of operation.

### 3.3.1 Dynamic behaviors of digesters

The temporal analysis of this entire period during AD provides a novel and non-invasive information on the system dynamic behaviors [28]. Fig. 3 shows the temporal dynamic changes of methane production obtained upon digesters at different S/I ratios. As seen in Fig.3A and Table 2, the producing methane process of batch digesters could well be explained according to the modified first-order Gompertz model ($R^2$ above 0.98). The values of predicted methane yields computing by the dynamic model were close to the experimental values observed. The greatest methane production rate ($R_m$) decreased from 15.04 mL/d/g VS<sub>added</sub> (2:3 of S/I ratio) to 4.70 mL/d/g VS<sub>added</sub> (4:1 of S/I ratio), which was in agreement with the experimental result (Fig. 1A). The period of methane production was allowed to proceed from 32 days at S/I ratio of 2:3 to 60 days at S/I ratio of 4:1 and the adaptation phase was found clearly in digesters at S/I ratio of 3:1 and 4:1(Fig. 3A).The digesters at S/I ratio of 2:3, 1:1 and 2:1 produced steadily and the lag phase ($\lambda$) ranged from 0.39d to 0.79d. In contrast, digesters at S/I ratios of 3:1 and 4:1 stopped producing methane at the beginning of fermentation and the $\lambda$ value was delayed for 19.81-23.62 day. The longer adaptation period was also observed during SS-AD inoculated by high S/I ratios in the previous literature [42]. As shown in Fig.3B and
Table 2, the shortest technical digestion time ($T_{80}$) was prolonged gradually with increasing S/I ratio, and 80% of cumulative methane production to total methane production of digesters at different S/I ratios occurred in 16, 17, 19, 37, and 42 day, respectively. The result suggested that the adaptive capacity of microorganisms to the complex environment of AD could be expedited by enlarging inoculum size at the start-up stage.

### 3.3.2 Variations in pH, VFAs, and daily biogas production of digesters

Digestion process failure and low methane yield can be caused due to imbalances of hydrolytic, fermentative, and acetogenic bacteria, and methanogenic archaea [35]. Usually, these imbalances are to be because the unfavourable conditions, such as the insufficient buffering capacity of AD system and the accumulation of VFAs [4]. Subsequently, the dramatic drop and fluctuation of pH will happen, which inhibits the activities of microbes with different metabolic functions, especially for methanogenic archaea being more sensitive to pH value, and finally disrupts the stability of AD process and reduces methane production [8]. Therefore, pH and VFAs are important factors used to estimate the performance of AD.

![Changes of pH value, volatile fatty acids (VFAs), and daily biogas production of digesters with RS and DM at different S/I ratios.](image)

The changes of pH, VFAs, and daily biogas production during AD are shown in Fig. 4. A first peaks of daily biogas production occurred in all digesters on the first day and daily biogas production decreased with increasing of S/I ratios. It suggested that the microbes of inoculum used in this study through acclimation of adaptability had high activity, and inoculum dose could be a limiting factor in the initial biogas production from transformation of the soluble matters. Digesters at S/I ratios of 1:2, 1:1, and 2:1 produced biogas steadily, the pH value of three digesters remained above 6.9 throughout the whole AD period, and the total content of VFAs was below 20.0 g/kg. Whereas, digesters at S/I ratios of 3:1 and 4:1 failed in producing biogas when the pH value decreased to less than 6.2, and the VFAs content was above 20.0 g/kg. The consecutive peak of daily biogas production was not observed until the pH value recovered to above 6.4 and the VFAs content reduced to below 20.0 g/kg. The ideal pH value range of 6.5-8.2 for an efficient AD was reported by Lee et al. [43], and Zheng et al. [8] found that the stable performance of digesters at S/I ratios. Acetogenesis and methanogenesis were intense in digesters with S/I ratios of 2:3, 1:1, and 2:1 during the first 20 days limiting factor in the initial biogas production from transformation of the soluble matters. Digesters at S/I ratios of 1:2, 1:1, and 2:1 produced biogas steadily, the pH value of three digesters remained above 6.9 throughout the whole AD period, and the total content of VFAs was below 20.0 g/kg. Whereas, digesters at S/I ratios of 3:1 and 4:1 failed in producing biogas when the pH value decreased to less than 6.2, and the VFAs content was above 20.0 g/kg. The consecutive peak of daily biogas production was not observed until the pH value recovered to above 6.4 and the VFAs content reduced to below 20.0 g/kg. The ideal pH value range of 6.5-8.2 for an efficient AD was reported by Lee et al. [43], and Zheng et al. [8] found that the stable performance of digesters at S/I ratios. Acetogenesis and methanogenesis were intense in digesters with S/I ratios of 2:3, 1:1, and 2:1 during the first 20 days.
period. However, this phenomenon was not observed in digester with S/I ratios of 3:1 and 4:1, because their VFAs concentrations were accumulated to 22.4-25.3 g/kg and 24.6-26.2 g/kg on day 3-12, particularly HPr concentration reached up 4.6-5.8 g/kg and 5.1-6.4 g/kg, respectively, which is more inhibitory to the methanogens than HAc [1]. Boon and Xun [46] indicated that HPr concentration over 3g/L have been shown to cause digester failure and Chen et al. [47] found that HPr concentration of 1g/L inhibited methane production, which was lower than in present study. The differences might be due to the buffer capacity of AD at different operational parameters, such as feedstocks, TS and inoculum. Therefore, monitoring of HPr might be an important indicator to demonstrate the process stability of digesters at high S/I ratios as an increase in VFAs could be indicative of an overload of the organic loading rate.

3.4 Analysis of the microbial community
The above results showed that digesters with different S/I ratios changed the stability of AD process. High S/I ratios resulted in over-accumulation of VFAs, striking drop of pH and delayed the methane production period. So far, studies on microbial community dynamics in SS-AD are relatively few compared to the numerous ones in W-AD [7]. Moreover, the inconsistent conclusions about the SS-AD microbial communities were found in the existing studies. Microbes in SS-AD are very sensitive to the many digestion factors including substrate types, size and source of inoculum, and operating conditions, and thereby the microbial community structure can be affected by these factors. In order to elucidate the plausible correlations between the communities of bacteria and archaea with the performance of digesters at different S/I ratios, high throughput analyses and qPCR were carried out. The samples of microbiological analysis were taken from digesters with S/I ratios of 2:3, 2:1, and 4:1 on day 12 for several reasons. Firstly, the highest SMPR and VMPR was achieved at S/I ratio 2:3 and 2:1, respectively, and then the inversely values were found at S/I ratio of 4:1. Secondly, the status of digestion and daily biogas production differed in digesters at the three S/I ratios on day 12. Specifically, on day 12, 2:3 digesters were in a period of steady decline, 2:1 digesters were on the third peak of daily biogas production, and 4:1 digesters were in the period of stopping biogas production and excess VFAs accumulation.

3.4.1 Bacterial community
A total of 238,470 sequences (29,809 sequences per each samples on average) were obtained for the four samples analyzed after the quality check, and 110,487 sequences were identified as bacterial. The alpha diversity of microbial communities (bacteria and archaea) in samples from digesters with three representatives S/I ratios on day 12 and inoculum samples is presented in Table 3. The index of coverages was superior to 99% for all samples, hence confirming the representativeness of the OTU set. A total of 540 to 700 OTUs of bacteria were found. The greatest number of OTUs was obtained in the inoculum samples, following by the samples from digesters at S/I ratios of 2:3, 2:1 and 4:1. The similar trends were found in Simpson and Shannon diversity indices. The results indicated that a high quality activated seed sludge to be used in this study through acclimating, and the strong acid and low pH environment might be go against the growth of hydrolytic and acidification bacteria during HSS-AD process.

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Digesters</th>
<th>Reads</th>
<th>Observed OTUs</th>
<th>Coverage (%)</th>
<th>Simpson</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Inoculum</td>
<td>25,461</td>
<td>700</td>
<td>99.36</td>
<td>0.97</td>
<td>6.68</td>
</tr>
<tr>
<td></td>
<td>S/I=2:3</td>
<td>27,853</td>
<td>668</td>
<td>99.14</td>
<td>0.96</td>
<td>6.19</td>
</tr>
<tr>
<td></td>
<td>S/I=2:1</td>
<td>30,381</td>
<td>570</td>
<td>99.28</td>
<td>0.95</td>
<td>6.10</td>
</tr>
<tr>
<td></td>
<td>S/I=4:1</td>
<td>26,792</td>
<td>540</td>
<td>99.30</td>
<td>0.93</td>
<td>5.85</td>
</tr>
<tr>
<td>Archaea</td>
<td>Inoculum</td>
<td>32,117</td>
<td>44</td>
<td>99.98</td>
<td>0.69</td>
<td>2.61</td>
</tr>
<tr>
<td></td>
<td>S/I=2:3</td>
<td>31,016</td>
<td>47</td>
<td>99.99</td>
<td>0.71</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td>S/I=2:1</td>
<td>32,759</td>
<td>48</td>
<td>99.98</td>
<td>0.71</td>
<td>2.57</td>
</tr>
<tr>
<td></td>
<td>S/I=4:1</td>
<td>32,091</td>
<td>51</td>
<td>99.98</td>
<td>0.73</td>
<td>2.60</td>
</tr>
</tbody>
</table>

Based on the classification results of total OTUs at 97% sequence similarity in Silva database, the relative abundance of thirteen major bacterial phyla with inoculum and different S/I ratios is shown in Fig.5A. About 98% of the total sequences could be classified into a known bacterial phylum in inoculum, with Firmicutes (accounting for 38.3% of the sequences) represented the most prevalent phylum, followed by Bacteroidetes (27.5%), Proteobacteria (11.6%), Spirochaetae (9.1%), Tenericutes (2.8%), Synergistetes (1.8%), Chloroflexi (1.4%) and Planctomycetes (1.3%). Other phyla were each represented by <1% of total sequences. These major bacterial phyla have also been found in other related studies [8, 35, 48], so they are likely universal in anaerobic digesters fed with lignocellulosic feedstocks. In consideration of the inoculum being collected from a well-run digester with high methane production from rape straw and dairy manure, these bacteria may play an important role in degrading lignocellulosic substrates during AD. After 12 days of digestion, the proportions of major phylum in three digesters with increasing S/I ratio showed a marked variation as follows: Firmicutes decreased gradually (55.8%, 45.0% and 32.9%); Bacteroidetes increased significantly (25.2%, 32.8% and 43.9%); Spirochaetae first decreased and then increased (3.0%, 2.2% and 3.3%); Fibrobacteres first increased and then decreased (1.2%, 2.3%, and 1.1%); Synergistetes, Tenericutes, Cyanobacteria, and Actinobacteria increased slightly. It indicated that Firmicutes and Bacteroidetes were the two most predominant phylum in all the digesters, which was consistent with the study on SS-AD digesters fed corn stover at different ratios of feedstock to effluent of liquid anaerobic digestion effluent [48]. Some studies have reported that Firmicutes and Bacteroidetes containing many known bacteria are capable of hydrolyzing and fermenting fiber into organic acids, and some species are positively correlated to VFAs concentration [8, 48]. Therefore, more abundances of Firmicutes and Bacteroidetes in digesters fed on rape straw and dairy manure at optimal S/I ratios resulted in greater degradation of cellulose and hemicellulose components and higher production of...
VFAs during hydrolysis and acidification, which provided more carbon nutrients for archaea and improved correspondingly methane production. Interestingly, the largest phylum shifted gradually from *Firmicutes* in digesters with S/I ratios of 2:3 and 2:1 to *Bacteroidetes* in digesters with S/I ratio of 4:1 in the initial phase of HSS-AD. It could be explained that some *Firmicutes* species was might depressed at higher VFAs concentration and lower pH in digester with S/I ratio of 4:1, and some *Bacteroidetes* species had stronger resistance to the acidic environment.

In total, 127,983 quality-checked archaeal sequences were obtained, and 44 to 51 OTUs were detected in the samples from inoculum and digesters at S/I ratios of 2:3, 2:1, and 4:1 on day 12. The diversity indices of Simpson and Shannon were from 0.69 to 0.73 and from 2.57 to 2.66, respectively (Table 3). The similar diversity estimates among these samples showed that archaea community successions but not attractive changes in species richness or evenness. Furthermore, the two diversity indices were lower than those of bacteria in the three digesters, indicating a more diversified species of bacteria degraded the complex components and carbohydrate structures of lignocellulosic biomass compared to archaea. However, the minor archaea are believed to be responsible for the methanogenesis reaction [18]. As shown in Fig. 6A, all these OTUs were classified to 11 known species (at 97% sequence similarity). Among them, *Methanoseta* genus belongs to *Methanosetaeae* (Mst) family; two genera of *Methanosarcina* and *Methanimicrococcus* belong to *Methanosarcinales* (Msc) family; three genera of *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera* belong to *Methanobacteriales* (MBT) order, *Methanobacteriaceae* family; five genera belong to *Methanomicrobiales* (MMB) order, including *Methanoculleus*, *Methanocorpusculum*, *Methanobollis*, *Methanogenium*, and *Methanospirillum*. The predominant archaeal genera in the inoculums and HSS-AD digesters were *Methanoseta*, *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus* and *Methanosphaera*. As shown in Fig. 6A, although the main types of archaea obtained were similar in these samples, the relative abundances differed clearly between the healthy digesters (2:3 and 2:1 of S/I ratio) and the sour digesters (4:1 of S/I ratio). After 12 days of digestion, the digesters with S/I ratios of 2:3 and 2:1, which produced biogas steadily, had a similar microbes profile with that of the inoculums. It suggested that the acclimated seed sludge to be used in our study had high methane producing activity, which was important for efficient SS-AD system start-up and stable methane production [7, 48]. The most predominant archaeal genus was *Methanoseta* in the samples from digesters at S/I ratio of 2:3 (55.6%) and 2:1 (53.2%), which was higher \((p<0.05)\) than that of 4:1 (39.8%). Although the relative abundance of *Methanosarcina* and *Methanoculleus* was lower than *Methanoseta* in the three digesters, the variation trend of both methanogens was similar with *Methanoseta*. Li et al. [48] found that *Methanosarcina* dominated the methanogen community in solid-state anaerobic digesters fed with corn stalk, which differed with the present results. The difference might be due to the different operational factors such as TS, feedstocks, and seeding, because it has been confirmed that these factors have affected the microbiome community during AD [49]. It is well known that *Methanoseta* and *Methanosarcina* are acetoclastic methanogens, which play an important role in acetoclastic methanogenesis. Zhao et al. [35] indicated that *Methanoseta* could improve methane production of digesters under conditions with high acetic acid content. In our study, the digesters with S/I of 2:3 and 2:1 on day 12 of fermentation contain high concentrations of acetic, lactic, and propionic acid (Fig. 4). Intriguingly, in the digesters at S/I ratio of 4:1, which had excess accumulation VFAs (25.4 g/L) and low pH (5.9) conditions, the relative abundance...
of hydrogenotrophic Methanobacterium in the sour digesters (22.5%) increased sharply compared to that of 2:3 (7.9%) and 2:1 S/I ratio (10.2%). The other two hydrogenotrophic methanogens including Methanobrevibacter and Methanosphaera had the similar change tendency with Methanobacterium. Our results are consistent with Blume et al. [50], who reported that Methanobacteriales had higher abundance than the families of Methanosarcinaceae, Methanomicrobiaceae and Methanosetaeaceae in environments with high total acid concentrations and low pH. Similar results were found that Methanoseta and Methanosarcina were strongly inhibited as a significant increase in members of the Methanobacteriales at acetate concentration up to 8 g/L [51]. Furthermore, several reporters found that Methanobacteriales were even able to grow at a pH below 5.0 [52-53]. In present study, the hydrogenotrophic Methanobacteriales order including the genera of Methanobacterium, Methanobrevibacter, and Methanosphaera was the most abundant methanogen, and despite resistance of this archaea to acidic and low pH environment, methanogenesis was inhibited. This inhabitation resulted from more accumulation of VFAs and acetate acid at concentration above 9.0 g/L. Therefore, methanogens were highly influenced by the S/I ratio through VFAs accumulation and pH lowering during HSS-AD.

![Fig. 6](image_url) Relative abundance in archaeal genus (A) and population (B) of inoculum and samples collected on day 12 of digestion substrates at different S/I ratios.

To better understand archaeal population changes in digesters with different S/I ratios during HSS-AD processes, four species of archaea including Mst, Msc, MBT, and MMB, which are generally considered important to biogas production, were quantified using qPCR (Fig. 6B). In general, the abundances determined by qPCR was perfect agreement with the predominance represented by the Illumina sequencing data for all the genera quantified. The highest amount of each methanogen was obtained at S/I ratio of 2:3, and decreased gradually as the S/I ratio increased. Mst was the most abundant family in all the samples, ranging from 1.5×10⁹ copies/g VS at S/I ratio of 2:3 to only 8.8 ×10⁶ copies/g VS at S/I ratio of 4:1. However, the decrease degree of population among these methanogen had significantly differences: the amount of Mst, Msc, MBT, and MMB was about 170, 180, 70, and 1400 times lower in the 4:1 digesters than in the 2:3 digesters. It suggested that MBT had the strongest ability of acid tolerance and conversely for MMB. Moreover, in coincidence with the sequencing data, acetoclastic Mst and hydrogenotrophic MBT were found most predominant in the rancid samples from the 4:1 digesters. Leclerc et al. [54] showed that the most frequent archaeal sequences were affiliated to Methanoseta and Methanobacterium in 84% and 73% of the digesters, respectively, which located in eight different countries and fed with seven different characteristics of waste. In the present study, considering of stopped producing methane in the 4:1 digesters on 12 days of fermentation, the role and importance of the two different methanogenesis pathways of methanogens was probably related with the resistance to acids and recovering methane production afterwards.

Hemi-solid state anaerobic digestion of lignocellulosic feedstocks is a complex process carried out by the synergistic microbes with different functions to produce methane. A balance among VFAs and acetotrophic and hydrogenotrophic methanogens is crucial for improvement methane production according to the optimum S/I ratios. Generally, the S/I ratio is expressed in the terms of VS ratio in most previous studies. However, it is not an accurate criterion because both substrate characters and microbial structure have synergistic impacts on the productions of VFAs and methane in the initial HSS-AD. Therefore, a better indicator should be considered in further study, such as the contents of easily digestible matters from feedstocks and the activities of bacteria and methanogens from inoculum (e.g., the ratio of bacteria to methanogens). Moreover, in order to improve the digestion performance of rape straw, further research is needed to optimize the physical, chemical, and biological growth conditions of bacteria and archaea, and identify the individual function of microbes during HSS-AD system by using metagenomics.

4. Conclusion

The ratio of substrate to inoculum (S/I, based on VS) affected the process stability and methane production from co-digestion of RS and DM in HSS-AD system. Different S/I ratios resulted in differences in methane production and microbial communities. Increasing S/I ratio could shorten the start-up period of HSS-AD. The highest methane yield of 209.1 mL/g VS_{added} and the highest volumetric methane production of 0.4 L/(L·d) was achieved at S/I ratio of 2:3 and 2:1, respectively. In digesters with S/I ratio of 4:1 on day 12, the amounts of bacteria and archaea were significantly inhibited as a consequence of excess VFAs accumulation and low pH. At the same time point, the main dominant bacteria had gradually shifted from Firmicutes of digester at S/I ratio of...
2:3 to Bacteroidetes of digester at S/I ratio of 4:1. The predominant archaea was affiliated to acetoclastic Methanoseta in all digesters, but the relative abundances of hydrogenotrophic Methanobacterium increased as the increasing of S/I ratio.

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