Effects of Organic Loading Rate on Methane Production from Switchgrass in Batch and Semi-Continuous Stirred Tank Reactor System

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Abstract

Long-term anaerobic digestion of switchgrass (SG) was investigated in batch and semi-continuous reactors operating at different Substrate to Inoculum (S:I) ratios and organic loading rates (OLRs) by monitoring process stability, microbial community structure, and investigating kinetic modeling. The highest methane yield of 204 $mLCH_4$/gVS was observed when the batch system was operated at the S:I ratio of 1.1. Methane yields in the semi-continuous configuration were around 148, 157, and 60 $mLCH_4$/gVS at OLRs of 0.75, 1.0, and 1.5 gVS/(L.d), respectively. A drastic decrease in methane yields was observed at the highest OLR value of 1.5 gVS/(L.d). The optimum OLR in the semi-continuous configuration was decided as 1.0 gVS/(L.d) where 35% of the SG theoretical methane yield and 38% energy recovery were attained. Several methanogenic archaea, *Methanosarcina*, *Methanosaeta*, *Methanothrix,* and *Methanomethylovorans,* together with bacteria species, *Chloroflexi*, *Firmicutes, Spirochaetes*, and *Actinobacteria* were observed in the semi-continuous configuration after 270 days of operation. The dominant bacterial phyla of s-CSTR were mostly members of *Spirochaetes* phylum, which plays a considerable role in acetate oxidation under either mesophilic or thermophilic conditions.

Keywords: Anaerobic Digestion, Lignocellulose, Methane, Microbiome, Modeling, Switchgrass

1. Introduction

Easy access to energy is among the most important needs that directly affects the living standard, and it is a requirement for sustainable economic growth. In recent decades, many studies were carried out worldwide to develop sustainable energy production processes from renewable sources as a replacement for fossil fuels. The primary motivation for these studies was to reduce the dependence on fossil fuels while preventing environmental problems caused by the use of fuels as much as possible [1,2,3]. Among the alternative energy sources, biomass energy stands out with its remarkable potential as a biofuel feedstock. Notably, most recent studies focused on utilizing lignocellulosic biomass sources due to their wide availability and suitable biochemical composition for biofuel production.

One of the prairie grass species native to North America, switchgrass, attracted much attention from scientists due to its high potential as a biofuel feedstock and its agricultural advantages. Switchgrass (SG) (*Panicum virgatum L.*) is a promising lignocellulosic substrate owing to its agricultural yield (up to 14 tons/acre), easy adaptation to wide ranges of unfavorable conditions, long lifespan, positive environmental benefits like erosion preventive, renewable and well-developed deep root system, tolerance to drought conditions, soil protection against degradation, low nutritional requirements, high durability, and high carbon content [4,5]. Since SG was selected as a model perennial energy crop by the U.S. Department of Energy in 1991 [6], Canada, some European countries, and China started research programs on SG in 1990 - 2000 [7]. Turkey initiated a research program on energy crops, including SG, in 2015. Seeds of different varieties of SG, which are not native to Turkey, were brought

from America. Adaptation studies were carried out in different regions with varying climate and soil conditions across Turkey by the Ministry of Agriculture and Forestry.

Studies have been broadly focused on the conversion of SG into various biofuels, especially bioethanol, along with the application of a pre-treatment process to reach the sugar platform. However, the production of ethanol requires the combination of costly up- and downstream processing in series. Combining many processes in series and optimizing the selected pre-treatment processes to minimize sugar loss results in an uneconomical ethanol production route from SG [8,9]. Integrating methane production into this route was found to increase the total energy recovery [10]. About the anaerobic digestion of SG, a few studies have focused on methane yield for different harvesting seasons [11–14], improving the methane yield via pre-treatment and ensiling [15–18] or co-digestion [7,19,20], and batch anaerobic digestion kinetic modeling [21–24]. Very limited attention has been paid to SG's digestion in a continuous anaerobic bioreactor with process stability [25–28] in the literature even though it has many advantages like retention time and reaction volume than the batch system.

In the AD of lignocellulosic biomass, although lignin's existence slows down the process, eventually, microorganisms can reach cellulose and hemicellulose fractions and hydrolyze them by using external enzymes [29]. Along with the many advantages, the AD process has some common complications such as operational instabilities, pH drops caused by volatile fatty acid accumulations, C/N ratios deficiency, and homogenous mixing difficulties due to solids accumulation in the digester. Some of the process complications can be avoided by the monitoring of pH, volatile fatty acids (VFA), and alkalinity continuously [24, 25]. Furthermore, reactor types, feeding regime, macro and micronutrients, and OLR are directly influential on the biomass to methane

95 conversion efficiency [30,31,32]. Particularly, there is a significant research gap in
96 determining the effect of various reactor configurations and OLRs on long term AD of
97 raw SG. Hence, this study aims at filling this gap by comparing the effect of different
98 reactor configurations and OLRs on methane production from SG.

99 In the study, the AD of raw SG was comparatively evaluated in two different
100 reactor configurations, i.e. batch (fed once) reactors and daily fed and continuously
101 stirred semi-continuous stirred tank reactor (s-CSTR) fed under three different OLRs,
102 0.75, 1.0, and 1.5 gVS/(L.d). During the AD process, pH and alkalinity to volatile fatty
103 acid ratio (Ripley ratio) were monitored to assess the process's stability. Obtained
104 methane production results of batch system were modeled using Cone, Reaction Curve,
105 First Order Kinetic, and Modified Gompertz models. Furthermore, denaturing gradient
106 gel electrophoresis (DGGE) analysis was performed to identify the microbial diversity
107 and dynamics in the s-CSTR reactor. Finally, methane productions and the energy
108 equivalents were compared with the theoretical methane yield and direct combustion
109 energy of SG, respectively, to determine the process efficiency.

110 **2. Materials and Methods**

111 **2.1. Substrate and Seed Sludge**

112 Kanlow variety of SG was cultivated in Konya, Turkey. The samples which
113 were harvested at 2nd year, received as field dried under the sun. Switchgrass variety
114 was chosen considering high biomass yield [33] and methane potential [10]. The dried
115 samples were milled to a size of 0.5 mm before using in experiments. The particle size
116 of 0.5 mm was selected to increase the hydrolysis rate while not causing any mixing
117 problems. The grinding process was carried out with a hammer mill, and milled samples
118 were stored in the plastic bags at room temperature.

119 The anaerobic seed sludge was obtained from a local biogas plant (Rani Farm)
120 near Manavgat, Turkey. The plant produces biogas from cattle manure in the AD
121 process. The main reason for obtaining anaerobic seed sludge from a cattle manure
122 processing plant was the cattle manure's microbial structure, which contains species that
123 can enzymatically hydrolyze raw lignocellulosic biomass along with acidogenic and
124 methanogenic microorganisms. Furthermore, since microorganisms in the cattle manure
125 seed were already adapted to hydrolyzing cellulose, it was assumed that the system
126 could reach the steady-state conditions faster. The sludge which contains 53.26% total
127 solids (TS) and 37.03% volatiles solids (VS) was used as the inoculum.

128 **2.2. Characterization of Switchgrass**

129 The characterization analyzes performed on raw SG were total solids (TS),
130 volatile solids (VS), soluble reducing sugar (DNS), soluble protein, acid-soluble and
131 insoluble lignin, van Soest detergent fiber, total organic carbon (TOC), lower heating
132 value (LHV), and elemental analysis.

133 TS and VS analyzes were carried out by following the Standard Method 2450-C
134 [34]. 3,5-dinitrosalicylic acid (DNS) method [35] and Lowry method [36] were used to
135 measure soluble reducing sugar and soluble protein contents of SG, respectively. Acid
136 soluble-insoluble lignin analyses were performed by following the National Renewable
137 Energy Laboratory's (NREL) procedure TP-510-42618 [37]. Structural carbohydrates
138 were measured digesting SG in an acid solution and then analyzing the pH neutralized
139 sample with an ultra-high performance liquid chromatography (Thermo Scientific,
140 UltiMate® 3000 UHPLC) [37]. Van Soest Detergent Fiber analysis [38] was conducted
141 to specify the cellulose, hemicellulose, and acid detergent lignin contents of SG. TOC

142 of SG was determined with Shimadzu TOC-L equipped with a solid sample module
143 (SSM-5000A).

144 In addition, elemental composition (C, H, O, N) analysis was performed by
145 CHNS elemental analyzer (LECO, CHNS-932). Trace element composition (Ca, K, Na
146 Mg, Al, Fe, Cu, Mn, Sr, and Se) was determined by ICP-OES – PerkinElmer 7000DV
147 and ICP-MS PerkinElmer Elan DRC-e. Moreover, the sample's lower heating value
148 (LHV) was analyzed by following the ASTM D5865-10A procedure. The higher
149 heating value (HHV) was also calculated by Dulong Equation [39].

150 Buswell Equation, which is a stoichiometric equation, was utilized to calculate
151 the theoretical methane potential by using carbon (C), hydrogen (H), oxygen (O) and
152 nitrogen (N) contents of SG measured in the elemental analysis [40]. This formula was
153 created by Symons and Buswell (1933) by using theoretical and practical studies on
154 anaerobic digestion of carbohydrates, and the formula assumes that the organic material
155 (e.g., $C_aH_cO_cN_d$) is completely converted into CH_4 and CO_2. It was aimed to compare
156 the methane yield obtained from batch and s-CSTR processes and the theoretical
157 methane potential to determine the process efficiency. Although it is advantageous to
158 use Buswell Equation to determine the theoretical methane potential due to quick to
159 measure nature of the elemental composition, the values calculated by the equation are
160 always higher than the practical methane potential of the substrate.

161 **2.3. Batch Anaerobic Digestion**

162 In batch reactors, S:I values were selected to correspond to the organic loading
163 rates specified in the continuous reactor. In order to investigate the methane potential of
164 SG at different substrate to inoculum ratios (S:I as gVS/gVS) of 1.1, 1.47, and 2.2,
165 batch tests were performed according to the biochemical methane potential (BMP) test

procedure provided by Perendeci et al., (2018) [41]. Batch tests were performed in reactors with 400 mL working volume and 100 mL headspace. The seed concentration of the reactors was adjusted to 3 g/L. In accordance with the method, sodium bicarbonate buffer (2.6 g/L) and micro-macro nutrients were added to reactors to keep the pH stable and to provide nutrients to the seed sludge, respectively. Table 1 presents the amounts of macro and micro-nutrients added. After all the components were added, the liquid volume of the reactors was adjusted to 400 mL with deionized water. A gas mixture containing 70% N_2 and 30% CO_2 was used to purge the reactors to eliminate the oxygen in the headspace. The reactors were sealed with septum caps and kept in a temperature-controlled incubator at 36±1°C. The batch reactors were incubated for 83 days until the methane production ceased. Methane productions on the 60^{th} day were taken as the ultimate results. Control reactors without substrate were also run to determine the biogas production from the seed. The average amount of methane production from the control reactors was subtracted from those of other reactors to determine the normalized biogas production directly from the used substrate. A total of 16 times biogas measurements were conducted simultaneously from all the reactors during the 60 days incubation. The residence time for anaerobic digestion of lignocellulosic materials is generally taken between 30-60 days depends on the material degradation [42]. Digestion of lignocellulose takes more time than hemicellulose structure. Some publications has reported the SRT of switchgrass digestion as follows: Frigon et al. 37 days [43], Niu et al. 45 days [44] and Zheng et al. 30 days [7]. In our experiments, we preferred 60 days for batch digestion as well as semi-CSTR digestion.

189 **Table 1.** Concentrations of micro and macro nutrients added to each batch reactor

Macro Nutrients	Conc. (mg/L)	Micro Nutrients	Conc. (mg/L)	Micro Nutrients	Conc. (mg/L)
NH_4Cl	172	$FeCl_2.4H_2O$	20	H_3BO_3	0.5
KH_2PO_4	65	$CoCl_2.6H_2O$	5	Na_2SeO_3	0.5
$MgCl_2.6H_2O$	39	$MnCl_2.4H_2O$	1	$CuCl_2.2H_2O$	0.4
$CaCl_2.2H_2O$	19	$ZnCl_2$	0.5	$Na_2MoO_4.2H_2O$	0.1

190

191 A liquid-gas displacement setup was used to measure the total volume of biogas
192 produced in the reactors. In the displacement setup, to avoid dissolution of biogas in the
193 liquid, an acidic salt solution (pH 1) was used as the displacement liquid that was
194 prepared according to Standard Method 2720 [34]. The composition of biogas (CH_4 and
195 CO_2) was determined by using Varian CP-4900 Micro-gas chromatography (micro-GC)
196 equipped with a thermal conductivity detector (TCD) and PPQ column (10 m). Column
197 and injector temperatures were set at 70°C and 110°C, respectively. The flow rate of
198 carrier gas, Helium, was adjusted to 25 mL/min. Specific methane yield was expressed
199 as mL CH_4/g VS.

200 **2.4. Semi-Continuous Stirred Tank Reactor (s-CSTR) Digestion**

201 **2.4.1. Operational conditions**

202 A 5 L stainless steel reactor (Bioprocess Control 5S) with a heating jacket and
203 mechanical stirrer (100 rpm) was used as the s-CSTR in the experiments. The heating
204 jacket was connected to a water bath (JSR JSIB-22T) to maintain the system at a
205 mesophilic temperature (36±1°C). pH and temperature were monitored by using the
206 same probe (Hamilton Polilyte Plus Arc120) and a control screen (Ikarus Tech.). Total
207 biogas production was measured by a gas flow meter (Bioprocess Control μFlow), and

biogas was collected in a gas collection bag (Supelco Tedlar Bag 5L) to determine the gas composition. System configuration of s-CSTR is presented in Fig. 1. Initially, the digester was filled with seed sludge (5.4% TS) and fed every-other-day with SG depending on the OLR (0.75, 1.0, and 1.5 gVS/(L.d)). The same amount of effluent was withdrawn to provide semi-continuous reactor conditions. Sludge retention time (SRT) of 60 days was used for each OLR value. The first 30 days were assumed as the adaptation period to reach a steady-state condition of examined OLR. Reactors were kept operational for 90 days for each OLR. After the initial 30 days, average biogas production yields measurements were started and continued until the 90th day.

1	Completely stirrer tank reactor	5	Reactor inlet for SG feeding
2	pH and temperature sensor	6	Gas flowmeter
3	pH and temperature monitoring	7	Gas sampling bag
4	Reactor mixer	8	Water bath

Fig. 1. System configuration of s-CSTR

2.4.2. Analytical methods for semi-Continuous Stirred Tank Reactor

222 Temperature and pH were monitored daily. TS and VS of the reactor contents
223 were measured continuously by using the method presented under Section 2.2. The total
224 volume of the produced biogas and its composition were measured daily. The daily
225 volume of the biogas production (mL) was monitored by a gas flow meter, which was
226 connected to a gas collection bag. The composition of biogas (CH_4 and CO_2) was
227 measured by following the same method used for the batch reactors described in Section
228 2.3. Alkalinity represents the pH buffering capacity, which has a substantial role in
229 digester stability. Total alkalinity was measured by a titrimetric method (2320-B) [34]
230 twice a week.
231 VFA compositions of the bioreactor media were measured by Varian GC-430
232 Gas Chromatograph equipped with a flame ionization detector (FID). The separation of
233 VFAs was performed by WCOT Fused Silica 25 m x 0.32 mm ID, 0.3µm coating
234 FFAP-CB capillary column. When samples were directly used in the VFA analysis
235 method according to APHA 5560C [34], fouling and clogging were observed in the
236 Varian GC-430 liner and column, and the obtained results were not consistent.
237 Therefore, to isolate the VFAs from the samples that were containing excessive
238 impurities, methyl *tert*-butyl ether (MTBE) liquid-liquid extraction method was applied
239 as described by Banel and Zygmunt (2011) [45]. Extracted samples were automatically
240 injected into the chromatography with an injection volume of 5 µL by autosampler.
241 Injector, oven, and detector temperatures were set to 250°C, 220°C, and 250°C,
242 respectively. The oven temperature program was starting at 60°C (2 min) and then
243 heating to 220°C (1 min) with a 10°C/min heating rate. The total analysis time for a
244 sample was 19 min. Helium was used as the carrier gas with a 28.0 mL/min flow rate.
245 H_2 and airflow were set to 30 mL/min and 300 mL/min, respectively. Split ratio and

split time were selected as 50 and 1.2 min. A VFA standard solution mixture (SupelCo Analytical) was used to generate the calibration curve for acetic acid, butyric acid, formic acid, heptanoic acid, hexanoic acid, isobutyric acid, isovaleric acid, 4-methyl valeric acid, propionic acid, and valeric acid. Using the VFA results, the Ripley ratio (VFA/ALK) was calculated as an indicator of the anaerobic digester stability.

2.5. Kinetic Modelling of Anaerobic Digestion

The prediction of substrates' energy potential is essential to determine the plant capacity and substrate loading rates in operation. There are few other ways to predict the energy potential as the BMP test, determination of theoretical methane potential via elemental fraction, lab-scale continuous reactor systems, or comparing with data of similar plants. Nevertheless, available kinetic model data are the easiest way to predict the energy potential of a substrate.

In this study, four different kinetic models as Modified Gompertz [46], Reaction Curve [47], First Order [48], and Cone [47] models were used, which are widely utilized in the literature. AQUASIM 2.1 simulation tool was used to perform model simulations. Lag phases, maximum methane production rates, and methane production potentials were determined by using Modified Gompertz and Reaction Curve models. In biochemical reactions, the lag phase is one of the critical parameters, and it is desired to be short. Modified Gompertz and Reaction Curve equations were represented in Equation 1 and Equation 2, respectively.

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

$$M = P.\left[1 - exp\left(-\frac{R_m(t-\lambda)}{P}\right)\right] \qquad (Eq.2)$$

In the given equations, *M, P, R_m*, λ, *t*, and *e* represent cumulative methane production, the maximum methane production potential of the substrate, the maximum methane production rate, lag phase duration (*d*), reaction time (*d*), and the Euler number (2.7183), respectively.

In order to calculate the hydrolysis rate and the cumulative methane yield, First Order and Cone models were used. The hydrolysis of cellulosic materials is mainly the rate-limiting step for anaerobic digestion. Therefore, the hydrolysis rate is crucial for the efficiency of the process. The equations of First Order and Cone models were represented, respectively.

$$M = P.\left[1 - e^{(-kt)}\right] \qquad (Eq.3)$$

$$M = \frac{P}{1 + (kt)^{-n}} \qquad (Eq.4)$$

In Equation 3 and Equation 4, *M*, *P*, *k*, *t*, *n*, and *e* are cumulative methane production, the maximum methane production potential of the substrate, hydrolysis rate constant (d^{-1}), reaction time (*d*), shape factor, and the Euler number (2.7183), respectively.

2.6. Microbial Community of Anaerobic Digestion

Denaturing gradient gel electrophoresis (DGGE) of 16s rRNA genes was carried out for analyzing the diversity and dynamics of the microbial community in the s-CSTR. The DNA isolation of bacteria and archaea in liquid samples obtained from s-CSTR at different periods was performed with ZR Soil Microbe DNA MiniPrep DNA extraction kit (Zymo Research) according to the manufacturer's instructions. For polymerase chain reaction (PCR) runs with the crude DNA samples, 907r (5′-CCGTCAATTCMTTTGAGTTT-3′) and GC-BacV3f (5′-CCTACGGGAGGCAGCAG-3′) forward primers were used for bacterial DNA, besides 915r (5'-

GTGCTCCCCCGCCAATTCCT-3′) reverse primers and GC-Arc344f (5'-ACGGGGYGCAGCAGGCGCGA-3′) forward primer were used for archaea [49]. At the 5′ end of forward primers, a GC clamp was added to stabilize the DNA fragments' melting behavior throughout the subsequent DGGE process. PCR amplification, DGGE analysis, and monitoring of the DNA were carried out according to Sahinkaya et al., (2013) [50]. DGGE runs were performed by mixing 45 µL of PCR product aliquots with 9 µL 6x Loading Dye and loading the mixture onto Polyacrylamide gels (6% (w/v) acrylamide:bisacrylamide stock solution in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA (Ethylenediaminetetraacetic acid), pH 8.3) with denaturing gradient ranging from 30% to 70% (100% denaturant contains 40% formamide and 7 M urea). After DGGE runs, SYBR Gold dye (100 µL/L in 1x TAE) was used for 30 minutes to stain the polyacrylamide gel, and then it was monitored with Gel Documentation Imaging System (Vilber Lourmat Quantum St4). Significant bands were excised with a razor blade, eluted into 20 µL sterile water, stored at 4°C overnight, and re-amplified by using the same PCR program mentioned above with the primers without GC clamp. DNA purification and Sanger sequencing reactions were done at Medsantek Ltd. Co. (Istanbul, Turkey), and similarities of the DNA sequences were compared with the sequence database of GenBank by using BLAST (Basic Local Alignment Search Tool) program.

2.7. Determination of Process Efficiency

Two theoretical values, i.e. theoretical methane yield and energy recovery, were comparatively used for determining the process efficiency. The calculation of the theoretical methane yield was presented in Section 2.2. Methane production efficiency was calculated by comparing the results with the theoretical yield. On the other hand,

316 energy recovery calculations were performed by comparing the energy equivalent of
317 methane produced with the combustion energy of raw SG. The mathematical expression
318 of the energy recovery was presented in Equation 5. The values of 39.8 MJ/m^3 and
319 15.11 MJ/kg used in the equation represent methane's and SG's calorific values, which
320 was obtained by characterization analyses (Table 2), respectively.

321 $$\text{Energy recovery (\%)} = \frac{\text{m}^3\ \text{CH}_4\ \text{produced x 39.8 MJ/m}^3}{\text{kg TS of substrate used x 15.11MJ/kg}} \text{x100} \qquad (Eq.5)$$

322

323 **3. Results and Discussion**

324 **3.1. Raw Switchgrass Characteristics**

325 SG samples were dried and milled prior to characterization analyses.
326 Characterization results are presented in Table 2. TS and VS results for the field dried
327 SG were found as 937 g/kg and 864 g/kg, respectively. Low moisture content indicates
328 that SG does not require any other drying methods than field drying. SG contains
329 36.24% hemicellulose, 35.94% cellulose, 20.45% lignin, 38.18% TOC, and 41.95% C.
330 Having high cellulose, hemicellulose, and carbon contents make SG a suitable raw
331 material for biofuel production processes. As a result of structural carbohydrates
332 analysis, SG was found to contain around 40% glucose and 21% xylose. More detailed
333 characterization results of the substrate were given in a previous study [10].

334 The chemical composition of the SG is found as $C_{140}H_{244}O_{109}N_1$ regarding the
335 elemental analysis results. The Buswell Equation was used to calculate the B_U value,
336 which is defined as the theoretical methane yield, as 442.8 mL CH_4/g VS for raw SG.
337 The theoretical methane potential of SG was reported in the literature as 500 mL CH_4/g
338 VS [51], 448 mL CH_4/g VS [52], and 530 mL CH_4/g VS [26], which are consistent with
339 the result of this study. Also, the C/N ratio was found as 120. Lastly, the higher heating

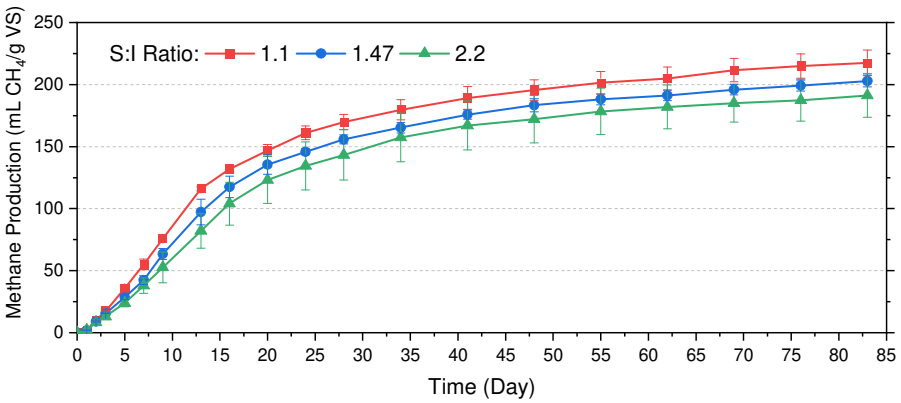
value of SG was calculated as 15.11 MJ/kg TS (3610.13 kcal/kg TS) using the Dulong Equation [39].

Table 2. Characterization results of raw SG

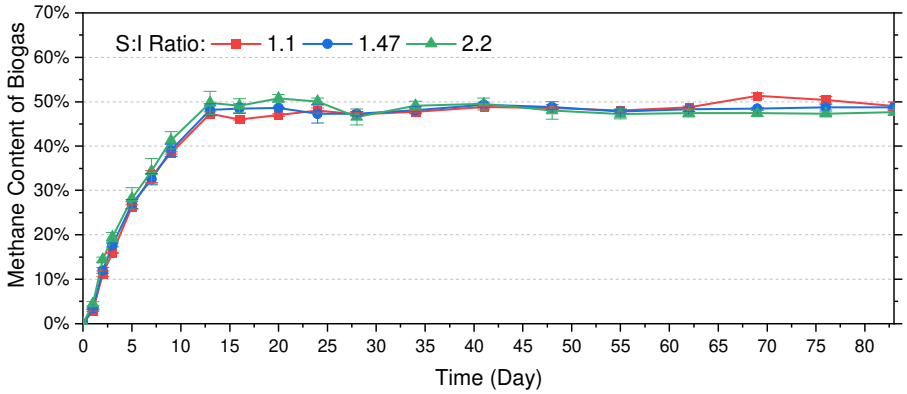
Analysis	Unit	Result
Total solid, TS	(g/kg Sample)	937.28 ± 1.90
Volatile solid, VS	(g/kg Sample)	863.96 ± 2.18
Soluble reducing sugar	(mg/g VS)	40.19 ± 6.29
Soluble protein	(mg/g VS)	59.81 ± 1.31
Total organic carbon, TOC	(%)	38.18 ± 0.1
Hemicellulose (HEMI)	(%)	36.24 ± 0.08
Cellulose (CELL)	(%)	35.94 ± 0.06
Acid soluble lignin, ASL	(%)	2.46 ± 0.04
Acid insoluble lignin, AIL	(%)	17.99 ± 056
Elemental Analysis		
C	(%)	41.95
H	(%)	6.14
N	(%)	0.35
S	(%)	-
Trace Elements		
Mg	(ppm)	1427
Al	(ppm)	60.2
Fe	ppm	69.4
Cu	ppm	1.51
Mn	ppm	27.4
Sr	ppm	21.4
Se	ppm	0.23
Ca	ppm	10.62
Na	ppm	2.735
K	ppm	8.643
Lover Heating Value, LHV	(MJ/ kg TS)	14.55
Higher Heating Value, HHV	(MJ/kg TS)	15.11

3.2. Batch Anaerobic Digestion

The cumulative methane production and the percentage of methane in biogas at different S:I ratios over time are given in Fig. 2 (a) and (b). The methane potential of seed sludge, 55.76 mL CH_4/g VS, was subtracted from all batch test results for normalization.



(a)



(b)

Fig. 2. Cumulative methane production (a) and methane percentage of the biogas (b) at different S:I ratios in the batch system

At the end of 60 days of incubation, the cumulative methane productions were 203.9, 190.5, and 181.0 mL CH_4/g-VS for S:I values of 1.1, 1.47 and 2.2, respectively. As the S:I ratios and corresponding loading rate increased, a decrease in the specific amount of methane production was observed.

For S:I of 1.1, 79% of the total methane amount (203.9 mL CH_4/g VS) was obtained in the first 24 days, which claims that the retention time for batch AD of SG can be considered 24-25 days in terms of economic methane production. Methane percentage of biogas increased in the first 13 days and continued at a constant percentage without any significant change till the end of the tests (Fig. 2b). The amount of biogas and methane produced in batch tests are presented in Table 3.

Table 3. Anaerobic batch digestion performance at different S:I ratios

(S:I ratio)	Biogas Yield (mL/g VS)	Methane Yield (mL CH_4/g VS)
1.1	422.15	203.90
1.47	394.41	190.50
2.2	373.20	181.00

When the batch digestion performance of SG in the literature was reviewed, it was found that the methane potential of SG was reported as 135.31 mL CH_4/g VS at an S:I ratio of 1:12 for 44 days of digestion [44], 112.4 mL CH_4/g VS at F/M ratio of 1:2 for 38 days retention time [13], 216 mL CH_4/g VS at an S:I ratio of 1:4.5 for 58 days retention time [53], 111.0 mL CH_4/g VS for 30 days retention time [54] and 131.1 mL CH_4/g VS for 30 days retention time [7] when SG was co-digested with manure. The results of batch methane production performance in this study for different S:I ratios were consistent with the literature.

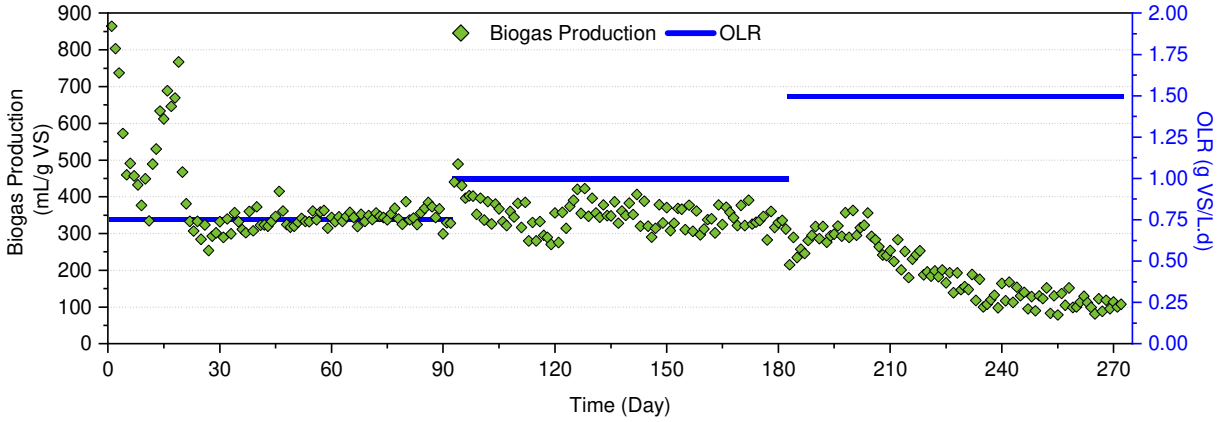
3.3. Semi-Continuous Stirred Tank Reactor (s-CSTR) Digestion

3.3.1. Performance of the Digester

For different OLRs, average biogas productions, daily biogas production rates, methane content of biogas, and biogas - methane yields in the s-CSTR system are given in Table 4.

The biogas production rates were 255.58, 347.2, and 213.81 mL/L·day with the average biogas yields of 340.81, 347.34, and 142.55 mL/g-VS for 0.75, 1.0, and 1.5 gVS/(L.d) OLR values, respectively. In the s-CSTR system, a significant decrease in the biogas yield was observed for the highest OLR of 1.5 gVS/(L.d), along with slightly lower (3%) the biogas' methane content compared to that at 1 gVS/(L.d). High carbohydrate content of SG caused low methane content (50%). The highest biogas and methane yields of 347.34 mL/g VS and 156.9 mL CH_4/g VS were achieved at 1.0 g VS/(L.d) OLR. For the highest OLR, a homogeneous mixing in the s-CSTR was not possible due to SG accumulation, which may be considered one of the main reasons observing lower biodegradation performance. Methane generation decreased by 38.2% with increasing OLR from 1.5 g VS/(L.d) to 1 g VS/(L.d) OLR, which may be due to solids build-up in the bioreactor, mixing limitation and nutrient deficiency at higher OLR.

Methane yields for 0.75 and 1.0 gVS/(L.d) OLRs were quite similar (Fig. 3) and 1.0 gVS/(L.d) would be preferable OLR in terms of using the reactor volume efficiently and higher methane production.



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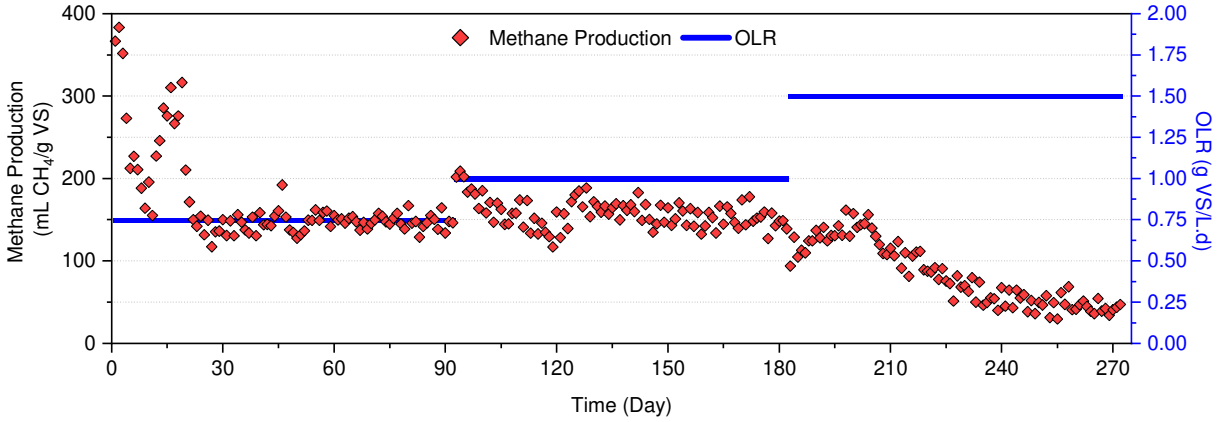
415 **(a)**



416
417 **(b)**

418 **Fig. 3.** For different OLRs, (a) biogas and (b) methane yields of SG in the s-CSTR
419 system

420 **Table 4.** Biogas and methane production performances for different OLRs in the s-
421 CSTR system. a: percentage of s-CSTR results to batch system results

OLR (gVS/(L.d))	Biogas Production Rate (mL/(L.d))	Biogas Yield (mL/g VS)	Methane Content (%)	Methane Yield (mL/g VS)	Biogas Yield[a] (%)	Methane Yield[a] (%)
0.75	255.58	340.81±22.7	43.3±1.7	147.56±12.7	80.73	72.37
1.00	347.20	347.34±31.9	45.16±1.1	156.85±13.6	88.07	82.33
1.50	213.81	142.55±43.9	42.17±3.2	60.11±21.21	38.20	33.21

422

There are some contradictory findings in the limited literature. Jin et al. (2012) studied the co-digestion of SG and sewage sludge (72% primary and 28% secondary sludge) at 1.7-2.23 kg VS/(m^3.day) OLRs (2% and 4% fine grind) [27]. They obtained 0.261 and 0.270 m^3 CH_4/kg $VS_{SGadded}$ methane yields for 1.7 and 2.23 kg VS/(m^3.day) OLRs values, respectively. In the other studies of Jin and teams [20 21], the same substrates, SRT, OLR and feeding type were used during the 63 days operation of the bioreactor. They reported SG specific methane potentials of 296 and 14 mL CH_4/g VS for fine-grind (2 mm) and coarse-grind (7.8 mm) samples, respectively [25]. However, contrary to these findings, when the same fine-grind (2 mm) and coarse-grind (7.8 mm) SG samples were digested with local dairy seed sludge, 252 and 328 mL CH_4/g VS methane yields were obtained, respectively [25]. Operational issues such as solids accumulation, inefficient mixing, or floating were not reported during the bioreactor operation used in the study. In our study, the average methane productions for long-term (270 days) reactor operation are lower than those reported in the studies of Jin et al. On the other hand, these results indicate the importance of using cattle manure as seed for the anaerobic digestion of raw lignocellulosic biomass. Cattle manure seed, which is also used in this study, contains naturally adapted microorganisms to process raw plant material that can hydrolyze lignocellulose using cellulolytic enzymes such as cellulase and β-glucosidase. Liang et al. (2018) examined the residence time effect on semi-continuous digestion of SG between 20-3.3 day. Progressing from RT=20 days to 3.3 days, methane concentration decreased slightly from 50.5±1.0 to 46.7±0.7% and CO2 increased from 42.6±2.2 to 45.9±1.5% which is also another clue for longer residence time of lignocellulosic materials [28]. May (2020) suggested to intermittent co-treatment to increase the biogas production, but the microbiome was able to tolerate

447 daily milling of 50% of the digester volume [56]. Frigon et al. (2012) demonstrated the
448 co-digestion of switchgrass with dairy manure with laboratory-scale digesters, which
449 shows an increase of 86% in methane production compared with a digester fed only
450 with manure at a similar loading of fresh material [43].

451 **3.3.2. Stability of Digester**

452 The digester output samples were collected twice a week for the whole period
453 and analyzed for alkalinity and VFAs. The system's pH, monitored every day, averaged
454 7.3, 7.1, and 7.1 for 0.75, 1.0, and 1.5 gVS/(L.d) OLR, respectively. For 0.75 gVS/(L.d)
455 OLR, the pH values were stable; however, with the increases of OLR, the pH values
456 decreased on some days. In AD, pH levels lower than ~5 are considered unsuitable for
457 methanogenic *Archaea*. Therefore, pH was adjusted back to 7.0 by 0.5 M NaOH to
458 avoid any possible inhibition. Daily pH data and the occurrence of pH drops are
459 presented in Fig. 4.

460 The initial average alkalinity value was 4472.6 mg $CaCO_3$/L for 0.75 gVS/(L.d)
461 OLR, then a decrease to an average value of 3610.8 mg $CaCO_3$/L was observed when
462 the OLR was increased to 1.0 gVS/(L.d). At the OLR of 1.5 gVS/(L.d), the system's
463 alkalinity increased to an average value of 4588.5 mg $CaCO_3$/L. The optimum range of
464 alkalinity for the anaerobic digestion varies between 2000 and 5000 mg $CaCO_3$/L, and
465 the lower limit is 1000 mg $CaCO_3$/L to provide a convenient buffer capacity [57]. The
466 alkalinity range of the s-CSTR reactor in this study was found in a suitable range for
467 anaerobic digestion.

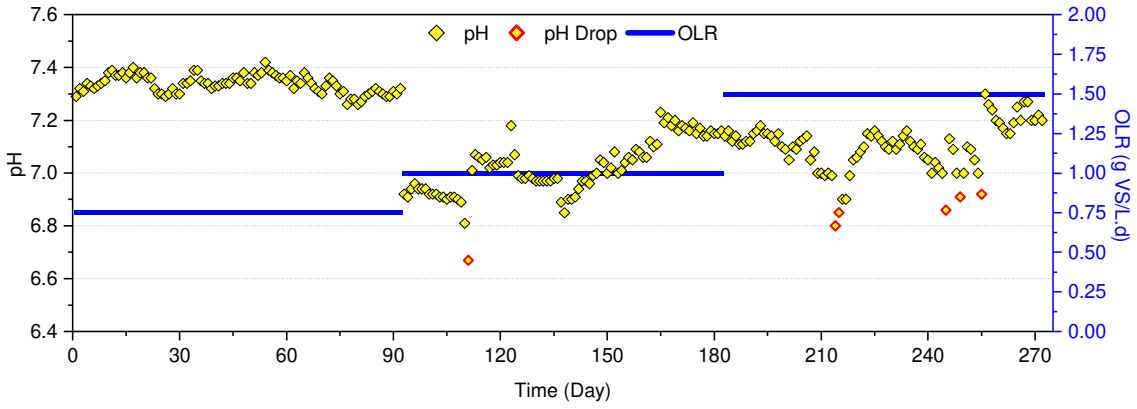


Fig. 4. pH changes for different OLR values of anaerobic digestion of SG at s-CSTR

Acetic acid (HAc) has been identified as the most observed organic acid in total volatile organic acids. The average HAc/VFA ratios were 50.1, 85.3, and 65.3% for 0.75, 1, and 1.5 gVS/(L.d) OLRs, respectively. Hence, acetic acid was the most abundant VFA, as expected. VFA accumulation occurs in cases where the symbiotic relationship between methanogenic and acetogenic microorganisms in a digester is disturbed due to the reactor overloading, microbial washout, or inhibition in the AD. In the anaerobic treatment systems, high VFA/ALK ratios caused by an increase in VFAs or a decrease in alkalinity lead to a sudden decrease in pH [20]. This pH decrease may affect the system's microbial balance due to the inhibition effect of VFAs on methanogens. In this study, VFA/ALK ratios were observed lower than 0.1 for all OLRs during the operation.

3.4. Kinetic Models

The kinetic parameters were presented in Table 5. The statistical indicator, R^2, was also calculated and presented in Table 5. Predicted methane potential values were plotted against experimental data to compare visually, as illustrated in Fig. 5. All kinetic models showed high prediction capacity with high R^2 values.

Higher values of the maximum methane production rate are an indication of a higher methane potential. As may be noted from the kinetical parameters given in Table 5, the highest R_m value was observed as 9.3 mL CH_4/g VS.d according to the Modified Gompertz model for S:I ratio of 1.1. The increase in S:I ratio resulted in decreases in R_m. For 1.47 and 2.2. S:I ratios, on the other hand, R_m values were 8 mL CH_4/g VS.d and 7.1 mL CH_4/g VS.d, respectively. In accordance with the maximum methane production rate, the lag phase increased with the increasing S:I ratio. λ values were estimated by the model as 1.68 days, 1.86 days, and 2.18 days for 1.1, 1.47, and 2.2 S:I ratios, respectively. El-Mashad, (2013) reported the maximum methane production rate and lag phase duration as 6.96 mL CH_4/g VS.d and 2.11 day for SG [22]. High R^2 values were determined for all experiments as 0.992, 0.994, and 0.996 for 1.1, 1.47, and 2.2 S:I ratios, respectively. Differences in the measured and the calculated values were relatively higher and calculated as 5.3%, 4.1%, and 3.3% for 1.1, 1.47, and 2.2 S:I ratios, respectively.

The maximum methane production rates, R_m, were determined as 12.6 mL CH_4/g VS.d, 10.8 mL CH_4/g VS.d and 9.5 mL CH_4/g VS.d for S:I ratios of 1.1, 1.47, and 2.2, respectively, for reaction curve model. Lag phase durations were calculated as 1.01 days, 1.03 days, and 1.21 days for S:I ratios of 1.1, 1.47, and 2.2, respectively. The trend for R_m and λ values was in parallel with the findings of the Modified Gompertz model. Determination coefficients, R^2, were 0.994, 0.993, and 0.993 for 1.1, 1.47, and 2.2 S:I ratios, respectively. Differences in measured and the calculated values were quite low as 0.3%, 0.3% and 0.2% for S:I ratios of 1.1, 1.47, and 2.2, respectively.

Another critical biochemical reaction kinetic parameter is the hydrolysis rate constant (k), which indicates the biodegradation and methane production rates. The k

values were observed as 0.054 1/day, 0.050 1/day and 0.046 1/day for S:I ratios of 1.1, 1.47, and 2.2, respectively. El-Mashad (2013) found the hydrolysis rate as 0.05 1/day for SG, which is similar to this study's findings [22]. The hydrolysis rate decreased via increasing S:I ratios. The decrease in hydrolysis rate was parallel with methane production. The R^2 value was calculated as 0.993 for all experiments.

Cone model uses hydrolysis rate constant (k) and shape factor (n) kinetic parameters to estimate the methane production. The calculated k values were 0.080 1/day, 0.073 1/day and 0.067 1/day for S:I ratios of 1.1, 1.47, and 2.2, respectively. The decrease in the hydrolysis rate of Cone model with increasing S:I ratio was parallel with those of the First Order model. The R^2 value was calculated as 0.999 for all experiments. The highest prediction capacity for anaerobic digestion of SG was obtained for the Cone model.

Table 5. Predicted model parameters

Model	**Parameter**	**S:I = 1.1**	**S:I = 1.47**	**S:I = 2.2**
Experimental	M (mL CH_4/g VS_{added})	203.9	190.5	181.0
First Order Kinetic	k (1/Day)	0.054	0.050	0.046
	P (mL CH_4/g VS_{added})	203.7	189.7	178.7
	R^2	0.993	0.993	0.993
	Difference (%)	-0.1	-0.4	-1.3
Cone	k (1/Day)	0.080	0.073	0.067
	N	1.7	1.8	1.9
	P (mL CH_4/g VS_{added})	198.9	186.2	177.1
	R^2	0.999	0.999	0.999

	Difference (%)	-2.5	-2.3	-2.2
Modified Gompertz	R_m (mL CH_4/g VS_{added}.Day)	9.3	8.0	7.1
	λ (Day)	1.68	1.86	2.18
	P (mL CH_4/g VS_{added})	193.6	183.0	175.2
	R^2	0.992	0.994	0.996
	Difference (%)	-5.3	-4.1	-3.3
Reaction Curve	R_m (mL CH_4/g VS_{added}.Day)	12.6	10.83	9.45
	λ (Day)	1.01	1.03	1.21
	P (mL CH_4/g VS_{added})	203.3	191.1	180.5
	R^2	0.994	0.993	0.993
	Difference (%)	-0.3	0.3	-0.2

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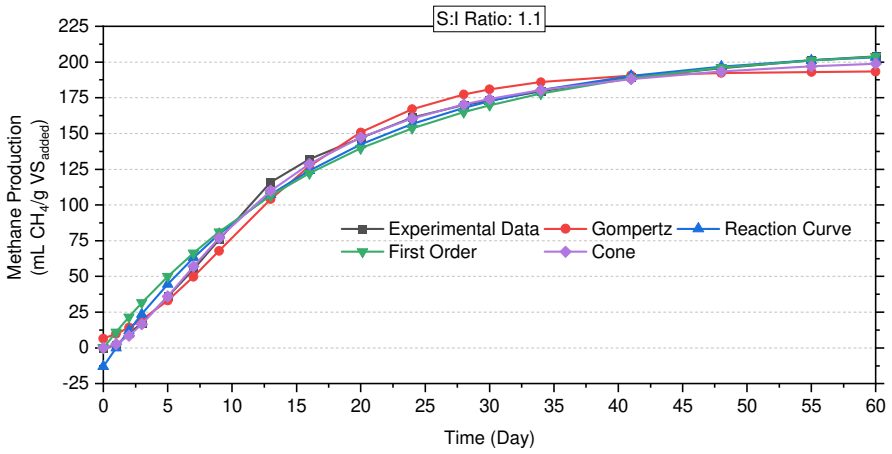
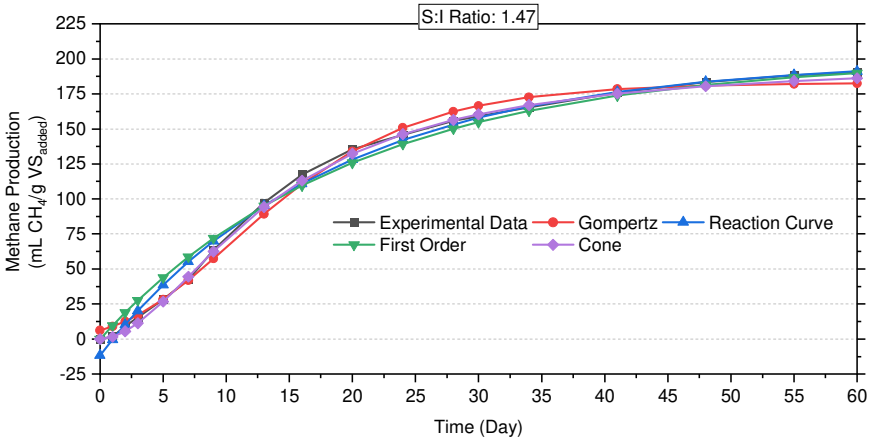


(a)



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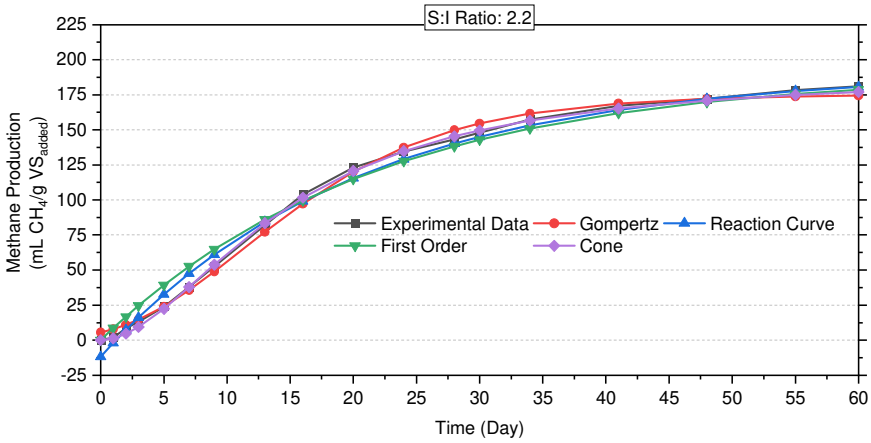

(c)

Fig. 5. Methane productions and model predictions in the batch system for **(a)** S:I = 1.1 (**b**) S:I = 1.47, and **(c)** S:I = 2.2

3.5. Microbial Community Structure

Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA genes was carried out for characterizing microbial community structures of s-CSTR for 0.75, 1.00, and 1.50 OLRs and for seed culture as well. Comparison of gene sequences with GenBank databases for detecting reactor clones' similarity to the reference strains was performed with the BLAST program. The microbial community of the s-CSTR was found to be stable according to the similarity search.

Several methanogenic archaea, including *Methanosarcina*, *Methanosaeta*, *Methanothrix,* and *Methanomethylovorans* genus, as well as *Chloroflexi*, *Firmicutes, Spirochaetes*, and *Actinobacteria,* were observed in s-CSTR (Table 6). Besides, some uncultured bacteria were also obtained in samples.

Table 6. Blast results of the sequences

	Phylogenic phylum/genus	Similarity (%)	Accession No.
Bacteria	Spirochaetes	89	GQ347015.1
	Chloroflexi	93	CU921102.1
	Firmicutes	97	KX367894.1
	Actinobacteria	97	NR_146663.1
Archea	Methanosarcina	98	NR_148758.1
	Methanosaeta	93	NR_113571.1
	Methanothrix	99	NR_102903.1
	Methanomethylovorans	94	NR_102454.1

One of the dominant bacterial phyla of s-CSTR are members of *Spirochaetes* phylum, that play an important role in acetate oxidation. These bacteria can be commonly found in natural environments, engineered habitats, and anaerobic digesters. In municipal waste sludge processing anaerobic digesters, *Spirochaetes* play a significant role in acetate oxidation under both mesophilic and thermophilic conditions, in liaison with hydrogenotrophic methanogenic archaea (i.e., *Methanobacteriales*, *Methanomicrobiales*, or *Methanococcales*) [58].

The other Bacteria identified in s-CSTR are the members of *Firmicutes* and *Actinobacteria* phylum. *Clostridia* class of *Firmicutes* phylum include *Clostridium* and *Terrisporobacter* genera, which are endospore-forming strictly anaerobic bacteria. *Clostridium* is responsible for lignocellulose degradation with its cellulolytic enzymes [59]. Besides, they perform as acetate oxidation during methane production from the digestion of organic substances [60]. *Terrisporobacter* can also act as an acetic acid

producer by utilizing simple carbohydrates during anaerobic digestion [61]. Ziganshin et al. also stated that, throughout the agro-industrial waste treatment, *Firmicutes* (mainly *Clostridia*) were the major bacterial phyla [61, 62]. The role of *Actinobacteria* and *Chloroflexi* in methanogenic habitats is yet to be characterized, but they are reputed to hydrolyze carbohydrates and ferment sugars [64]. The higher populations of hydrolyzing bacteria produce more organic acids during the hydrolysis stage, providing more carbon sources for archaea to generate more biogas.

Methanosarcina genus can metabolize a wide range of substrates for methane production, unlike the other methanogens. Some strains of this genus isolated from anaerobic sewage sludge are model organisms for methane production [65]. In biogas plants, the genus is one of the highly dominant species of archaeal communities because of their acetate consumption potential as a carbon and energy source via acetoclastic pathway. The other member of *Methanosarcinales* order is *Methanosaeta* genus, which is also a methane producer via acetoclastic pathway. Unlike *Methanosarcina*, they are obligate acetate utilizer for methanogenesis [64]. *Methanothrix* is an obligate anaerobic archaea which can be isolated from mesophilic sewage digesters. They decarboxylate acetate to produce methane and become dominant in the population when acetate concentration is low. With an increase in acetate concentration, they leave the dominance to *Methanosarcina* genus [66]. The *Methanomethylovorans* genus members are methyl group consuming and methane-producing archaea, which can be isolated from the slurry obtained from eutrophic pond sediments [67].

Meanwhile, very limited microbial community studies have also been performed from the anaerobic reactor digesting SG [19]. Zhong et al. (2020) stated that bacterial phyla of *Bacteroidetes* and *Firmicultes* and archaeal genera of *Methanobrevibacter,*

581 *Methanosarcina, and Methanobacterium* were abundant in the co-digestion of SG and
582 agricultural waste [19].
583
584 **3.6. Methane and Energy Yields**
585 Comparisons of average methane productions to theoretical methane potential
586 and energy equivalents of methane produced to direct combustion energy of SG were
587 performed to determine the process efficiency. The theoretical methane yield of SG was
588 calculated as 408.2 mL CH_4/g TS by using the Buswell Equation equal to 16.2 MJ/kg
589 TS, whereas the higher heating value, calculated by using the Dulong Equation, was
590 found 15.11 MJ/kg TS. Methane productions in the batch and s-CSTR systems,
591 presented in Table 3 and Table 4, were multiplied by 39.8 MJ/m^3, which is methane's
592 calorific value under normal temperature and pressure, to determine the average energy
593 recoveries in the form of methane. Although the energy equivalent of the theoretical
594 methane yield and the SG's calorific value were found close, the theoretical methane
595 yield's energy equivalent was slightly higher due to the differences in the calculation
596 methods. Energy recovery and methane production yields are presented in Fig. 6.

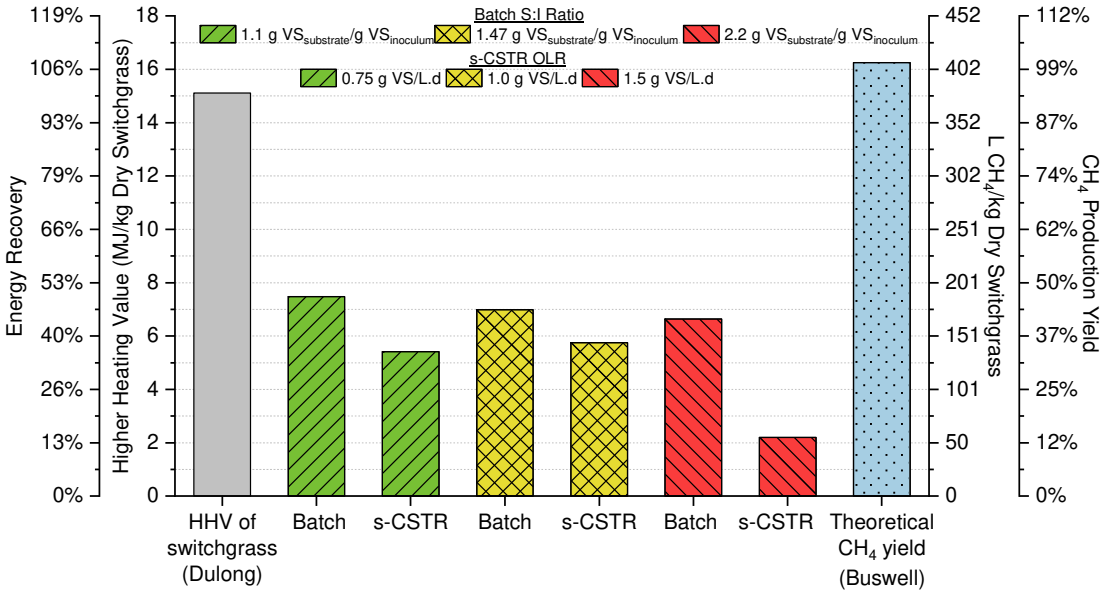

Fig. 6. Energy recoveries and methane yields reached in different reactor systems

Methane production results of the batch system for S:I ratios of 1.1, 1.47, and 2.2 were 187.95, 175.6, and 166.84 L/kg TS, respectively. The highest methane yield, obtained at S:I ratio of 1.1, represents 46.1% of the theoretical methane yield, and it reached around 50% energy recovery. Increasing the S:I ratios to 1.47 and 2.2 in the batch system caused a slight decrease in energy recovery to 46.25% and 43.95%. On the other hand, for 0.75, 1.0, and 1.5 gVS/(L.d) OLRs in the s-CSTR system, methane production yields were 33.32%, 35.42%, and 13.57% of the theoretical yield, representing energy recovery of 35.83%, 38.08%, and 14.59%, respectively. Although energy recovery results seem to be low compared to the direct combustion of SG, when the robust biochemical structure of lignocellulose is considered, it can be seen that anaerobic digestion was successful in converting raw lignocellulosic biomass into methane. Moreover, after fermentation, the remaining media can be used in soil amendment, or it can be dried and combusted for further energy recovery.

4. Conclusions

Specific methane potential of SG was investigated in batch and s-CSTR. According to the batch results, the energy recovery was around 50%, 46%, and 44% for the S:I ratios of 1.1, 1.47, and 2.2, respectively. The s-CSTR system's energy recovery was around 36%, 38%, and 15% for the OLRs of 0.75, 1.0 and 1.5 gVS/(L.d), respectively. Low methane production from 1.5 OLR was mostly due to SG accumulation, causing mixing problems and high C/N ratio, VFA accumulation, which led to pH decrease from time to time. Even though the results showed that the methane production from the s-CSTR process at 1.0 gVS/L·day of OLR can reach 82 % of the batch process, the optimal choice for industrial applications should be the usage of s-CSTR from the point of the working volume and the hydraulic retention time. In the long-term operated s-CSTR, several methanogenic archaea, including *Methanosarcina*, *Methanosaeta*, *Methanothrix,* and *Methanomethylovorans* genus were detected. The nutrient deficiency was found as the main reason behind the low digestion performance in this study. Therefore, we recommend future studies to supply micronutrients to the SG anaerobic digestion processes.

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637 **References**

638 [1] M. Sterner, Bioenergy and renewable power methane in integrated 100%
639 renewable energy systems. Limiting global warming by transforming energy
640 systems, 2010.

641 [2] Z. Romanowska-duda, S. Szufa, M. Grzesik, K. Piotrowski, R. Janas, The
642 Promotive Effect of Cyanobacteria and Chlorella sp. Foliar Biofertilization on
643 Growth and Metabolic Activities of Willow (Salix viminalis L.) Plants as
644 Feedstock Production, Solid Biofuel and Biochar as C Carrier for Fertilizers via
645 Torrefaction Proce, Energies. 14 (2021).
646 https://doi.org/https://doi.org/10.3390/en14175262.

647 [3] S. Szufa, P. Piersa, Ł. Adrian, J. Czerwinska, A. Lewandowski, W.
648 Lewandowska, J. Sielski, M. Dzikuc, M. Wróbel, M. Jewiarz, A. Knapczyk,
649 Sustainable drying and torrefaction processes of miscanthus for use as a
650 pelletized solid biofuel and biocarbon-carrier for fertilizers, Molecules. 26
651 (2021). https://doi.org/10.3390/molecules26041014.

652 [4] K. David, A.J. Ragauskas, Switchgrass as an energy crop for biofuel production:
653 A review of its ligno-cellulosic chemical properties, Energy Environ. Sci. 3
654 (2010) 1182–1190. https://doi.org/10.1039/b926617h.

655 [5] A. Głowacka, B. Szostak, R. Klebaniuk, Effect of biogas digestate and mineral
656 fertilisation on the soil properties and yield and nutritional value of switchgrass
657 forage, Agronomy. 10 (2020) 1–22. https://doi.org/10.3390/agronomy10040490.

658 [6] L. Wright, A. Turhollow, Switchgrass selection as a “ model” bioenergy crop: A

history of the process, Biomass and Bioenergy. 34 (2010) 851–868. https://doi.org/10.1016/j.biombioe.2010.01.030.

[7] Z. Zheng, J. Liu, X. Yuan, X. Wang, W. Zhu, F. Yang, Z. Cui, Effect of dairy manure to switchgrass co-digestion ratio on methane production and the bacterial community in batch anaerobic digestion, Appl. Energy. 151 (2015) 249–257. https://doi.org/10.1016/j.apenergy.2015.04.078.

[8] N. Akbarian-Saravi, M. Mobini, M. Rabbani, Development of a comprehensive decision support tool for strategic and tactical planning of a sustainable bioethanol supply chain: Real case study, discussions and policy implications, J. Clean. Prod. 244 (2020) 118871. https://doi.org/10.1016/j.jclepro.2019.118871.

[9] M. Rabbani, S. Momen, N. Akbarian-Saravi, H. Farrokhi-Asl, Z. Ghelichi, Optimal design for sustainable bioethanol supply chain considering the bioethanol production strategies: A case study, Comput. Chem. Eng. 134 (2020) 106720. https://doi.org/10.1016/j.compchemeng.2019.106720.

[10] I.A. Başar, E. Kökdemir Ünşar, H. Ünyay, N.A. Perendeci, Ethanol, methane, or both? Enzyme dose impact on ethanol and methane production from untreated energy crop switchgrass varieties, Renew. Energy. 149 (2020) 287–297. https://doi.org/10.1016/j.renene.2019.12.025.

[11] D. Massé, Y. Gilbert, P. Savoie, G. Bélanger, G. Parent, D. Babineau, Methane yield from switchgrass and reed canarygrass grown in Eastern Canada, Bioresour. Technol. 102 (2011) 10286–10292. https://doi.org/10.1016/j.biortech.2011.08.087.

[12] R.A. Labatut, L.T. Angenent, N.R. Scott, Biochemical methane potential and biodegradability of complex organic substrates, Bioresour. Technol. 102 (2011) 2255–2264. https://doi.org/10.1016/j.biortech.2010.10.035.

[13] J.C. Frigon, P. Mehta, S.R. Guiot, Impact of mechanical, chemical and enzymatic pre-treatments on the methane yield from the anaerobic digestion of switchgrass, Biomass and Bioenergy. 36 (2012) 1–11. https://doi.org/10.1016/j.biombioe.2011.02.013.

[14] M. Kupryś-Caruk, S. Podlaski, D. Kotyrba, Influence of double-cut harvest system on biomass yield, quality and biogas production from C4 perennial grasses, Biomass and Bioenergy. 130 (2019) 105376. https://doi.org/10.1016/j.biombioe.2019.105376.

[15] X. Zhao, J. Liu, J. Liu, F. Yang, W. Zhu, X. Yuan, Y. Hu, Z. Cui, X. Wang, Effect of ensiling and silage additives on biogas production and microbial community dynamics during anaerobic digestion of switchgrass, Bioresour. Technol. 241 (2017) 349–359. https://doi.org/10.1016/j.biortech.2017.03.183.

[16] H. Khalid, F. Cai, J. Zhang, R. Zhang, W. Wang, G. Liu, C. Chen, Optimizing key factors for biomethane production from KOH-pretreated switchgrass by response surface methodology, Environ. Sci. Pollut. Res. 26 (2019) 25084–25091. https://doi.org/10.1007/s11356-019-05615-y.

[17] J. Shen, J. Zhang, W. Wang, G. Liu, C. Chen, Assessment of pretreatment effects on anaerobic digestion of switchgrass: Economics-energy-environment (3E) analysis, Ind. Crops Prod. 145 (2020) 111957.

https://doi.org/10.1016/j.indcrop.2019.111957.

[18] İ.A. Başar, Ö. Çoban, M.Y. Göksungur, Ç. Eskicioğlu, N.A. Perendeci, Enhancement of lignocellulosic biomass anaerobic digestion by optimized mild alkaline hydrogen peroxide pretreatment for biorefinery applications, J. Environ. Manage. 298 (2021). https://doi.org/10.1016/j.jenvman.2021.113539.

[19] Y. Zhong, R. Chen, J.P. Rojas-Sossa, C. Isaguirre, A. Mashburn, T. Marsh, Y. Liu, W. Liao, Anaerobic co-digestion of energy crop and agricultural wastes to prepare uniform-format cellulosic feedstock for biorefining, Renew. Energy. 147 (2020) 1358–1370. https://doi.org/10.1016/j.renene.2019.09.106.

[20] S. Uma, A.K. Thalla, C.P. Devatha, Co-digestion of Food Waste and Switchgrass for Biogas Potential: Effects of Process Parameters, Waste and Biomass Valorization. 11 (2020) 827–839. https://doi.org/10.1007/s12649-018-0508-2.

[21] D. Jackowiak, J.C. Frigon, T. Ribeiro, A. Pauss, S. Guiot, Enhancing solubilisation and methane production kinetic of switchgrass by microwave pretreatment, Bioresour. Technol. 102 (2011) 3535–3540. https://doi.org/10.1016/j.biortech.2010.11.069.

[22] H.M. El-Mashad, Kinetics of methane production from the codigestion of switchgrass and Spirulina platensis algae, Bioresour. Technol. 132 (2013) 305–312. https://doi.org/10.1016/j.biortech.2012.12.183.

[23] A.S. Ciggin, Anaerobic co-digestion of sewage sludge with switchgrass: Experimental and kinetic evaluation, Energy Sources, Part A Recover. Util. Environ. Eff. 38 (2016) 15–21. https://doi.org/10.1080/15567036.2015.1060549.

[24] X. Tao, P. Zhang, G. Zhang, M. Nabi, S. Wang, J. Ye, S. Bao, Q. Zhang, N. Chen, Carbide slag pretreatment enhances volatile fatty acid production in anaerobic fermentation of four grass biomasses, Energy Convers. Manag. 199 (2019) 112009. https://doi.org/10.1016/j.enconman.2019.112009.

[25] G. Jin, T. Bierma, P.M. Walker, Low-heat, mild alkaline pretreatment of switchgrass for anaerobic digestion, J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 49 (2014) 565–574. https://doi.org/10.1080/10934529.2014.859453.

[26] G. Jin, T. Bierma, Low-heat alkaline pretreatment of biomass for dairy anaerobic codigestion, J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes. 49 (2014) 786–796. https://doi.org/10.1080/03601234.2014.929912.

[27] G. Jin, T. Bierma, P. Walker, Biogas production from switchgrass under experimental conditions simulating U.S. digester operations, J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 47 (2012) 470–478. https://doi.org/10.1080/10934529.2012.646150.

[28] X. Liang, J.M. Whitham, E.K. Holwerda, X. Shao, L. Tian, Y.W. Wu, V. Lombard, B. Henrissat, D.M. Klingeman, Z.K. Yang, M. Podar, T.L. Richard, J.G. Elkins, S.D. Brown, L.R. Lynd, Development and characterization of stable anaerobic thermophilic methanogenic microbiomes fermenting switchgrass at decreasing residence times, Biotechnol. Biofuels. 11 (2018) 1–18. https://doi.org/10.1186/s13068-018-1238-1.

[29] A. Seesatat, S. Rattanasuk, K. Bunnakit, P. Maneechot, P. Sriprapakhan, R.

747 Artkla, Biological degradation of rice straw with thermophilic lignocellulolytic
748 bacterial isolates and biogas production from total broth by rumen
749 microorganisms, J. Environ. Chem. Eng. (2020) 104499.
750 https://doi.org/10.1016/j.jece.2020.104499.

751 [30] N.A. Perendeci, F.Y. Ekinci, J.J. Godon, Monitoring the performance and
752 microbial diversity dynamics of a full scale anaerobic wastewater treatment plant
753 treating sugar factory wastewater, Environ. Sci. Process. Impacts. 15 (2013) 494–
754 502. https://doi.org/10.1039/c2em30597f.

755 [31] H. Sun, S. Wu, R. Dong, Monitoring Volatile Fatty Acids and Carbonate
756 Alkalinity in Anaerobic Digestion: Titration Methodologies, Chem. Eng.
757 Technol. 39 (2016) 599–610. https://doi.org/10.1002/ceat.201500293.

758 [32] J.C. Gaby, M. Zamanzadeh, S.J. Horn, The effect of temperature and retention
759 time on methane production and microbial community composition in staged
760 anaerobic digesters fed with food waste, Biotechnol. Biofuels. 10 (2017) 1–13.
761 https://doi.org/10.1186/s13068-017-0989-4.

762 [33] K.P. Vogel, R.B. Mitchell, Heterosis in switchgrass: Biomass yield in swards,
763 Crop Sci. 48 (2008) 2159–2164. https://doi.org/10.2135/cropsci2008.02.0117.

764 [34] APHA, Standard Methods for the examination of water and wastewater., Am.
765 Public Heal. Assoc. Water Work. Assoc. Environ. Fed. Washingt. DC. (2005).

766 [35] G.L. Miller, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing
767 Sugar, Anal. Chem. (1959). https://doi.org/10.1021/ac60147a030.

768 [36] O.H. LOWRY, N.J. ROSEBROUGH, A.L. FARR, R.J. RANDALL, Protein
769 measurement with the Folin phenol reagent., J. Biol. Chem. 193 (1951) 265–275.

770 [37] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D.C. Nrel,
771 Determination of Structural Carbohydrates and Lignin in Biomass Determination
772 of Structural Carbohydrates and Lignin in Biomass, 2011 (2012).
773 https://doi.org/NREL/TP-510-42618.

774 [38] P.J. Van Soest, Use of detergent in the analysis of fibrous feeds. A rapid method
775 for the determination of fibre and lignin, J. Assoc. Anal. Chem. 46 (1963) 829–
776 835.

777 [39] G. Tchobanoglous, H. Theisen, S. Vigil, Integrated Solid Waste Management.
778 Engineering Principles and Management Issues, International edition. Singapore:
779 McGraw- Hill., 1993.

780 [40] G.E. Symons, A.M. Buswell, The Methane Fermentation of Carbohydrates1,2, J.
781 Am. Chem. Soc. 55 (1933) 2028–2036. https://doi.org/10.1021/ja01332a039.

782 [41] N.A. Perendeci, S. Gökgöl, D. Orhon, Impact of alkaline H2O2 pretreatment on
783 methane generation potential of greenhouse crop waste under anaerobic
784 conditions, Molecules. 23 (2018). https://doi.org/10.3390/molecules23071794.

785 [42] S. Wang, Anaerobic Digestion of Poultry Processing Wastes for Bioenergy and
786 Nutrients Recovery, The University of Georgia, 2015.

787 [43] J.C. Frigon, C. Roy, S.R. Guiot, Anaerobic co-digestion of dairy manure with
788 mulched switchgrass for improvement of the methane yield, Bioprocess Biosyst.

789 Eng. 35 (2012) 341–349. https://doi.org/10.1007/s00449-011-0572-5.

790 [44] H. Niu, X. Kong, L. Li, Y. Sun, Z. Yuan, X. Zhou, Analysis of Biogas Produced
791 from Switchgrass by Anaerobic Digestion, BioResources. 10 (2015) 7178–7187.
792 https://doi.org/10.15376/biores.10.4.7178-7187.

793 [45] A. Banel, B. Zygmunt, Application of gas chromatography-mass spectrometry
794 preceded by solvent extraction to determine volatile fatty acids in wastewater of
795 municipal , animal farm and landfill origin Anna Banel and Bogdan Zygmunt,
796 (2011) 590–597. https://doi.org/10.2166/wst.2011.204.

797 [46] L. Jiunn-Jyi, L. Yu-You, T. Noike, Influences of pH and moisture content on the
798 methane production in high-solids sludge digestion, Water Res. 31 (1997) 1518–
799 1524. https://doi.org/10.1016/S0043-1354(96)00413-7.

800 [47] A. Donoso-Bravo, S.I. Pérez-Elvira, F. Fdz-Polanco, Application of simplified
801 models for anaerobic biodegradability tests. Evaluation of pre-treatment
802 processes, Chem. Eng. J. 160 (2010) 607–614.
803 https://doi.org/10.1016/j.cej.2010.03.082.

804 [48] G. Zhen, X. Lu, T. Kobayashi, G. Kumar, K. Xu, Anaerobic co-digestion on
805 improving methane production from mixed microalgae (Scenedesmus sp.,
806 Chlorella sp.) and food waste: Kinetic modeling and synergistic impact
807 evaluation, Chem. Eng. J. 299 (2016) 332–341.
808 https://doi.org/10.1016/j.cej.2016.04.118.

809 [49] G. Muyzer, T. Andreas, C.O. Wirsen, H.W. Jannasch, Phylogenetic relationships
810 of Thiomicrospira species and their identification in deep-sea hydrothermal vent

811 samples by denaturing gradient gel electrophoresis of 16S rDNA fragments, Arch
812 Microbiol. 164 (1995) 165–172.

813 [50] E. Sahinkaya, A. Kilic, B. Calimlioglu, Y. Toker, Simultaneous bioreduction of
814 nitrate and chromate using sulfur-based mixotrophic denitrification process, J.
815 Hazard. Mater. 262 (2013) 234–239.
816 https://doi.org/10.1016/j.jhazmat.2013.08.050.

817 [51] J.N. Himmelsbach, D.R. Raman, R.P. Anex, R.T. Burns, C.R. Faulhaber, Effect
818 of ammonia soaking pretreatment and enzyme addition on biochemical methane
819 potential of switchgrass, Trans. ASABE. 53 (2010) 1921–1927.

820 [52] Y. Li, R. Zhang, G. Liu, C. Chen, Y. He, X. Liu, Comparison of methane
821 production potential, biodegradability, and kinetics of different organic
822 substrates, Bioresour. Technol. 149 (2013) 565–569.
823 https://doi.org/10.1016/j.biortech.2013.09.063.

824 [53] L. Barbanti, G. Di Girolamo, M. Grigatti, L. Bertin, C. Ciavatta, Anaerobic
825 digestion of annual and multi-annual biomass crops, Ind. Crops Prod. 56 (2014)
826 137–144. https://doi.org/10.1016/j.indcrop.2014.03.002.

827 [54] D. Brown, B. Engineering, Comparison of Solid-State to Liquid Phase Anaerobic
828 Digestion of Lignocellulosic Biomass for Biogas Production, Bioresour. Technol.
829 124 (2012) 379–386.

830 [55] G. Jin, T. Bierma, Low-heat alkaline pretreatment of biomass for dairy anaerobic
831 codigestion, J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes.
832 49 (2014) 786–796. https://doi.org/10.1080/03601234.2014.929912.

[56] A.J. May, Cotreatment Enhanced Anaerobic Digestion of Lignocellulosic Biomass, The Pennsylvania State University, 2020.

[57] G. Tchobanoglous, F.L. Burton, H.D. Stensel, Metcalf & Eddy, Inc. Wastewater Engineering Teatment and Reuse, J. Wastewater Eng. (2003).

[58] S.H. Lee, J.H. Park, S.H. Kim, B.J. Yu, J.J. Yoon, H.D. Park, Evidence of syntrophic acetate oxidation by Spirochaetes during anaerobic methane production, Bioresour. Technol. 190 (2015) 543–549. https://doi.org/10.1016/j.biortech.2015.02.066.

[59] W. Suksong, P. Kongjan, P. Prasertsan, S. O-Thong, Thermotolerant cellulolytic Clostridiaceae and Lachnospiraceae rich consortium enhanced biogas production from oil palm empty fruit bunches by solid-state anaerobic digestion, Bioresour. Technol. 291 (2019) 121851. https://doi.org/10.1016/j.biortech.2019.121851.

[60] D. Karakashev, D.J. Batstone, E. Trably, I. Angelidaki, Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae, Appl. Environ. Microbiol. 72 (2006) 5138–5141. https://doi.org/10.1128/AEM.00489-06.

[61] X. Zuo, H. Yuan, A.C. Wachemo, X. Wang, L. Zhang, J. Li, H. Wen, J. Wang, X. Li, The relationships among sCOD, VFAs, microbial community, and biogas production during anaerobic digestion of rice straw pretreated with ammonia, Chinese J. Chem. Eng. 28 (2020) 286–292. https://doi.org/10.1016/j.cjche.2019.07.015.

[62] A.M. Ziganshin, J. Liebetrau, J. Pröter, S. Kleinsteuber, Microbial community

855 structure and dynamics during anaerobic digestion of various agricultural waste
856 materials, Appl. Microbiol. Biotechnol. 97 (2013) 5161–5174.
857 https://doi.org/10.1007/s00253-013-4867-0.

858 [63] S. Dyksma, L. Jansen, C. Gallert, Syntrophic acetate oxidation replaces
859 acetoclastic methanogenesis during thermophilic digestion of biowaste,
860 Microbiome. 8 (2020) 1–14. https://doi.org/10.1186/s40168-020-00862-5.

861 [64] A. Sikora, A. Detman, A. Chojnacka, M.K. Blaszczyk, Anaerobic Digestion: I. A
862 Common Process Ensuring Energy Flow and the Circulation of Matter in
863 Ecosystems. II. A Tool for the Production of Gaseous Biofuels, 2017.
864 https://doi.org/10.5772/64645.

865 [65] J.G. Ferry, Methanosarcina acetivorans: A Model for Mechanistic Understanding
866 of Aceticlastic and Reverse Methanogenesis, Front. Microbiol. 11 (2020) 1–12.
867 https://doi.org/10.3389/fmicb.2020.01806.

868 [66] M.S.M. Jetten, A.J.M. Stams, A.J.B. Zehnder, Methanogenesis from acetate: a
869 comparison of the acetate metabolism in Methanothrix soehngenii and
870 Methanosarcina spp., FEMS Microbiol. Lett. 88 (1992) 181–197.
871 https://doi.org/10.1016/0378-1097(92)90802-U.

872 [67] B.P. Lomans, R. Maas, R. Luderer, H.J.M. Op Den Camp, A. Pol, C. Van Der
873 Drift, G.D. Vogels, Isolation and characterization of Methanomethylovorans
874 hollandica gen. nov., sp. nov., isolated from freshwater sediment, a
875 methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol,
876 Appl. Environ. Microbiol. 65 (1999) 3641–3650.

https://doi.org/10.1128/aem.65.8.3641-3650.1999.

Graphical Abstract