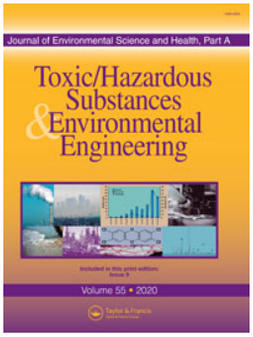

Taylor & Francis
Taylor & Francis Group

Journal of Environmental Science and Health, Part A
Toxic/Hazardous Substances and Environmental Engineering

ISSN: 1093-4529 (Print) 1532-4117 (Online) Journal homepage: https://www.tandfonline.com/loi/lesa20

Microbial community dynamics during anaerobic co-digestion of corn stover and swine manure at different solid content, carbon to nitrogen ratio and effluent volumetric percentages

Gail Joseph, Bo Zhang, Scott H. Harrison, Joseph L. Graves Jr., Misty D. Thomas, Renuka Panchagavi, Jude Akamu J. Ewunkem & Lijun Wang

To cite this article: Gail Joseph, Bo Zhang, Scott H. Harrison, Joseph L. Graves Jr., Misty D. Thomas, Renuka Panchagavi, Jude Akamu J. Ewunkem & Lijun Wang (2020) Microbial community dynamics during anaerobic co-digestion of corn stover and swine manure at different solid content, carbon to nitrogen ratio and effluent volumetric percentages, Journal of Environmental Science and Health, Part A, 55:9, 1111-1124, DOI: 10.1080/10934529.2020.1771975

To link to this article: https://doi.org/10.1080/10934529.2020.1771975

Published online: 27 May 2020.

JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART A
2020, VOL. 55, NO. 9, 1111–1124
https://doi.org/10.1080/10934529.2020.1771975

Microbial community dynamics during anaerobic co-digestion of corn stover and swine manure at different solid content, carbon to nitrogen ratio and effluent volumetric percentages

Gail Joseph[a], Bo Zhang[b], Scott H. Harrison[c], Joseph L. Graves , Jr.[d], Misty D. Thomas[c], Renuka Panchagavi[e], Jude Akamu J. Ewunkem[f], and Lijun Wang[b]

[a]Department of Energy and Environmental Systems, North Carolina Agricultural and Technical State University, Greensboro, North Carolina, USA; [b]Department of Natural Resources and Environmental Design, North Carolina Agricultural and Technical State University, Greensboro, North Carolina, USA; [c]Department of Biology, North Carolina A&T State University, Greensboro, North Carolina, USA; [d]Department of Nanoengineering, Joint School of Nanoscience & Nanoengineering, North Carolina A&T State University and UNC Greensboro, Greensboro, North Carolina, USA; [e]Dept of Computational Science and Engineering, North Carolina A&T State University, Greensboro, North Carolina, USA; [f]Department of Nanoscience, Joint School of Nanoscience & Nanoengineering, North Carolina A&T State University and UNC Greensboro, Greensboro, North Carolina, USA

ABSTRACT

The methane production and the microbial community dynamics of thermophilic anaerobic co-digestion (AD) of corn stover, swine manure and effluent were conducted at total solid (TS) content of 5%, 10% and 15%, the carbon to nitrogen ratio (C/N) of 20, 30 and 40 and the effluent volumetric percentage (EVP) of 20%, 40% and 60%. For batches with 5% TS, the highest methane yield of 238.5–283.1 mL g^{-1} volatile solid (VS) and the specific methane productivity of 138.5–152.2 mL g^{-1} initial VS were obtained at the C/N ratios of 20 and 30. For the mixtures with 10% and 15% TS, the highest methane yield was 341.9 mL g^{-1} VS and 351.2 mL g^{-1} VS, respectively, when the C/N ratio of 20% and 60% EVP conditions were maintained. Co-digestion of swine manure with corn stover caused an obvious shift in microbial population, in which the archaeal population changed from 0.3% to 2.8% and the bacterial community changed from 97.2% to 99.7%. The experimental batches with the highest relative abundance of the archaeal population (2.00% of total microbial population for 5% TS, 1.74% for 10% TS and 2.76% for 15% TS) had the highest rate of methanogenesis subsequently enhancing methane production (283.08 mL g^{-1} VS for 5% TS, 341.91 mL g^{-1} VS for 10% TS and 351.23 mL g^{-1} VS for 15% TS). The results of microbiome analysis enabled understanding the key populations in biomethane generation.

ARTICLE HISTORY
Received 7 March 2020
Accepted 15 May 2020

KEYWORDS
Anaerobic co-digestion; swine manure; corn stover; 16S metagenomic sequencing; anaerobic microbial community

Introduction

Anaerobic co-digestion (AD) converted wastes into energy and could be used as a solution for environmental pollution.[1,2] AD may be categorized as mono-digestion and co-digestion. Mono-digestion included the use of a single substrate that often caused nutrient imbalance resulting in lower biogas production.[3] AD included digestion of multiple substrates with complementary characteristics.[4] Co-digestion resulted in positive synergism such as balanced carbon to nitrogen (C/N) ratio, optimal pH value, and the percent dilution of toxic compounds and supplement of trace elements (TS%).[5–7] C/N ratios of 20/1 to 30/1 had reported to produce optimum biogas.[8] Low C/N ratios in animal manures were unable to satisfy the AD requirements. Hence, to conduct an effective AD, there should be another carbon-rich substrate to be co-digested with manure to compensate for its carbon deficiency and improve its characteristics for AD.[9] AD of swine manure with rice straw at a C/N ratio of 8, 20 and 30 showed that the methane production was low at C/N of 8 and 20 while batches with C/N of 30 showed stable methane production.[10] Corn stover was a low cost, abundant lignocellulosic biomass that had the potential for energy production via AD.[11]

Several factors had an effect on biomethane production such as the substrate type, operating temperature, pH and pre-treatment methods.[12] Most commercial anaerobic digesters ran at a mesophilic temperature between 20 and 40 °C.[13] There has been a growing interest in setting up anaerobic digesters at thermophilic temperatures (50–60 °C).[14] Thermophilic AD offered advantages over mesophilic AD such as decreased retention time and higher biogas production. When AD batches were run at TS% between 10% and 35%, the biomethane production

CONTACT Gail Joseph gailjoseph8@gmail.com Department of Energy and Environmental Systems, North Carolina Agricultural and Technical State University, 1601 East Market Street, Greensboro, NC 27411, USA; Lijun Wang lwang@ncat.edu Department of Natural Resources and Environmental Design, North Carolina Agricultural and Technical State University, 1601 East Market Street, Greensboro, NC 27411, USA.
This article has been republished with minor changes. These changes do not impact the academic content of the article.

decreased as the TS% increased from 10% to 25% and methane production was inhibited at TS of 35%.[15]

AD consisted of four steps, that is, hydrolysis, acidogenesis, acetogenesis and methanogenesis, in which specific groups of microorganisms were involved at each step.[16] Microorganisms play a crucial role in the digester, and the efficiency of AD depended on the activity of the microbial community.[17] The first three phases involved bacterial communities while the last step of methanogenesis involved archaeal populations.[18] Microbial diversity was best understood for the latter stage as archaea had low diversity and hence the change in the population could be easily ascertained. Due to the high diversity among the bacterial population, the identification of changes can be difficult,[19] and the presence or absence of certain groups of microbes could serve as a benchmark for either well-operating reactors or for process failure. Classes such as *Clostridia* (which degrades proteins and cellulose) and *Bacilli* (which degraded fat and carbohydrates) were shown to be indicators of effectively operating anaerobic digesters.[16,19,20] The *Methanosarcina* genus consisted of diverse group of archaea and the species could grow either by one of the four catabolic pathways – either by obligate CO_2 reduction with H_2 or acetoclastic fermentation of acetate or methylotrophic catabolism of methanol, methylated amines and dimethylsulfide, or methyl reduction with H_2.[21] However, the overall microbial communities in AD was not well understood because of the complexity of microbial communities and their metabolic pathways.[22] It was necessary to analyze this community to improve the efficiency of the process. Restricting the study to just culture-dependent techniques would give an incomplete picture of the microbial ecology and physiology associated with an AD process. Also, it would create a biased analysis as the environmental factors that could influence the comparative diversity and activities of different microbial communities would be obscured in favor of those differences ascribable to growth in the laboratory environment.[23] An accurate model of microbial community structure in the AD process will help identify the driving forces of diversity and metabolic capability. These findings will enable us to rationally design microbial communities to promote higher efficacy of an AD system.[19] In this study, we will also utilize next-generation sequencing, a high throughput sequencing process, to identify and characterize the microbial community structure.[24] The objective of this study, therefore, was to evaluate the AD performance with different TS content, C/N and EVP values. Corn stover, swine manure and effluent were anaerobically co-digested for 21 days at 55 °C. Effects of different TS%, C/N ratio and EVP on the biomethane yield were investigated. Next-generation sequencing was used to characterize the microbiome and the abundance of different microorganisms involved in biogas generation and process failure.

Material and methods

Feedstock and characterization

Fresh swine manure was collected from the North Carolina Agricultural and Technical State University (NCAT) swine research unit (Greensboro, NC, USA) on the day of the experiment and used on a wet basis. Corn stover was harvested from the University farm and dried in an Isotemp oven for a minimum of 24 h at 105 °C until a constant weight was achieved. Using a Thomas Model 4 Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) the dried corn stover was ground to a particle size ranging from 0.2 to 0.5 mm then stored in an airtight container at room temperature for future use. The effluent containing microorganisms essential for the AD process was collected from a previously running anaerobic digester.[25] This anaerobic digester digested swine manure with a solid content of 1–3 wt% under continuous agitation at 250 rpm at 55 °C. The effluent was collected in sterile glassware and transferred immediately to the laboratory from the farm and stored in a shaker at 55 °C. Fresh effluent was collected on the day of the experiment and the unused effluent was autoclaved at 121 °C for 30 min and then discarded.

Total solids (TS) and volatile solids (VS) were determined using the laboratory procedures (LAPs) developed by the National Renewable Energy Laboratory (NREL). The C/N ratio of all materials was calculated. The elemental analysis was conducted using a PE 2400 II CHNS/O analyzer (Perkin Elmer, Waltham, MA, USA).

AD of corn stover, swine manure and effluent

AD experiments were carried out with dried corn stover, swine manure and effluent in a 500 mL flask with a working volume of 300 mL. Water was added to adjust the TS content to 5%, 10% and 15%. The C/N ratio was controlled at levels of 20, 30 and 40, and the effluent volume to total volume percentage of 20%, 40% and 60%. The pH value was adjusted to 7.10 using 0.1 M potassium phosphate buffer as it was the optimum pH.[26] The AD experiments were carried out using the Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control, Sweden), which measures and records the biomethane potential on a daily basis.[27] After the addition of corn stover, swine manure and effluent the bottles were purged with mixed inert gas (80% nitrogen and 20% carbon dioxide) for 10 min to remove any oxygen present and create anaerobic conditions. All experiments were carried out in triplicate at 55 °C for a total period of 21 days. On day 21, the cumulative biomethane volume was recorded from the AMPTS II and the biogas composition was recorded using Biogas 5000 analyzer (Landtech North America, Dexter, MI). Samples of about 50 mL were drawn to check the pH. Another 50 mL from the digester flasks were transferred to a sterile centrifuge tube with 50% glycerol and stored at −80 °C for metagenomic analysis. Prior to AD, corn stover was stored in an oven at 105 °C for 24 h to avoid any contaminants or microorganism. All glassware was autoclaved and sterilized at 121 °C for 30 min before use.

Experimental design and data analysis

To study the effect of experimental variables on AD, a three-level full factorial design was applied. As shown in

Table 2, three factors of the TS content, the C/N ratio and the effluent volume to total volume percentage were considered, each at three levels. Therefore, a total of 27 experimental conditions was studied, and AD experiments were repeated three times under each condition. Experimentally, AD experiments directly yielded the following results, including cumulative CH_4 productivity (unit: mL), the weight loss of VS (unit: gram), pH and biogas composition. The removal ratio of VS (%), the specific methane productivity ($mL\,g^{-1}$ initial VS)[28] and the methane yield ($mL\,g^{-1}$ VS) were calculated via Eqs. (1)–(3), respectively

$$\text{The removal ratio of VS } (\%) = \frac{\text{Weight loss of VS}}{\text{Initial VS}} \times 100\% \quad (1)$$

$$\text{Specific methane productivity } (mLg^{-1}/\text{g initial VS}) = \frac{\text{Cumulative methane productivity}}{\text{Initial VS}} \quad (2)$$

$$\text{Methane yield } (mLg^{-1}/\text{g VS}) = \frac{\text{Cumulative methane productivity}}{\text{Weight loss of VS}} \quad (3)$$

Among these three results, the removal ratio of VS and the specific methane productivity were directly derived from the experimental results, while the methane yield was a combination of two experimental results and did not have a direct relationship with experimental variables. Regression analysis was used to statistically estimate the relationship between the removal ratio of VS or the specific methane productivity and experimental variables.[29] The experimental data were fitted to the second-degree polynomial Eq. (4)

$$Y = B_0 + \sum_{i=1-3}^{n} BiXi + \sum_{i<j}^{n} BijXiXj + \sum_{j=1-3}^{n} BjjXj^2 \quad (4)$$

where Y = removal ratio of VS or specific methane productivity, X_1 = effluent to total volume percentage, X_2 = carbon to nitrogen ratio, $X3$ = total solid percentage, B_0 = constant, Bi, Bij, Bjj = linear, interaction and quadratic coefficients, respectively.

The statistical significance of each item listed in Eq. (4) was assessed by using analysis of variance (ANOVA) based on $P \leq 0.05$.[30]

DNA extraction

DNA was isolated from the AD samples using Qiagen QIAamp® PowerFecal® Kit (Catalog No. 12830-50; Qiagen, Hilden, Germany) following the manufacturer's protocol. The extracted DNA was stored at −20 °C for downstream applications.

Library construction and 16S rRNA gene sequencing

The 16S Metagenomic Sequencing Library protocol (Illumina, San Diego, CA) was followed for library preparation. The 16S protocol was designed to amplify the V4 hypervariable region of the 16S ribosomal RNA gene (16S rRNA) using primer pair sequences 515F–806R purchased from Integrated Device Technology (San Jose, CA). The primers were modified from the original 515F–806F primer pair in the following way:

Forward (with 515F)

5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA

Reverse (with 806R)

5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT

The underlined regions were the locus-specific sequences, and the overhangs from the Illumina 16S protocol were not underlined. With the primer pair, the only V4 region was targeted and the expected final libraries were around 300–400 bp.[31]

In the first round of PCR, the 16S Locus was amplified in a 25 µL reaction from a 5 ng μL^{-1} template DNA for 25 cycles using 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a thermal cycler (Master Cycler gradient Eppendorf, USA) by following the 16S Metagenomic Sequencing Library protocol (Illumina, San Diego, CA). This protocol consisted of an initial denaturation step at 95 °C for 3 min, which was followed by 25 cycles of denaturation at 95 °C for the 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, and the final extension at 72 °C for 5 min. The products from the first PCR run served as a template for the second PCR run. In the second PCR protocol, the same protocol was used with a change in the annealing temperature set to 50 °C. The PCR products were purified with AMPure XP beads (Beckman Coulter, CA, USA) and the expected library size was in the range of 300–400 bp. The quality of the final library was verified using D1000 Screen Tape (Agilent Technologies, CA, USA) following the manufacturer's instruction. Library concentration was measured using the fluorometric quantification using the dsDNA binding dye. Purified amplicons were then pooled in equimolar concentrations and mixed with 20% PhiX control library. Sequencing was performed on an Illumina MiSeq (Illumina, San Diego, CA) using the MiSeq v3 reagent kit.

Bioinformatics analyses

At the end of the sequencing, the metadata mapping file was available in the FASTQ file format. The sequencing data were processed and analyzed using bioinformatics pipeline prepared in the Quantitative Insights into Microbial Ecology (QIIME and QIIME 2) software package.[32] The pipeline involved steps such as joining of the forward and reverse reads, checking mapping files for errors, demultiplexing and quality filtering sequences, and operational taxonomic units (OTUs) picking based on 97% sequence similarity. Operational Taxonomic Unit (OUT) picking was carried out by searching reads against the Greengenes database.[33] OTU picking strategy used was closed reference. Bray–Curtis index was used as a metric for Beta-diversity. The alignment was carried out using the UCLUST algorithm. The Greengenes reference taxonomy that was used for the analysis was the August 2013 release of gg_13_8_99 and

Table 1. Characteristics of swine manure, corn stover and effluent.

Sample name	Swine manure[a]	Corn stover[a]	Effluent[a]
TS[b] (wt%)	51.11 ± 0.7	94.56 ± 3.0	0.20 ± 0.6
VS[c] (wt%, dry basis)	78 ± 0.4	93.90 ± 0.7	58.5 ± 1.0
Carbon %	38.19 ± 0.92	47.36 ± 0.7	66.25 ± 6.0
Nitrogen %	3.15 ± 0.74	0.57 ± 0.01	2.17 ± 0.2
C/N ratio[d]	12.12	83.09	30.53

[a]Values are means ± standard deviation ($n = 3$).
[b]TS = total solid.
[c]VS = volatile solids.
[d]C/N = carbon to nitrogen ratio.

contained 202,421 bacterial and archaeal sequences. The archaeal and bacterial taxonomy was assigned from phylum to genus level.

Results and discussion

Cumulative biomethane production

The physical and chemical characteristics of swine manure, corn stover and effluent were as mentioned in Table 1.

Corn stover was anaerobically co-digested with swine manure and effluent for a total period of 21 days at 55 °C. A total of 27 batches were run altering the TS, C/N and EVP ratio. The set of TS values of 5%, 10% and 15% was chosen because previous studies had reported a decline in methane production when the TS% was increased to more than 15%.[34,35]] For TS 5%, 10% and 15%, the maximum cumulative methane yield was 2441.6, 4808.05, and 5663.6 N mL, respectively and are summarized in Table 2. The VS removal ratios of 5%, 10% and 15% batches were 36.2–58.2%, 23.5–45% and 6–33.8%, respectively.

We observed that the highest methane yield for AD batches with TS of 5% was in the range of 238–283 mL g^{-1} VS, while the specific methane productivity was 138–152 mL g^{-1} initial VS. The EVP of 20%, 40% and 60% did not have a noticeable effect on methane production. The batches with C/N ratio of 30 showed a small increase in biomethane volume on comparison to the C/N of 20 and 40. The batches with C/N 40 produced low biomethane and were considered as failed AD batches. Out of the nine batches run at TS of 10%, the batches with C/N ratio of 20 showed a methane yield in the range of 306–342 mL g^{-1} VS, while batches with C/N 30 and 40 showed lower methane production. For TS 10%, the batch with C/N 20 and EVP of 60 produced the highest methane volume (341.91 mL g^{-1} VS) and the highest specific methane productivity (153 mL g^{-1} initial VS). At 15% TS, only the batches with the C/N ratio of 20 produced biomethane while batches with C/N ratio of 30 and 40 produced lower biomethane. The highest methane yield of 351.23 mL g^{-1} VS was obtained from the batch with C/N 20, EVP 60% and TS 15%.

The optimum pH for AD was between 6.4 and 7.6.[36] A stable pH played a crucial role in anaerobic digesters. In the current study, all the experimental batches had the initial pH adjusted to 7.1 on day 0 of the experiment. The pH was measured at the end of the experiments (day 21) and the batches that had the lowest biomethane production yield showed an acidic pH of nearly 5.0. Batches with TS content of 5% with a C/N ratio of 40 showed an acidic pH (~5.0). Batches with TS 10% showed a pH between 5.4 and 5.5 when C/N ratio was 30 and 40. These batches exhibited the lowest yield of biomethane as well. A similar trend was noticed with batches with TS 15% and C/N 30 or 40, which showed an acidic pH and no methane yield.

The biogas composition was determined using the Biogas analyzer. Batches with TS of 5% with C/N of 20 and 30 had the methane content higher than 60 vol%. Batches with TS 10% and 15% showed a methane yield of more than 60% vol only when the C/N ratio was at 20. For all AD batches with C/N of 40 had the H_2S value of more than 5000 ppm and that led to low or no methane production. Swine manure was one of the main sources for the accumulation of H_2S and improper balance of C/N ratio could have led to high volume of H_2S. The presence of oxygen in the batches could be due to the addition of corn stover, swine manure and effluent to the bottle in the presence of air. However, the bottles were purged with inert gas to get rid of the oxygen, it could be possible that some oxygen maybe trapped in. The CO_2 was in the range of 8.7–45% (Table 2).

Regression analysis

The regression analysis was applied to study the relationships between the dependent variables (the removal ratio of VS and the specific methane productivity) and three independent variables (TS%, C/N ratio and EVP). After statistical regression analysis of the experimental data, two second-order polynomial Eqs. (5) and (6) were obtained

$$\begin{aligned}\text{Removal ratio of VS } (Y,\ \text{unit}:\ \%) &= 116.6572 \\ &+0.2426X_1 - 2.8798X_2 - 3.8592X_3 - 0.0054X_1X_2 \\ &-0.0129X_1X_3 - 0.038X_2X_3 + 0.001{X_1}^2 + 0.04171{X_2}^2 \\ &+0.1080{X_3}^2 \end{aligned} \quad (5)$$

For the removal ratio of VS, R^2 and adjusted R^2 values of 0.938 and 0.905 were obtained, respectively. Therefore, the variance of the equation's errors was at least 90.5% less than the variance of the dependent variable.[37] The F value of regression was 1.52E − 08, which indicated that this equation was statistically significant. ANOVA tables, the residual output, the probability output and the normal probability plot are provided in Appendix A. The comparison between predicted values and experimental values is shown in Figure 1(a). The P values for the EVP (X_1), C/N ratio (X_2), TS% (X_3), X_1X_2, X_1X_3, X_2X_3, ${X_1}^2$, $X_2$2 and ${X_3}^2$ were 0.639, 0.048, 0.0749, 0.4829, 0.4016, 0.2239, 0.8525, 0.0666 and 0.2215, respectively. If a statistical significance level cutoff was chosen as $P = 0.05$, it was concluded that the effect of the C/N ratio (X_2) was statistically significant

$$\begin{aligned}\text{Specific methane productivity } (Y,\ \text{unit}: \text{mL/g initial VS}) &= 551.0281 - 0.3314X_1 - 16.7711X_2 - 25.969X_3 \\ &-0.00272X_1X_2 + 0.007826X_1X_3 + 0.10652X_2X_3 \\ &+0.004688{X_1}^2 + 0.1597{X_2}^2 + 0.8174{X_3}^2 \end{aligned} \quad (6)$$

For the specific methane productivity, R^2 and adjusted R^2 values of 0.8065 and 0.704 were obtained, respectively. So, the standard deviation of the errors was 70.4% less than the

Table 2. Biomethane production and biogas composition for all the anaerobic digestion batches on day 21.

AD no.	TS%	C/N	EVP %	Cumulative methane yield (N mL)[a]	Specific methane productivity (mL g^{-1} initial VS)	Removal ratio of VS (%)	Methane yield (mL g^{-1} VS)[a]	pH[a]	CH_4%	CO_2 %	O_2%	H_2S ppm	Balance
1	5	20	20	2402.23 ± 75	150.69 ± 4.6	55.67	270.670 ± 8.4	7.4 ± 0.5	64.2 ± 1.9	24.4 ± 0.7	0.4 ± 0.4	170 ± 1.0	11.0 ± 7.6
2	5	30	20	2441.55 ± 182	147.64 ± 11.1	52.16	283.084 ± 21.1	7.2 ± 0.2	61.0 ± 2.6	21.3 ± 4.3	0.6 ± 0.2	133 ± 96	17.1 ± 0.6
3	5	40	20	193.93 ± 7	11.51 ± 0.4	43.01	26.752 ± 1.0	5.4 ± 0.1	9.0 ± 10.0	14.4 ± 5.4	11.9 ± 1.0	>5000	64.7 ± 9.7
4	5	20	40	2262.43 ± 291	148.02 ± 18.1	57.25	258.560 ± 33.3	7.5 ± 0.3	60.5 ± 6.4	21.0 ± 1.3	0.5 ± 0.0	207 ± 84	15.0 ± 8.5
5	5	30	40	2419.30 ± 198	152.22 ± 12.4	56.63	268.811 ± 22.0	7.6 ± 0.2	60.5 ± 3.2	30.6 ± 0.4	0.2 ± 0.3	125 ± 76	8.7 ± 7.4
6	5	40	40	214.03 ± 21	13.19 ± 1.3	36.22	36.426 ± 3.6	5.5 ± 0.5	12.2 ± 5.0	13.3 ± 3.6	9.1 ± 5.1	>5000	65.4 ± 3.0
7	5	20	60	2027.67 ± 189	138.59 ± 12.9	58.10	238.553 ± 22.3	7.5 ± 0.1	63.7 ± 5.1	23.2 ± 6.8	0.6 ± 0.2	188 ± 64	12.6 ± 4.8
8	5	30	60	2197.20 ± 114	144.07 ± 7.8	56.55	254.748 ± 13.3	7.5 ± 0.3	64.9 ± 4.8	27.4 ± 0.9	0.9 ± 0.4	211 ± 75	6.8 ± 2.9
9	5	40	60	288.87 ± 22	18.54 ± 1.4	41.71	44.446 ± 3.4	5.3 ± 0.1	20.7 ± 14.7	8.7 ± 12.1	10.9 ± 2.0	>5000	59.7 ± 0.6
10	10	20	20	4323.53 ± 83	132.87 ± 3.1	39.18	339.098 ± 6.5	7.6 ± 0.7	68.0 ± 0.5	29.2 ± 0.8	0.0 ± 0.1	242 ± 101	2.8 ± 1.2
11	10	30	20	297.02 ± 19	8.81 ± 1.1	23.78	37.04 ± 2.5	5.4 ± 0.1	17.7 ± 20.1	13.5 ± 14.3	14.8 ± 3.0	>5000	54.0 ± 16.8
12	10	40	20	283.52 ± 16	8.25 ± 0.9	23.51	35.11 ± 2.0	5.4 ± 0.5	10.9 ± 13.1	19.9 ± 0.9	6.3 ± 1.3	>5000	62.9 ± 11.0
13	10	20	40	4134.35 ± 263	129.67 ± 12.4	42.34	306.248 ± 19.5	7.5 ± 0.8	65.0 ± 5.0	31.6 ± 7.4	0.2 ± 0.4	267 ± 91	3.2 ± 1.6
14	10	30	40	288.10 ± 10	8.71 ± 0.7	25.95	33.565 ± 1.2	5.5 ± 0.1	10.2 ± 4.8	17.3 ± 3.2	9.6 ± 1.0	>5000	62.9 ± 5.1
15	10	40	40	166.32 ± 25	4.93 ± 1.6	24.85	19.851 ± 3.0	5.3 ± 0.3	15.5 ± 3.9	44.9 ± 2.0	4.4 ± 6.8	>5000	35.2 ± 3.6
16	10	20	60	4808.13 ± 319	153.97 ± 10.2	45.03	341.906 ± 22.7	7.6 ± 0.1	66.9 ± 3.9	29.0 ± 1.7	0.0 ± 0.7	307 ± 23	4.1 ± 3.0
17	10	30	60	381.23 ± 10	11.75 ± 0.3	31.22	37.649 ± 1.0	5.4 ± 0.2	11.5 ± 0.5	13.9 ± 0.9	10.0 ± 3.8	>5000	64.7 ± 3.1
18	10	40	60	217.55 ± 18	6.58 ± 0.5	26.08	25.223 ± 2.1	5.3 ± 0.3	10.8 ± 3.0	14.9 ± 1.5	8.2 ± 2.2	>5000	66.1 ± 3.4
19	15	20	20	5653.37 ± 294	115.05 ± 5.9	33.15	347.088 ± 18.4	7.4 ± 0.1	67.0 ± 4.2	28.7 ± 2.3	0.3 ± 0.5	189 ± 56	4.0 ± 0.3
20	15	30	20	396.20 ± 8	7.78 ± 0.3	11.17	69.679 ± 1.5	5.4 ± 0.7	19.4 ± 9.8	23.7 ± 10.2	6.7 ± 1.0	>5000	50.3 ± 10.1
21	15	40	20	204.60 ± 37	3.95 ± 0.9	12.06	32.736 ± 6.0	5.4 ± 0.6	3.0 ± 20.5	4.9 ± 14.3	8.4 ± 1.3	>5000	83.7 ± 19.0
22	15	20	40	5298.93 ± 276	109.30 ± 5.2	33.78	323.597 ± 16.9	7.6 ± 0.2	69.0 ± 2.4	26.1 ± 0.6	0.0 ± 0.1	355 ± 89	4.9 ± 1.2
23	15	30	40	333.22 ± 38	6.63 ± 0.8	9.70	68.349 ± 7.8	5.7 ± 0.8	10.1 ± 6.7	17.1 ± 0.9	11.7 ± 2.6	>5000	61.1 ± 3.4
24	15	40	40	217.43 ± 29	4.25 ± 0.5	9.03	47.005 ± 6.4	5.6 ± 0.4	1.4 ± 11.9	9.9 ± 8.4	13.8 ± 4.8	>5000	74.9 ± 7.4
25	15	20	60	5663.69 ± 194	118.42 ± 3.7	33.72	351.231 ± 12.0	7.3 ± 0.1	71.0 ± 0.3	27.0 ± 1.0	0.0 ± 0.2	235 ± 122	2.0 ± 0.5
26	15	30	60	232.26 ± 31	4.68 ± 0.6	5.97	78.414 ± 10.6	5.2 ± 0.3	20.3 ± 6.1	18.5 ± 6.0	9.0 ± 1.7	>5000	52.2 ± 5.5
27	15	40	60	224.30 ± 29	4.44 ± 0.5	6.67	66.459 ± 8.6	5.3 ± 0.4	9.6 ± 0.9	7.9 ± 8.2	15.1 ± 3.8	>5000	67.4 ± 4.2

[a]Values are means ± standard deviation ($n = 3$).

variance of the dependent variable. The F value of regression was 0.000154, so this equation was statistically significant. ANOVA tables, the residual output, the probability output and the normal probability plot are provided in Appendix A. The comparison between predicted values and experimental values is shown in Figure 1(b). The P values for the EVP (X_1), C/N ratio (X_2), TS% (X_3), X_1X_2, X_1X_3, X_2X_3, X_1^2, $X_2$2 and $X_3$2 were 0.9255, 0.0888, 0.0807, 0.9586, 0.9405, 0.6133, 0.8995, 0.29 and 0.1804, respectively. In this case, none of independent variables was statistically significant.

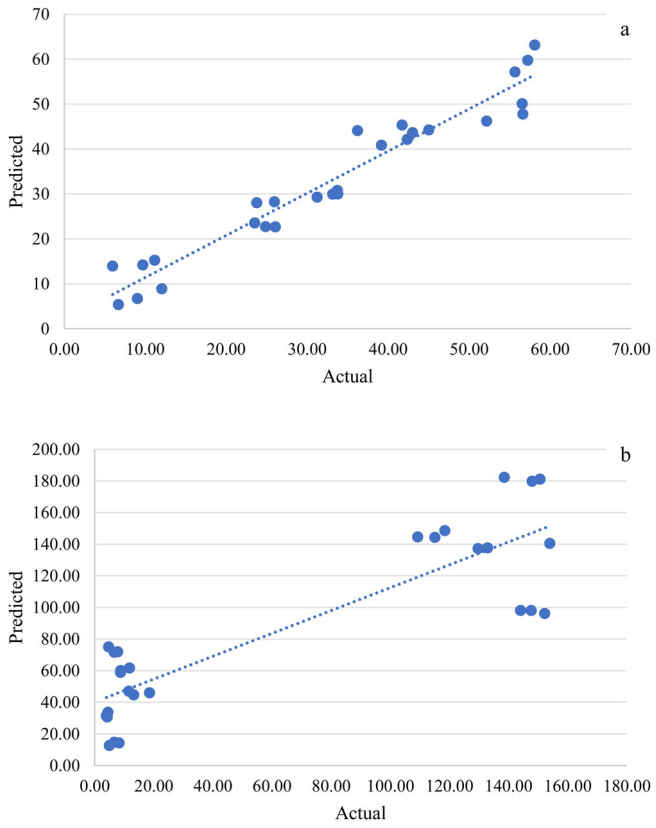

Figure 1. The comparison between the predicated values and the experimental values (a) the removal ratio of VS, (b) the specific methane productivity.

Dynamics of microbial communities

Microbial data analysis

The extracted DNA was quantified using the fluorometer and was in the range of 0.5–83 μg μL^{-1}. The extracted DNA was used for library preparation. The size of the library was determined using the Agilent 2200 Tapestation system. The size of the pooled libraries was in the range of 300–400 bp, which was the expected size since the primers target the V4 region only. Using the MiSeq reagent kit v3 and the amplicon DNA, full-length reads of V4 region was obtained in a single run at the end of approximately 65 h. The output of the MiSeq run was more than 20 million reads and generated more than 100,000 reads for each sample, which was enough size for data analysis. The MiSeq reagent kit v3 had the ability to double the amount of output per flow cell. Total of 27 batches was anaerobically digested. The MiSeq reagent kit v3 (600 cycles) can accommodate only 24 samples (Illumina). All batches with C/N of 40 produced low biomethane, hence only three of those batches at different TS % were considered to study the microbial community structure that caused the process failure. Total of 21 samples was selected out of the 27 batches and one batch with fresh swine manure and the other with effluent were analyzed as controls. AD process involved two groups of microorganisms – bacteria and archaea as shown in Table 3.

The control batch of effluent showed a balanced profile of the bacterial community, which consisted of Euryarchaea (14.3%), Firmicutes (14.3%), Thermotogae (14.3%), Proteobacteria (14.3%), Bacteroidetes (14.3%) and Spirochetes (28.5%). Euryarchaea phylum belonged to archaeal domain, while phyla of Firmicutes, Thermotogae, Proteobacteria, Bacteroidetes, and Spirochetes belonged to bacterial domain. Co-digestion of swine manure with corn

Table 3. Bacterial and Archaeal community structure in the AD batches, swine manure, and effluent on day 21

AD No.	TS%	CN Ratio	EVP&	Cumulative Methane Yield (N mL)	Bacteria %	Archaea %
1	5	20	20	2402.23 ± 75	99.198	0.802
2	5	30	20	2441.55 ± 182	98.000	2.000
4	5	20	40	2262.43 ± 291	99.441	0.559
5	5	30	40	2419.30 ± 198	98.140	1.860
6	5	40	40	214.03 ± 21	100.000	0.000
7	5	20	60	2027.67 ± 189	99.083	0.917
8	5	30	60	2197.20 ± 114	99.621	0.379
10	10	20	20	4323.53 ± 83	99.140	0.860
11	10	30	20	297.02 ± 19	100.000	0.000
13	10	20	40	4134.35 ± 263	99.437	0.563
14	10	30	40	288.10 ± 10	100.000	0.000
15	10	40	40	166.32 ± 25	100.000	0.000
16	10	20	60	4808.13 ± 319	98.261	1.739
17	10	30	60	381.23 ± 10	100.000	0.000
19	15	20	20	5653.37 ± 294	99.457	0.543
20	15	30	20	396.20 ± 8	100.000	0.000
22	15	20	40	5298.93 ± 276	98.611	1.389
23	15	30	40	333.22 ± 38	100.000	0.000
24	15	40	40	217.43 ± 29	100.000	0.000
25	15	20	60	5663.69 ± 194	97.205	2.795
26	15	30	60	232.26 ± 31	100.000	0.000
Effluent	NA	NA	NA	NA	85.714	14.286
Swine Manure	NA	NA	NA	NA	99.680	0.320

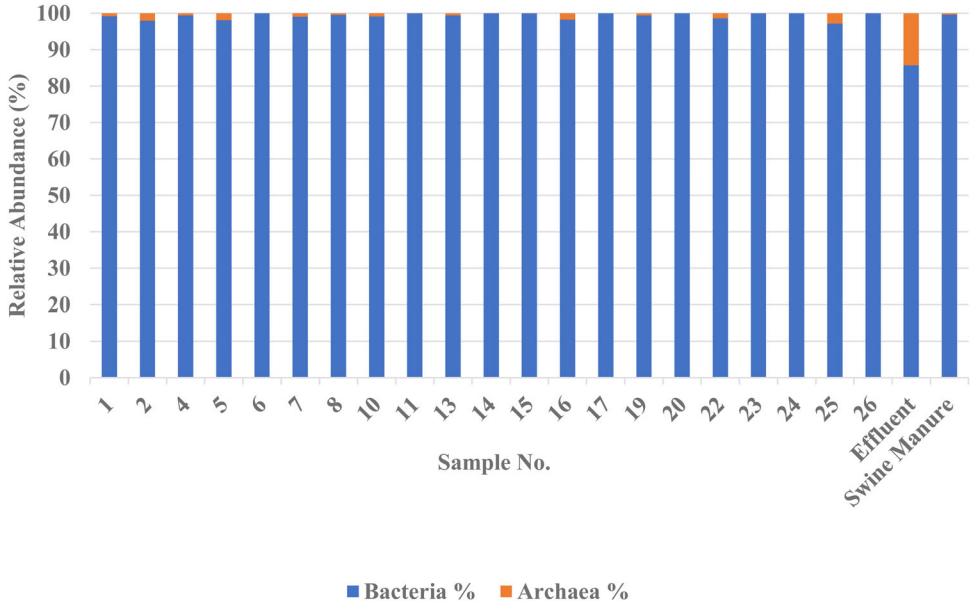

Figure 2. Bacterial and archaeal community structure of anaerobic digestion batches 1–26 along with effluent and swine manure.

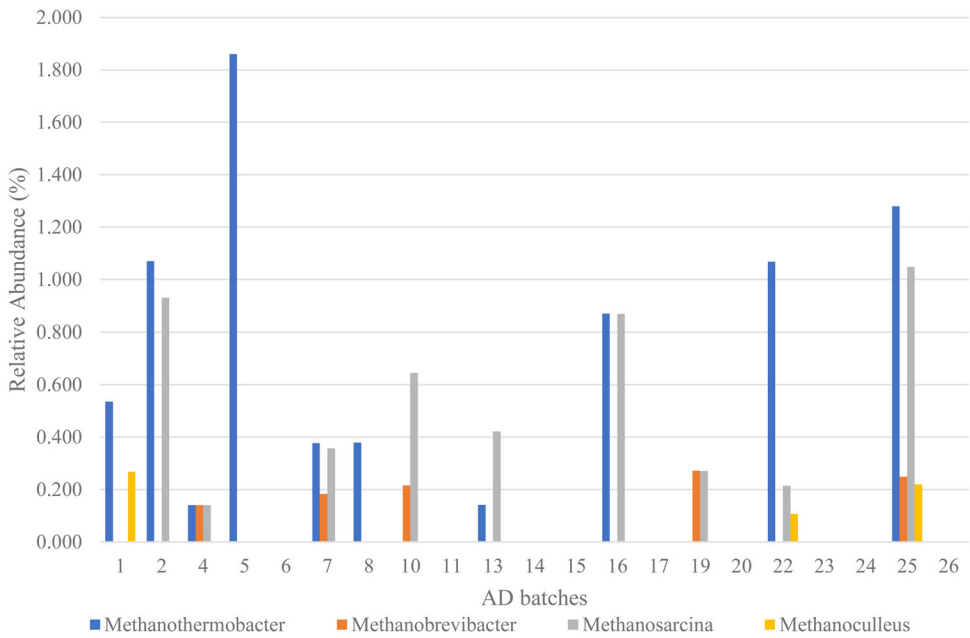

Figure 3. Comprehensive archaeal community structure at the genus level from all the anaerobic digestion batches.

stover caused an obvious shift in population, in which the archaeal population was reduced to 0.3–2.8% and the bacterial community was 97.2–99.7% (Figure 2). These results were consistent with previous studies.[38]

Archaeal community structure

The archaeal group consisted of methane producing microbes.[39] At the pH range of 5.0–7.0, the VFA was present in a non-dissociated form that could be lethal to the archaeal microbes.[40] In this study, the highest archaeal population was observed when the TS was 15%, C/N 20 and EVP 60%. For TS 5% and TS 10%, the highest archaeal population was observed when the C/N was 30 and 20, respectively. These results agreed with the biomethane production results as the higher relative abundance of the archaeal population could lead to a higher rate of methanogenesis. The batches with lower biomethane production showed a low presence or complete absence of the archaeal population. Phylum *Euryarchaeota* was the most abundant among the archaeal phyla with the relative abundance of close to 99%. Previous studies had reported the Euryarchaeota population between 50.4% and 87.3% of the total archaeal sequences.[41] The remaining 1% consisted of Phylum *Crenarchaeota*. Phylum *Euryarchaeta* consisted of two classes – *Methanobacteria* and *Methanomicrobia* while Phylum *Crenarchaeota* consisted of one class which remained unclassified.

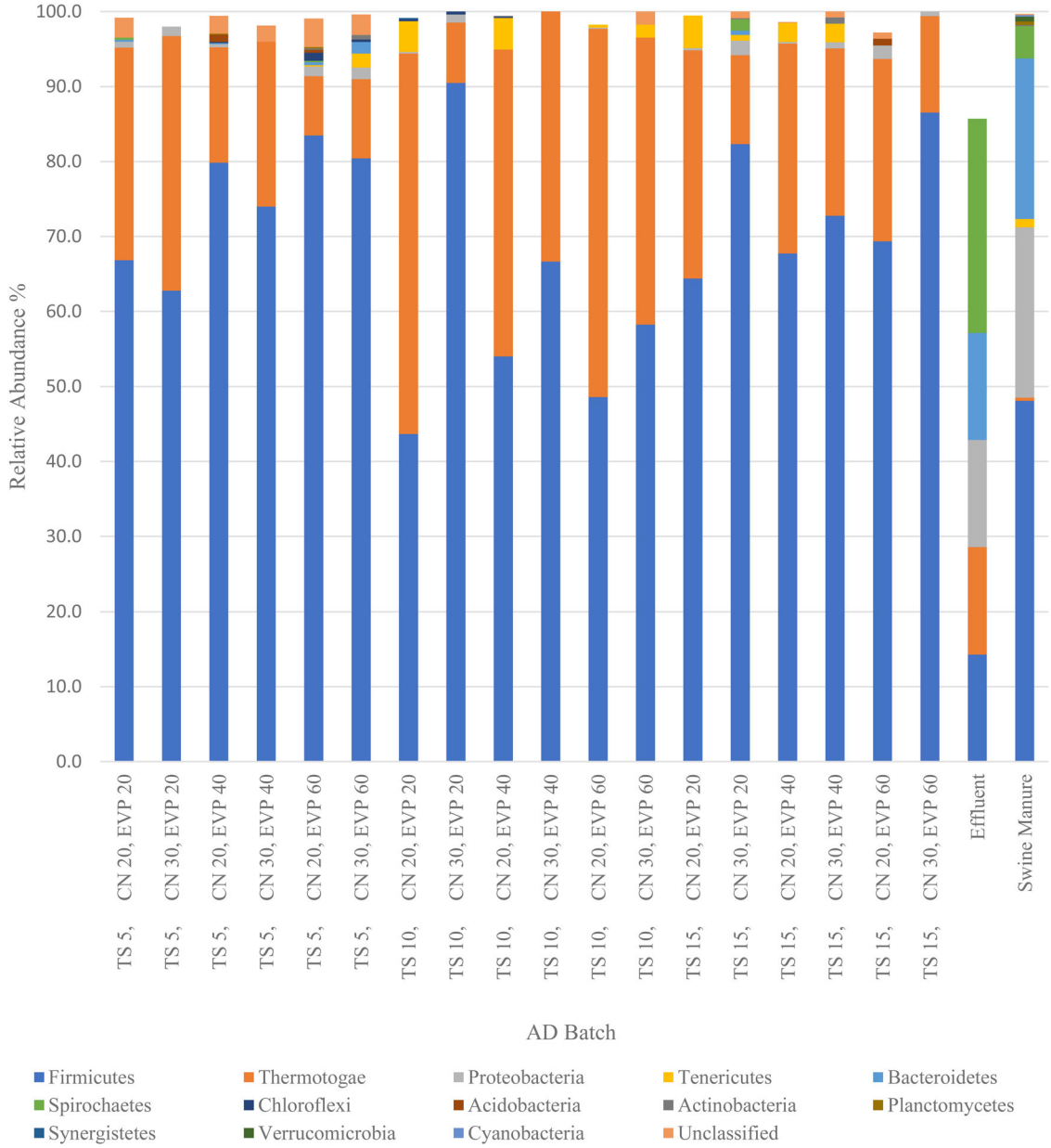

Figure 4. Taxonomic composition of bacterial communities at phyla level for anaerobic digestion batches along with swine manure and effluent.

The relative abundance of each genus in all the samples were calculated. The genus belonging to Phylum *Crenarchaeota* showed a relative abundance of less than 0.1%. Phylum *Euryarchaeta* consisted of genus *Methanothermobacter, Methanobrevibacter, Methanosarcina* and *Methanoculleus.* Methanogens belonging to genus *Methanothermobacter*, belong to the family *Methanobacteriaceae,* was thermophilic and had an optimum growth temperature between 55 °C and 65 °C and use CO_2 and H_2 as substrates for the production of methane.[42] *Methanobrevibacter* was strict anaerobic archaea that produced methane through reduction of carbon dioxide and hydrogen.[43] *Methanosarcina* was both acetoclastic (acetate utilizing) and hydrogenotrophic (hydrogen utilizing).[44] *Methanoculleus* was hydrogenotrophic methanogens.[41] *Methanothermobacter* was the most abundant genus (50% of the total archaeal population) followed by *Methanosarcina* with 35% of the total archaeal population (Figure 3). Batches 6, 11, 14, 15, 17, 20, 23, 24 and 26 did not show the presence of methanogens in the digester. The absence of a certain group of the microbial population could be due to the loss of species stratification and decrease in the evolution of new communities.[44]

Bacterial community structure

AD involved hydrolysis and acidification process that involves a large number of bacterial populations.[45] The taxonomic composition of the bacterial communities at the phyla level is shown in Figure 4. Thirteen major phyla were identified from all co-digestion batches. For all failed batches, *Firmicutes* was the most dominant phylum. In order to produce a fair amount of biogas, it seemed that the abundance of *Thermotogae* was required. Phylum such as

Proteobacteria, Tenericutes, Bacteroidetes, Spirochetes, Chloroflexi, Acidobacteria and *Actinobacteria* was not critical to a healthy AD of swine manure and corn stover.

Firmicutes were acetogenic and syntrophic bacteria that can degrade VFA. The predominance of bacteria belonging to Phylum *Firmicutes* proposed that it played a critical role in biomethane production.[45] *Firmicutes* participated in the acidogenesis phase of AD and produced H_2 and CO_2 throughout fermentation. *Firmicutes* had the ability to tolerate unfavorable environments and produced precursors for methanogenesis process.[41] *Firmicutes* produced extracellular enzymes such as cellulase, lipase and protease that metabolized cellulose, protein, lignin and lipids. Published work by other researchers had also reported that *Firmicutes* was the most abundant phyla.[46]

Class *Clostridia* belonging to *Firmicutes* was the most abundant class in all the AD batches. This could be caused by the cellulose degrading ability of *Clostridia* that could have led to the decomposition of cellulose, chitin and starch.[41,47] Bacteria belonging to Phylum *Thermotogae* was gram negative, thermophilic anaerobes and was capable of utilizing a great variety of carbohydrates and generating hydrogen.[48] Bacteria belonging to *Thermotogae* had the ability to metabolize organic substrates effectively and produced H_2 gas as a by-product. The bacteria also possessed thermostable enzymes that had gained importance for different biotechnological applications.[49]

Phylum *Proteobacteria* was not dominant in the study but it was present in the batches that produced methane. Phylum *Proteobacteria* was involved in the degradation of organic waste into propionate, butyrate and acetate.[41] *Spirochetes* fermented the carbohydrates or amino acids into acetate, H_2 and CO_2 and *Tenericutes* had reported to utilize lignin.[46] Both the phyla were present in the batches with a relative abundance between 0.1% and 4.4%.

Conclusion

Corn stover, swine manure and effluent were anaerobically co-digested for 21 days at 55 °C with varying TS (5–15 wt%), C/N (20–40) and EVP ratio (20–60 vol%). The VS removal ratios of 5%, 10% and 15% batches were 36.2–58.2%, 23.5–45% and 6–33.8%, respectively. The batches of TS 5% with a C/N ratio of 20 or 30 resulted in higher biomethane production as compared to that of C/N 40. For TS 10%, the batch with C/N 20 and EVP of 60% produced the highest methane volume, methane yield and specific methane productivity (4808.13 N mL, 341.9 mL g^{-1} VS and 153.9 mL g^{-1} initial VS). In general, TS 10% batches with C/N ratio 20 showed higher methane yield than batches with C/N 30 and 40. Batches of TS 15% showed a similar low C/N requirement. The highest methane yield of 351.2 mL g^{-1} VS was obtained from the batch with C/N 20, EVP 60% and TS 15%, while the batches with C/N 40 produced low biomethane and were considered as failed batches. The results of the VS removal ratios and the specific methane productivity were fit into two second-order polynomial equations which gave R^2 values of 0.938 and 0.8065, respectively.

The diversity of the bacterial and archaeal communities had a direct correlation with the digester performance and biomethane production. The archaeal community was in the range of 0.3–2.8% of the total microbial community. The highest archaeal population was observed when the TS was 15%, C/N 20 and EVP 60% which also had the highest biomethane production. For TS 5% and TS 10%, the highest relative number of archaea was observed when the C/N was 30 and 20, respectively. The results of microbiome analysis enabled understanding the key populations in biomethane generation

Acknowledgment

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIFA.

Funding

The publication was made possible by the United States Department of Agriculture (USDA NIFA award: NC.X-314-5-18-130-1).

ORCID

Gail Joseph http://orcid.org/0000-0002-8720-6460

Reference

[1] Joseph, G.; Zhang, B.; Mahzabin Rahman, Q.; Wang, L.; Shahbazi, A. Two-sStage Thermophilic Anaerobic co-Digestion of Corn Stover and Cattle Manure to Enhance Biomethane Production. *J. Environ. Sci. Health, Part A* **2019**, *54*, 452–459. DOI: 10.1080/10934529.2019.1567156.

[2] Xu, R.; Zhang, K.; Liu, P.; Khan, A.; Xiong, J.; Tian, F.; Li, X. A Critical Review on the Interaction of Substrate Nutrient Balance and Microbial Community Structure and Function in Anaerobic co-Digestion. *Bioresour. Technol.* **2018**a, *247*, 1119–1127. DOI: 10.1016/j.biortech.2017.09.095.

[3] Cardona, L.; Levrard, C.; Guenne, A.; Chapleur, O.; Mazéas, L. Co-Digestion of Wastewater Sludge: Choosing the Optimal Blend. *Waste Manag.* **2019**, *87*, 772–781. DOI: 10.1016/j.wasman.2019.03.016.

[4] El-Mashad, H. M.; Zhang, R. Biogas Production from Co-Digestion of Dairy Manure and Food Waste. *Bioresour. Technol.* **2010**, *101*, 4021–4028. DOI: 10.1016/j.biortech.2010.01.027.

[5] Esposito, G.; Frunzo, L.; Giordano, A.; Liotta, F.; Panico, A.; Pirozzi, F. Anaerobic Co-Digestion of Organic Wastes. *Rev. Environ. Sci. Biotechnol.* **2012**, *11*, 325–341. DOI: 10.1007/s11157-012-9277-8.

[6] Fernández, A.; Sanchez, A.; Font, X. J. B. E. J. Anaerobic Co-Digestion of a Simulated Organic Fraction of Municipal Solid Wastes and Fats of Animal and Vegetable Origin. *Biochem. Eng. J.* **2005**, *26*, 22–28. DOI: 10.1016/j.bej.2005.02.018.

[7] Davidsson, A.; Lövstedt, C.; Jansen, J. l C.; Gruvberger, C.; Aspegren, H. Co-Digestion of Grease Trap Sludge and Sewage Sludge. *Waste Manag.* **2008**, *28*, 986–992. DOI: 10.1016/j.wasman.2007.03.024.

[8] Velásquez Piñas, J. A.; Venturini, O. J.; Silva Lora, E. E.; Calle Roalcaba, O. D. Technical Assessment of Mono-Digestion and Co-Digestion Systems for the Production of Biogas from Anaerobic Digestion in Brazil. *Renew. Energy* **2018**, *117*, 447–458. DOI: 10.1016/j.renene.2017.10.085.

[9] Neshat, S. A.; Mohammadi, M.; Najafpour, G. D.; Lahijani, P. Anaerobic Co-Digestion of Animal Manures and Lignocellulosic Residues as a Potent Approach for Sustainable Biogas Production. *Renew. Sust. Energy Rev.* **2017**, *79*, 308–322. DOI: 10.1016/j.rser.2017.05.137.

[10] Riya, S.; Suzuki, K.; Terada, A.; Hosomi, M.; Zhou, S. Influence of C/N Ratio on Performance and Microbial Community Structure of Dry-Thermophilic Anaerobic Co-Digestion of Swine Manure and Rice Straw. *JOMB.* **2016**, *5*, 11–14. DOI: 10.12720/jomb.5.1.11-14.

[11] Katsimpouras, C.; Christakopoulos, P.; Topakas, E. Acetic Acid-Catalyzed Hydrothermal Pretreatment of Corn Stover for the Production of Bioethanol at High-Solids Content. *Bioprocess Biosyst. Eng.* **2016**, *39*, 1415–1423. DOI: 10.1007/s00449-016-1618-5.

[12] Civelek Yoruklu, H.; Korkmaz, E.; Manav Demir, N.; Ozkaya, B.; Demir, A. The Impact of Pretreatment and Inoculum to Substrate Ratio on Methane Potential of Organic Wastes from Various Origins. *J. Mater. Cycles Waste* Manag. **2018**, *20*, 800–809. DOI: 10.1007/s10163-017-0641-1.

[13] Mézes, L.; Bai, A.; Nagy, D.; Cinka, I.; Gabnai, Z. Optimization of Raw Material Composition in an Agricultural Biogas Plant. *Tr. Ren. Energy* **2017**, *3*, 61–75. DOI: 10.17737/tre.2017.3.1.0031.

[14] Gavala, H. N.; Yenal, U.; Skiadas, I. V.; Westermann, P.; Ahring, B. K. Mesophilic and Thermophilic Anaerobic Digestion of Primary and Secondary Sludge. Effect of Pre-Treatment at Elevated Temperature. *Water Res.* **2003**, *37*, 4561–4572. DOI: 10.1016/S0043-1354(03)00401-9.

[15] Abbassi-Guendouz, A.; Brockmann, D.; Trably, E.; Dumas, C.; Delgenès, J.-P.; Steyer, J.-P.; Escudié, R. Total Solids Content Drives High Solid Anaerobic Digestion via Mass Transfer Limitation. *Bioresour. Technol.* **2012**, *111*, 55–61. DOI: 10.1016/j.biortech.2012.01.174.

[16] Vanwonterghem, I.; Jensen, P. D.; Dennis, P. G.; Hugenholtz, P.; Rabaey, K.; Tyson, G. W. Deterministic Processes Guide Long-Term Synchronised Population Dynamics in Replicate Anaerobic Digesters. *ISME J.* **2014**, *8*, 2015–2028. DOI: 10.1038/ismej.2014.50.

[17] Riviere, D.; et al. Towards the Definition of a Core of Microorganisms Involved in Anaerobic Digestion of Sludge. *ISME J.* **2009**, *3*, 700.

[18] De Vrieze, J.; Saunders, A. M.; He, Y.; Fang, J.; Nielsen, P. H.; Verstraete, W.; Boon, N. Ammonia and Temperature Determine Potential Clustering in the Anaerobic Digestion Microbiome. *Water Res.* **2015**, *75*, 312–323. DOI: 10.1016/j.watres.2015.02.025.

[19] Carballa, M.; Regueiro, L.; Lema, J. M. Microbial Management of Anaerobic Digestion: Exploiting the Microbiome-Functionality Nexus. *Curr. Opin. Biotechnol.* **2015**, *33*, 103–111. DOI: 10.1016/j.copbio.2015.01.008.

[20] Sundberg, C.; Al-Soud, W. A.; Larsson, M.; Alm, E.; Yekta, S. S.; Svensson, B. H.; Sørensen, S. J.; Karlsson, A. 454 Pyrosequencing Analyses of Bacterial and Archaeal Richness in 21 Full-Scale Biogas Digesters. *FEMS Microbiol. Ecol.* **2013**, *85*, 612–626. DOI: 10.1111/1574-6941.12148.

[21] Maeder, D. L.; Anderson, I.; Brettin, T. S.; Bruce, D. C.; Gilna, P.; Han, C. S.; Lapidus, A.; Metcalf, W. W.; Saunders, E.; Tapia, R.; et al. The Methanosarcina barkeri Genome: Comparative Analysis with Methanosarcina acetivorans and Methanosarcina mazei Reveals Extensive Rearrangement within Methanosarcinal Genomes. *J. Bacteriol.* **2006**, *188*, 7922–7931. DOI: 10.1128/JB.00810-06.

[22] St-Pierre, B.; Wright, A-DGJBt. Metagenomic Analysis of Methanogen Populations in Three Full-Scale Mesophilic Anaerobic Manure Digesters Operated on Dairy Farms in Vermont, USA. *Bioresour. Technol.* **2013**, *138*, 277–284. DOI: 10.1016/j.biortech.2013.03.188.

[23] Manyi-Loh, C. E.; Mamphweli, S. N.; Meyer, E. L.; Okoh, A. I.; Makaka, G.; Simon, M. Microbial Anaerobic Digestion (Bio-Digesters) as an Approach to the Decontamination of Animal Wastes in Pollution Control and the Generation of Renewable Energy. *Int. J. Environ. Res. Public Health* **2013**, *10*, 4390–4417. DOI: 10.3390/ijerph10094390.

[24] Gonzalez-Martinez, A.; Rodriguez-Sanchez, A.; Muñoz-Palazon, B.; Garcia-Ruiz, M.-J.; Osorio, F.; van Loosdrecht, M. C. M.; Gonzalez-Lopez, J. Microbial Community Analysis of a Full-Scale DEMON Bioreactor. *Bioprocess Biosyst. Eng.* **2015**, *38*, 499–508. DOI: 10.1007/s00449-014-1289-z.

[25] Wei, Y.; Li, X.; Yu, L.; Zou, D.; Yuan, H. Mesophilic Anaerobic Co-Digestion of Cattle Manure and Corn Stover with Biological and Chemical Pretreatment. *Bioresour. Technol.* **2015**, *198*, 431–436. DOI: 10.1016/j.biortech.2015.09.035.

[26] Li, Y.; Merrettig-Bruns, U.; Strauch, S.; Kabasci, S.; Chen, H. Optimization of Ammonia Pretreatment of Wheat Straw for Biogas Production. *J. Chem. Technol. Biotechnol.* **2015**, *90*, 130–138. DOI: 10.1002/jctb.4297.

[27] Wang, B.; Nges, I. A.; Nistor, M.; Liu, J. Determination of Methane Yield of Cellulose Using Different Experimental Setups. *Water Sci. Technol.* **2014**, *70*, 599–604. DOI: 10.2166/wst.2014.275.

[28] Hussain, A.; Dubey, S. K. Specific Methanogenic Activity Test for Anaerobic Degradation of Influents. *Appl. Water Sci.* **2017**, *7*, 535–542. DOI: 10.1007/s13201-015-0305-z.

[29] Saleh, A. F.; Kamarudin, E.; Yaacob, A. B.; Yussof, A. W.; Abdullah, M. A. Optimization of Biomethane Production by Anaerobic Digestion of Palm Oil Mill Effluent Using Response Surface Methodology. *Asia-Pac. J. Chem. Eng.* **2012**, *7*, 353–360. DOI: 10.1002/apj.550.

[30] Zhang, B.; Zhang, Z.; von Keitz, M.; Valentas, K. Treatment Variable Effects on Supercritical Gasification of High-Diversity Grassland Perennials. *Appl. Biochem. Biotechnol.* **2009**, *154*, 59–66. DOI: 10.1007/s12010-009-8563-6.

[31] Li, Y.-F.; Shi, J.; Nelson, M. C.; Chen, P.-H.; Graf, J.; Li, Y.; Yu, Z. Impact of Different Ratios of Feedstock to Liquid Anaerobic Digestion Effluent on the Performance and Microbiome of Solid-State Anaerobic Digesters Digesting Corn Stover. *Bioresour. Technol.* **2016**, *200*, 744–752. DOI: 10.1016/j.biortech.2015.10.078.

[32] Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S. M.; Betley, J.; Fraser, L.; Bauer, M.; et al. Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *ISME J.* **2012**, *6*, 1621–1624. DOI: 10.1038/ismej.2012.8.

[33] Bornbusch, S. L.; Greene, L. K.; McKenney, E. A.; Volkoff, S. J.; Midani, F. S.; Joseph, G.; Gerhard, W. A.; Iloghalu, U.; Granek, J.; Gunsch, C. K.; et al. A Comparative Study of Gut Microbiomes in Captive Nocturnal Strepsirrhines. *Am. J. Primatol.* **2019**, *81*, e22986. DOI: 10.1002/ajp.22986.

[34] Motte, J.-C.; Escudié, R.; Bernet, N.; Delgenes, J.-P.; Steyer, J.-P.; Dumas, C. Dynamic Effect of Total Solid Content, Low Substrate/Inoculum Ratio and Particle Size on Solid-State Anaerobic Digestion. *Bioresour. Technol.* **2013**, *144*, 141–148. DOI: 10.1016/j.biortech.2013.06.057.

[35] Xu, F.; Wang, Z.-W.; Tang, L.; Li, Y. A Mass Diffusion-Based Interpretation of the Effect of Total Solids Content on Solid-State Anaerobic Digestion of Cellulosic Biomass. *Bioresour. Technol.* **2014**, *167*, 178–185. DOI: 10.1016/j.biortech.2014.05.114.

[36] Nazurally, N. Anaerobic Digestion of Fish Waste and Seagrass/Macroalgae: Potential Sustainable Waste Management for Tropical Small Island Developing States. *J. Mater. Cycles Waste Manag.* , **2018**, *20*, 1724–1735. DOI: 10.1007/s10163-018-0738-1.

[37] Ostertagová, E. Modelling Using Polynomial Regression. *Procedia Eng.* **2012**, *48*, 500–506. DOI: 10.1016/j.proeng.2012.09.545.

[38] Tian, Z.; Cabrol, L.; Ruiz-Filippi, G.; Pullammanappallil, P. Microbial Ecology in Anaerobic Digestion at Agitated and Non-Agitated Conditions. *PloS One.* **2014**, *9*, e109769. DOI: 10.1371/journal.pone.0109769.

[39] Shi, J.; Sharma-Shivappa, R. R.; Chinn, M.; Howell, N. Effect of Microbial Pretreatment on Enzymatic Hydrolysis and Fermentation of Cotton Stalks for Ethanol Production. *Biomass Bioenergy* **2009**, *33*, 88–96. DOI: 10.1016/j.biombioe.2008.04.016.

[40] Amani, T.; Nosrati, M.; Mousavi, S. M.; Kermanshahi, R. K. Study of Syntrophic Anaerobic Digestion of Volatile Fatty Acids Using Enriched Cultures at Mesophilic Conditions. *Int. J. Environ. Sci. Technol.* **2011**, *8*, 83–96. DOI: 10.1007/BF03326198.

[41] Tian, G.; Zhang, W.; Dong, M.; Yang, B.; Zhu, R.; Yin, F.; Zhao, X.; Wang, Y.; Xiao, W.; Wang, Q.; et al. Metabolic Pathway Analysis Based on High-Throughput Sequencing in a Batch Biogas Production Process. *Energy* **2017**, *139*, 571–579. DOI: 10.1016/j.energy.2017.08.003.

[42] Dworkin, M. The Prokaryotes: Archaea. *Bacteria: Firmicutes, Actinomycetes*; Springer Science & Business Media, Springer-Verlag: New York, **2006**.

[43] Dighe, A. S.; Jangid, K.; González, J. M.; Pidiyar, V. J.; Patole, M. S.; Ranade, D. R.; Shouche, Y. S. Comparison of 16S rRNA Gene Sequences of Genus Methanobrevibacter. *BMC Microbiol.* **2004**, *4*, 20. DOI: 10.1186/1471-2180-4-20.

[44] Li, Y.; Liu, C.; Wachemo, A. C.; Li, X. Effects of Liquid Fraction of Digestate Recirculation on System Performance and Microbial Community Structure during Serial Anaerobic Digestion of Completely Stirred Tank Reactors for Corn Stover. *Energy* **2018**, *160*, 309–317. DOI: 10.1016/j.energy.2018.06.082.

[45] Yi, J.; Dong, B.; Jin, J.; Dai, X. Effect of Increasing Total Solids Contents on Anaerobic Digestion of Food Waste under Mesophilic Conditions: Performance and Microbial Characteristics Analysis. *PloS One.* **2014**, *9*, e102548. DOI: 10.1371/journal.pone.0102548.

[46] Fu, S.-F.; Wang, F.; Shi, X.-S.; Guo, R.-B. Impacts of Microaeration on the Anaerobic Digestion of Corn Straw and the Microbial Community Structure. *Chem. Eng. J.* **2016**, *287*, 523–528. DOI: 10.1016/j.cej.2015.11.070.

[47] Sun, L.; Liu, T.; Müller, B.; Schnürer, A. The Microbial Community Structure in Industrial Biogas Plants Influences the Degradation Rate of Straw and Cellulose in Batch tests. *Biotechnol. Biofuels*. **2016**, *9*, 128–128. DOI: 10.1186/s13068-016-0543-9.

[48] Eriksen, N. T.; Riis, M. L.; Holm, N. K.; Iversen, N. H(2) Synthesis from Pentoses and Biomass in Thermotoga spp. *Biotechnol. Lett.* **2011**, *33*, 293–300. DOI: 10.1007/s10529-010-0439-x.

[49] Bhandari, V.; Gupta, R. S. The Phylum Thermotogae. In *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*, Rosenberg, E., et al., Eds.; Springer: Berlin, Heidelberg, **2014**; pp 989–1015.

Appendix A

Table A1. ANOVA test of the removal ratio of volatile solid (VS).

AD no.	*x*1: ER ratio	*x*2: C/N ratio	*x*3: Solid content	VS loss (%)
Original results				
1	20	20	5	55.67
2	20	30	5	52.16
3	20	40	5	43.01
4	40	20	5	57.25
5	40	30	5	56.63
6	40	40	5	36.22
7	60	20	5	58.10
8	60	30	5	56.55
9	60	40	5	41.71
10	20	20	10	39.18
11	20	30	10	23.78
12	20	40	10	23.51
13	40	20	10	42.34
14	40	30	10	25.95
15	40	40	10	24.85
16	60	20	10	45.03
17	60	30	10	31.22
18	60	40	10	26.08
19	20	20	15	33.15
20	20	30	15	11.17
21	20	40	15	12.06
22	40	20	15	33.78
23	40	30	15	9.70
24	40	40	15	9.03
25	60	20	15	33.72
26	60	30	15	5.97
27	60	40	15	6.67

Equation: The removal ratio of VS $(\%) = \frac{\text{Initial VS} - \text{Final VS}}{\text{Initial VS}} \times 100\%$

Summary output	
Regression statistics	
Multiple *R*	0.968547
R square	0.938084
Adjusted *R* square	0.905304
Standard error	5.215041
Observations	27

(*continued*)

ANOVA

	df	SS	MS	*F*	Significance *F*
Regression	9	7004.884	778.3204	28.61824	1.52E − 08
Residual	17	462.3431	27.19666		
Total	26	7467.227			

	Coefficients	Standard error	*t* Stat	*P* value	Lower 95%	Upper 95%
Intercept	116.6572	25.33061	4.605386	0.000252	63.21431	170.1001
*x*1	0.242665	0.508671	0.477057	0.639396	−0.83054	1.315867
*x*2	−2.8798	1.352117	−2.12985	0.048092	−5.73252	−0.02708
*x*3	−3.85924	2.034683	−1.89673	0.074988	−8.15205	0.433565
*x*1*x*2	−0.0054	0.007527	−0.71719	0.482998	−0.02128	0.010483
*x*1*x*3	−0.01295	0.015055	−0.86027	0.401613	−0.04471	0.018811
*x*2*x*3	−0.038	0.030109	−1.26215	0.223936	−0.10153	0.025522
*x*1*x*1	0.001005	0.005323	0.188742	0.85253	−0.01023	0.012234
*x*2*x*2	0.041719	0.02129	1.959548	0.066655	−0.0032	0.086638
*x*3*x*3	0.108065	0.085161	1.268941	0.221562	−0.07161	0.287739

Residual output

Observation	Predicted VS loss %	Residuals	Standard residuals
1	57.15486	−1.48295	−0.35167
2	46.23676	5.918683	1.403555
3	43.66255	−0.65387	−0.15506
4	59.7592	−2.51107	−0.59547
5	47.76142	8.865261	2.102306
6	44.10752	−7.88482	−1.8698
7	63.16722	−5.07041	−1.2024
8	50.08975	6.464728	1.533044
9	45.35616	−3.64556	−0.86451
10	40.86819	−1.68422	−0.39939
11	28.04999	−4.26677	−1.01182
12	23.57567	−0.06479	−0.01536
13	42.17745	0.164001	0.038891
14	28.27955	−2.32732	−0.5519
15	22.72554	2.12864	0.504785
16	44.29038	0.743359	0.17628
17	29.3128	1.908146	0.452497
18	22.67909	3.398945	0.806025
19	29.98476	3.163713	0.750242
20	15.26645	−4.09458	−0.97099
21	8.892015	3.164769	0.750493
22	29.99892	3.777429	0.895779
23	14.20092	−4.49954	−1.06702
24	6.746802	2.287414	0.542437
25	30.81677	2.900137	0.687738
26	13.93908	−7.96861	−1.88968
27	5.405265	1.269268	0.300994
27	−216.35	220.786	0.13882

Probability output

Percentile	VS loss %
1.851852	5.970465
5.555556	6.674533
9.259259	9.034216
12.96296	9.701379
16.66667	11.17187
20.37037	12.05678
24.07407	23.51088
27.77778	23.78322
31.48148	24.85418
35.18519	25.95224
38.88889	26.07804
42.59259	31.22094
46.2963	33.14847
50	33.71691
53.7037	33.77635
57.40741	36.2227
61.11111	39.18397
64.81481	41.71059
68.51852	42.34145
72.22222	43.00868
75.92593	45.03374
79.62963	52.15545
83.33333	55.67191
87.03704	56.55448
90.74074	56.62668
94.44444	57.24813
98.14815	58.09681

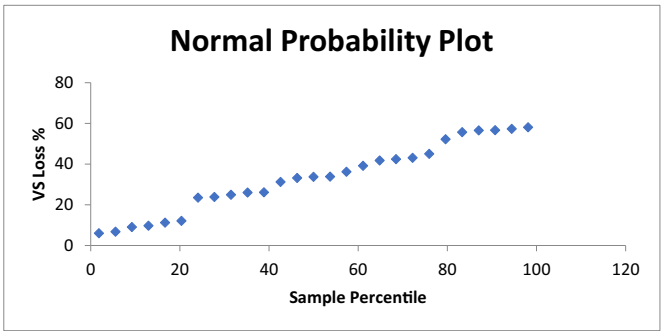

Table A2. ANOVA test of the specific methane productivity (mL g^{-1} initial VS).

AD no.	*x*1: ER ratio	*x*2: C/N ratio	*x*3: Solid content	Specific methane productivity (mL g^{-1} initial VS)
Original results				
1	20	20	5	150.69
2	20	30	5	147.64
3	20	40	5	11.51
4	40	20	5	148.02
5	40	30	5	152.22
6	40	40	5	13.19
7	60	20	5	138.59
8	60	30	5	144.07
9	60	40	5	18.54
10	20	20	10	132.87
11	20	30	10	8.81
12	20	40	10	8.25
13	40	20	10	129.67
14	40	30	10	8.71
15	40	40	10	4.93
16	60	20	10	153.97
17	60	30	10	11.75
18	60	40	10	6.58
19	20	20	15	115.05
20	20	30	15	7.78
21	20	40	15	3.95
22	40	20	15	109.30
23	40	30	15	6.63
24	40	40	15	4.25
25	60	20	15	118.42
26	60	30	15	4.68
27	60	40	15	4.44

Equation: Specific methane productivity $\left(\text{mL g}^{-1}\text{ initial VS}\right) = \frac{\text{Cumulative methane productivity}}{\text{Initial VS}}$

Summary output

Regression statistics

Multiple *R*	0.898054
R square	0.8065
Adjusted *R* square	0.704059
Standard error	35.84387
Observations	27

ANOVA

	df	SS	MS	*F*	Significance *F*
Regression	9	91033.78	10114.86	7.872821	0.000154
Residual	17	21841.31	1284.783		
Total	26	112875.1			

	Coefficients	Standard Error	*t* Stat	*P* value	Lower 95%	Upper 95%
Intercept	551.0281	174.1016	3.16498	0.005658	183.7059	918.3503
*x*1	−0.33145	3.496181	−0.0948	0.925579	−7.70775	7.044847
*x*2	−16.7711	9.293328	−1.80464	0.088876	−36.3783	2.836101
*x*3	−25.969	13.98472	−1.85695	0.080731	−55.4741	3.536232
*x*1*x*2	−0.00272	0.051736	−0.05259	0.958668	−0.11187	0.106433
*x*1*x*3	0.007826	0.103472	0.075631	0.940596	−0.21048	0.226133
*x*2*x*3	0.10652	0.206945	0.514728	0.61337	−0.33009	0.543135
*x*1*x*1	0.004688	0.036583	0.128159	0.899527	−0.07249	0.081872
*x*2*x*2	0.159787	0.146332	1.091946	0.290091	−0.14895	0.46852
*x*3*x*3	0.817478	0.585328	1.396616	0.180498	−0.41746	2.052412

Residual output				*Probability output*	
Observation	Predicted methane production (mL/int. VS)	Residuals	Standard residuals	Percentile	Methane production (mL/int. VS)
1	175.7053	−25.0182	−0.86318	1.851852	3.946909
2	92.66938	54.97435	1.896738	5.555556	4.246533
3	41.59078	−30.0851	−1.038	9.259259	4.435828
4	174.3966	−26.3758	−0.91003	12.96296	4.68168
5	90.81645	61.40229	2.118517	16.66667	4.933804
6	39.19365	−25.9992	−0.89703	20.37037	6.577664
7	176.8386	−38.2469	−1.3196	24.07407	6.630796
8	92.71428	51.35712	1.771936	27.77778	7.784447
9	40.54727	−22.0086	−0.75935	31.48148	8.25467
10	118.606	14.26608	0.492212	35.18519	8.710869
11	40.89607	−32.0868	−1.10707	38.88889	8.809304
12	−4.85651	13.11118	0.452365	42.59259	11.50568
13	118.0798	11.59	0.399881	46.2963	11.75437

(*continued*)

14	39.82571	−31.1148	−1.07353	50	13.19448
15	−6.47108	11.40488	0.393494	53.7037	18.53869
16	121.3044	32.66863	1.127141	57.40741	109.2993
17	42.5061	−30.7517	−1.061	61.11111	115.0544
18	−4.33489	10.91256	0.376508	64.81481	118.4242
19	102.3806	12.6738	0.437275	68.51852	129.6698
20	29.99666	−22.2122	−0.76637	72.22222	132.8721
21	−10.4299	14.37682	0.496032	75.92593	138.5917
22	102.637	6.662295	0.229864	79.62963	144.0714
23	29.70887	−23.0781	−0.79625	83.33333	147.6437
24	−11.2619	15.50844	0.535076	87.03704	148.0208
25	106.6441	11.78009	0.40644	90.74074	150.6872
26	33.17182	−28.4901	−0.98297	94.44444	152.2187
27	−8.34315	12.77898	0.440904	98.14815	153.9731