Effects of Slurry Reflux on the Stability and Microbial Community Structure of Corn Stalk Anaerobic Digestion System

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Abstract

Anaerobic digestion (AD) of corn stalks with slurry reflux and non-reflux was compared and evaluated to clarify the effects of slurry reflux on AD. It was found that slurry reflux increased cumulative methane production by 45.80% and improved system stability. With the increase of organic loading rate (OLR), pH value and oxidation-reduction potential (ORP) of reflux group remained 7.16±0.23 and -338.71±9.22. High-throughput sequencing results showed that slurry reflux slowed down the decrease of microbial community diversity, and the richness of bacterial community increased by 9.16%. The dominant microorganisms were *Bacteroidetes* and *Methanothrix* in reflux group, the relative abundances were 32.41% and 41.75%, respectively. The increase of organic loading rate (OLR) altered the main methane-producing pathway of the AD system, and slurry reflux can delay this trend.

Introduction

China was rich in crop stalk resources, with an annual output of approximately 850 million tons (Li et al., 2016). However, the utilization rate of these resources was relatively low (Du et al., 2017). Nearly 50% of the crop stalks were used for fuel and incineration annually, which caused environmental pollution as well as considerable wastage of resources. As an engineering solution to degrade organic waste and produce biogas, AD was considered as a sustainable and effective approach to treat waste, generated clean energy, and recycled organic matter by utilizing crop stalks (Yanjin et al., 2020). However, the production of methane by AD also produced a amount of slurry, which was rich in nitrogen, phosphorus, potassium, and other nutrients (Lu et al., 2010).The slurry would inevitably cause secondary pollution, if it was not properly utilized (Chen et al., 2013; Qiu et al., 2016). Slurry reflux was therefore an efficient and inexpensive way to reduce emissions from the AD system, which in turn, can effectively reduce the slurry discharge and the subsequent disposal cost (Wu et al., 2018). Compared with the AD system, where animal manure was used as the substrate, the AD system with crop stalks as lacked nutrients and buffering capacity (Zheng et al., 2020).The basicity of the slurry can be used to balance the acidity of the digestion liquid through slurry reflux, maintain the pH value within an appropriate range. Additionally, slurry reflux can improve the richness and vitality of microorganisms in AD system (Hu et al., 2014; Yang et al., 2016).

The biogas-production efficiency and system stability of the AD system depended largely on the activity of functional microorganism, while the diversity and richness of the microbial community can also affect its performance (Razaviarani & Buchanan, 2015). In AD, the stages of acid production, acetic acid production, and methane production were completed by different functional flora (Li et al., 2018). By studying the community structure of bacteria and archaea in AD system, the internal relationship and interaction between functional microorganism and AD performance can be obtained (Abbassi-Guendouz et al., 2012; Rui et al., 2015). Maria et al.(2017) has reported that *Firmicutes* and *Bacteroidetes* were the dominant microorganisms, and the dominant microorganisms of archaea were *Methanosarcina* (acidophilic and hydrogenotrophic methanogens) and *Methanobacteria* (hydrogenotrophic methanogens). Li et al. (2018) used corn stalks as a substrate with slurry reflux and found that the

system was enriched in *Bacteroidetes* and *Firmicutes*. Gulhane et al. (2017) reported that the recycling of sewage increased the number of hydrolyzing and digestion microbial communities that consumed VFAs (volatile fatty acids). Different substrates determined the species and abundance of microbial communities in the system, which would also lead to differences of various enzymes (Rui et al., 2015). Microbial community can not only provide an early warning of system stability but also reveal mechanism of AD system (Abbassi-Guendouz et al., 2012; Rui et al., 2015). Currently, there were lack of researches on the continuous AD of corn stalks, especially the relationship between microbial communities in AD system with the gradually increase of OLR. Therefore, this study examined system stability and the influence of the microbial community, setting an AD system, which incorporated slurry reflux. Corn stalks were used as the substrate, and various OLR were set, to aimed to provide a scientific basis for the application of slurry reflux in biogas engineering technology.

Materials And Methods

Substrates and Inoculum

Corn stalk was collected from the Beishan Scientific Research Base of Shenyang Agricultural University, which was crushed to 2–3 mm after natural drying. The inoculum was obtained from the AD pond, which used pig measure as feedstock, in Comprehensive Energy Demonstration Base of Shenyang Agricultural University. The physicochemical parameters of the corn stalk and inoculum were shown in Table 1.

Experimental Digester and Setup

All the AD experiments were performed in two continuous stirred tank reactors (CSTR) with total volume of 10 L (working volume was 8 L) (Fig. 1). The temperature was maintained at 37 ± 1°C, the total solid (TS) content was 10%, and the mixing ratio of inoculum and substrate was 1:3. Slurry was carried out once every 3 d. 75% slurry was mixed with corn stalk as the reflux to the reflux group (R2), while tap water was used to replaced slurry in non-reflux group (R1). The stirring was set for 20 min every 2 h, and the rotating speed was 30 rpm. The first 10 days of the process comprise the start-up period (no data). Slurry reflux started on day 11. According to the different OLR, the test was divided into three phases: Phase Ⅰ(11–37d), Phase Ⅱ(38–64d) and Phase Ⅲ(65–91d) with the OLR 2.0gTS/($L_{reactor}$·d), 3.0gTS/($L_{reactor}$·d) and 4.0gTS/($L_{reactor}$·d), respectively.

Analytical Techniques and Statistical Method

The pH value, total solid (TS) and volatile solid (VS) concentrations were measured according to the Standard Methods (APHA, 2012). The oxidation-reduction potential (ORP) was measured using a Hach HQ40D multi-parameter water quality analyzer. A portable biogas analyzer was used for proportion analysis of the biogas. VFA concentrations were determined using gas chromatography on FID detector, 19091N-133 (30m×250μm×0.26μm) column. Ammonia nitrogen was used for determination in the sodium reagent colorimetric method.

From the two groups of AD reactors with varying conditions, a total of six samples were selected for microbial community structure analysis, these samples represented pre-reflux material (R1-1;R2-1), the end of Phase Ⅱ (R2-2), the end of Phase Ⅲ (R1-3;R2-3), and the end of Phase Ⅳ (R2-4).

The extraction of sample DNA was conducted following the instructions of the OMEGA E.Z.N.A™ Mag-Bind Soil DNA Kit. The diluted DNA samples were amplified using PCR (T100™ Thermal Cycler). Microbial amplification primers were 341F: 5'-CCCTACACGACGCTCTTCCGA TCTG (BARCODE) CCTACGGGNGGCWGCAG-3' and 805R: 5'-GACTGGAGTTCCTTGGCACCCGAGAA TTCCAGACTACHVGGGTA TCTAA TCC-3'. Archaea expansion increased 349 f: primers for 5'-CCCTACACGACGCTCTTCCGA TCTN (barcode) GYGCASCAGKCGMGAAW-3 and 806 r:' 5-'GACTGGAGTTCCTTG-GCACCCGAGAA TTCCAGGACTACVSGGGTA TCTAA T-3'. PCR products with normal amplified fragments of bacteria and archaea above 400 bp were treated with 0.6 magnetic beads (Agencourt AMPure XP).After treatment, the samples were sent to Shanghai Sangon Bioengineering Co., Ltd for sequencing. The detection types were bacteria and archaea, the platform was Miseq2 ×300 bp, and the library was the NCBI16S database. Operational taxonomic unit (OTU) clustering and species annotation were performed for more than 97% of the sequences, and Alpha diversity analysis and relative abundance analysis were also performed.

Results

Influence of slurry reflux on methane production

Methane production rate and cumulative methane production were the important indicators to measure the performance of AD system. The average methane production rate and cumulative methane production at each phase in AD process were shown in Table 2.

The average methane production rate of the AD system, OLR was from 2 to 4gTS/($L_{reactor}$· d),showed a decreasing trend, from 171.23 and 220.22 to 100.44 mL/(gTS.3d) and 171.23 mL/(gTS.3d), respectively. The average volume methane production rate increased significantly from 1.03 and 1.32 to 1.20 and 2.05 L/($L_{reactor}$.3d), respectively. At the same time, the cumulative methane production in each phase also gradually increased, which in R1 and R2 were 74.00L, 84.88L and 86.76L, and 95.10L, 115.12L and 147.92L, respectively. The cumulative methane production of R2 increased by 28.6%, 35.6% and 70.5%, compared with R1 in three phases, respectively.

Influence of slurry reflux on system stability

Ammonia nitrogen concentration, inorganic carbon concentration, VFAs, pH value and ORP were the key factors of system stability. The variation indirectly reflected the stability of the AD system (Fig. 2).

The concentration of ammonia nitrogen decreased gradually from ~ 1100mg/L to ~ 200mg/L during the whole process of AD process. With the increase of OLR, the TIC concentration of R1 decreased from 883.45mg/L to 24.06mg/L; R2 decreased slowly but remained at 500 ± 100 mg/L. The variation trend of

VFAs concentration was similar to that of ammonia nitrogen, which decreased significantly, from 2310.99mg/L and 2101.90 mg/L to 14.5 and 29.1mg/L, respectively. At the end of phase Ⅱ, VFAs concentrations in both groups were low. VFAs concentration of R1 increased, when OLR was 4gTS/($L_{reactor}$·d), and pH value dropped to 5.92,which Inhibited methanogens metabolism. The pH of R2 decreased gradually from peak values of 7.50 to 7.02. The ORP of R1 and R2 increased, when OLR increased, and the variation of R1 was more significant from − 436.10 to -271.70 mV, and the average values of R2 in three phases, which maintained in suitable range, were − 347.90mV, -336.90mV and − 331.10mV respectively.

Microbiological analysis during biogas production process

Dynamics of microbial community richness and diversity

The microbial diversity parameters, including Shannon, Simpson, Ace and Chao 1, were estimated at different phases of the AD systems were shown in Table 3. The bacterial community diversity of the reflux group and the non-reflux system showed a downward trend, and decreased by 1.1% and 6.4%, respectively. The diversity of archaea increased by 39% and 20%, respectively. With the increase of OLR, the richness of R2 microbial community increased from 1357.67 to 1543.50.

Dynamics of bacterial populations at the phylum level

The relative abundance of bacterial communities at phylum level in samples at different phase were shown in Fig. 4. There were 6 dominant phyla in this study. They were *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Chloroflexi*, *Verrucomicrobia* and *Synergistetes*. The dominant phyla accounted for 83.14%-91.75%. *Firmicutes* and *Bacteroidetes* were the dominant in the two groups, and their relative abundance accounted for 39.55%-51.29%. During the AD process, the relative abundances of *Firmicutes* and *Bacteroidetes* were negatively correlated, the relative abundance of *Bacteroidetes* was from 18.07% (R2-2) to 32.41%(R2-4), *Firmicutes* decreased from 21.48–14.57%. Yuan et al. (2014) found that *Firmicutes* was the dominant in the unstable start-up stage of the AD system, which rapidly declined after the system stabilized, and *Bacteroidetes* became the dominant during the stability of the system.

The relative abundance of *Proteobacteria* decreased when OLR increased from 2gTS/($L_{reactor}$·d) to 4gTS/($L_{reactor}$·d). The peak value of *Verrucomicrobia* was 25.84% when OLR was 4 gTS/($L_{reactor}$·d), which increased 20.84% compared with 2gTS/($L_{reactor}$·d). The relative abundances of *Chloroflexi* under different OLR were 23.36%, 29.52% and 13.52%, respectively, which peaked at 3gTS/($L_{reactor}$·d). The relative abundance of each phylum changed significantly, indicating that the bacterial community structure was significantly affected by OLR (Jing et al., 2019).

Dynamics of archaeal populations at the phylum and genus level

Only 1% of the archaea in the system belonged to unknown phylum, and there were almost no unknown archaea resources in the AD system. Archaea at the phylum level (Fig. 5.a) had two main dominant

microorganisms, *Euryarchaeota* and *Crenarchaeota*. And the relative abundances of *Euryarchaeota* in different OLR were over 60%, and the second dominant was *Crenarchaeota*.

At the genus level (Fig. 5.b), the dominant microorganisms inclouded *Methanothrix*, *Methanospirillum*, *Methanobacterium*, *Methanosphaerula* and *Methanomassiliicoccus*. The relative abundances of *Methanothrix* at different OLR (R2-2, R2-3, R2-4) were, 45.16%, 38.76% and 41.75%, respectively. In the non-reflux group (R1-1, R1-3) the relative abundances were 58.24%, 15.34%, respectively.

Methanospirillum, *Methanobacterium*, *Methanosphaerula* and *Methanomassiliicoccus* were hydrotrophic methanogens (Bassani et al., 2017; Girma et al., 2017; Ilaria et al., 2015 and Lee et al., 2017). With the increase of OLR, the relative abundances were 21.10, 24.14 and 41.39%, respectively, showing an upward trend. Compared with R1(R1-3) and R2(R2-3), hydrotrophic methanogens in the non-reflux group (R1-3) were overwhelmingly dominant, with a relative abundance of 68.05%, higher than hydrotrophic methanogens in R2(R2-3) under the same OLR, which was 43.19%. When OLR increased to 4gTS/($L_{reactor}$·d), the relative abundance of hydrotrophic methanogens in R2 increased to 41.39%, which was similar to that of *Methanothrix* (41.75%).

Discussion

Methane production in AD system decreased with the increase of OLR, which could be effectively alleviated by slurry reflux. Methane production of R1 and R2 decreased by 41.3% and 22.2%, when OLR 2 to 4 gTS/($L_{reactor}$·d), respectively. Average volumetric methane production rate was an index reflecting reactor efficiency. Higher average volumetric methane production rate can improve the volume utilization rate of reactor and reduce the operating cost. Slurry reflux carried undegraded organic matter back to the AD system, reduced microbial loss and increases methane production, which was consistent with previous report (Li et al., 2018). However, Han et al. (2018) and Zhang et al. (2017) found that reflux inhibited AD and reduced methane production, which was mainly caused by nitrogen deficiency in the long-term AD process of corn stalk. In this test, 81d slurry reflux operation did not show a significant decrease in methane production.

The variation trend of VFAs was similar to ammonia nitrogen, which may be related to the excessively short start-up phase. In this experiment, only 10d start-up phase was set. The pH value was determined by ammonia nitrogen, TIC and VFAs, which were buffer systems (J et al., 2002). The decrease of TIC concentration and the increase of VFAs concentration led to the imbalance of R1 AD system.

Bacteria had higher microbial population richness and diversity than archaea in AD system, which was mainly caused by the diversity difference of bacterial and archaea genetic development (Guo et al., 2014). The diversity and richness of bacterial and archaea communities were correlated with the stability of the system. The disappearance of some bacterial population suggested that the loss of bacterial stratification and the decrease in the evolution of new communities, which was a successful manifestation of the biochemical activities of the anaerobic system (Li et al., 2018). The relative

abundance of *Firmicutes* in R1 was higher than that in R2. *Bacteroidetes* became the dominant bacterial community, indicating that slurry reflux can improve the stability of AD system (Yuan et al. 2014).

Euryarchaeota was the dominant microorganisms on phylum level, the results were supported by some previous researches (Li et al., 2018; Tian et al., 2017). The relative abundance of *Methanothrix* in reflux group (R2-3) was higher than that in non-reflux group (R1-3), which may be due to the continuous outflow of slurry in non-reflux group and the VFAs concentration was low. The relative abundance of hydrotrophic methanogens in R2 increased when OLR increased. This was because hydrotrophic methanogens were more dominant in AD systems under higher OLR (Ros et al., 2017).

In conclusion, slurry reflux improved the performance of AD system, reduces slurry emission and improved methane production rate. The stability of AD system was significantly improved by slurry reflux, and pH and ORP were maintained in the appropriate range. The results of high-throughput sequencing analysis showed that slurry reflux slowed down the decline of microbial community diversity and increased the richness of bacterial community. The dominant microorganisms were *Bacteroidetes* and *Methanothrix* in reflux group. The increase of OLR altered the main methane-producing pathway of AD system, and slurry reflux could delay it.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This manuscript does not contain any individual person's data.

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare no competing financial interest.

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Authors' contributions

LZ performed the experiments and analyzed the raw data. YG and JMZ conceived the study, designed the experiments and drafted the manuscript. JXS and ZW participated in the design of the study. JYL and WK helped to perform the experiments. All authors read and approved the final manuscript.

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Tables

Table 1

Physicochemical properties of corn stalks and inoculum

Parameters	Corn Stalk	Inoculum
TS (%)	90.23±0.07	17.46±0.12
VS (%)	84.62±0.04	6.12±0.08
C (%)	42.75±0.21	11.32±0.14
N (%)	0.75±0.07	0.77±0.09
pH Value	-	7.60±0.1

Table 2

Methane production performance of reactor in each phase

	Phase Ⅰ		Phase Ⅱ		Phase Ⅲ	
Ractor	R1	R2	R1	R2	R1	R2
Time (d)	11-37		38-64		65-91	
Organic load rate (gTS/$L_{reactor}$·d))	2.0	2.0	3.0	3.0	4.0	4.0
Average methane production rate (mL/(gTS.3d))	171.23	220.22	130.99	177.66	100.44	171.24
Average volumetric methane production rate (L/($L_{reactor}$.3d))	1.03	1.32	1.18	1.60	1.20	2.05
Cumulative methane production (L)	74.00	95.10	84.88	115.12	86.76	147.92

Table 3

Alpha diversity index of microbial community during anaerobic digestion process

	Groups	R1-1	R1-3	R2-1	R2-2	R2-3	R2-4
Bacterial	Seq num	50509	58538	55398	43665	22254	40155
	OTUs	1227	1133	1284	1105	1116	1354
	Ace	1494.53	1386.81	1468.03	1434.84	1992.30	1705.42
	Shannon	4.59	4.54	4.55	4.55	4.26	3.98
	Chao1	1406.35	1332.23	1414.02	1357.67	1514.35	1543.50
Archaea	Seq num	35502	72156	66784	79634	59710	66585
	OTUs	141	219	150	159	108	212
	Ace	271.08	420.89	294.22	234.75	273.60	356.38
	Shannon	1.59	2.21	1.63	1.92	1.95	2.02
	Chao1	220.50	304.00	211.22	209.32	174.00	277.54

Figures

Associated content

Figures (11)

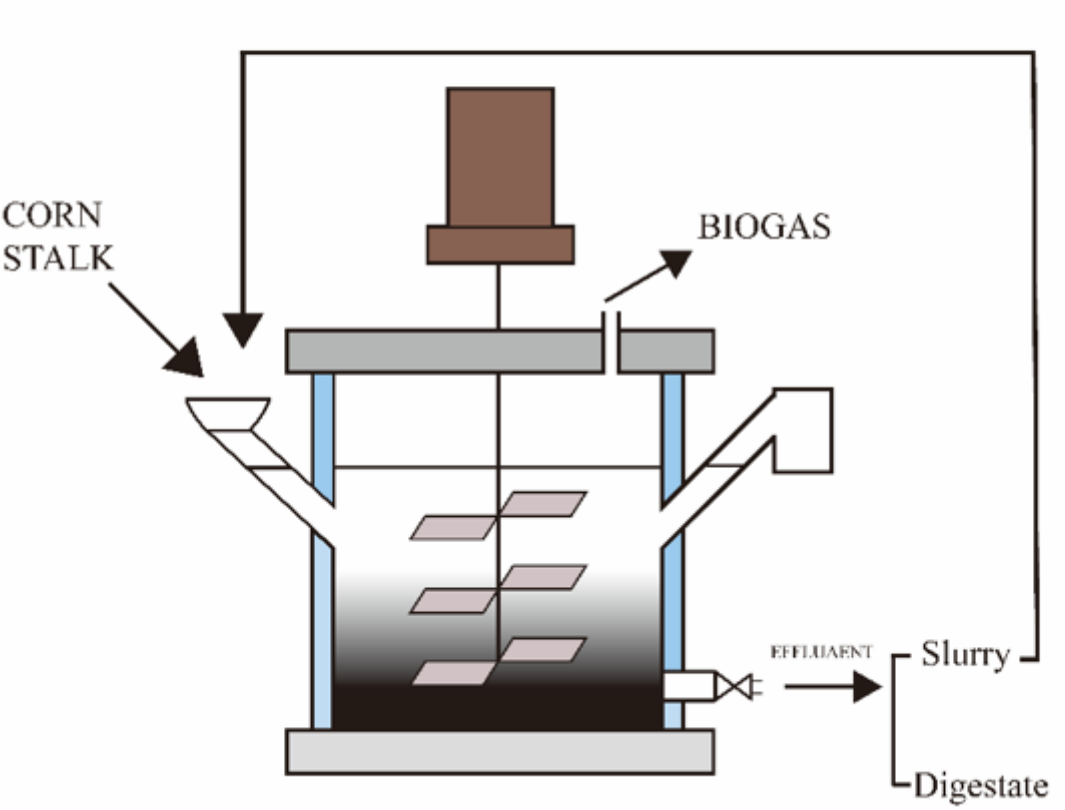

Figure 1

Schematic diagram of the CSTR

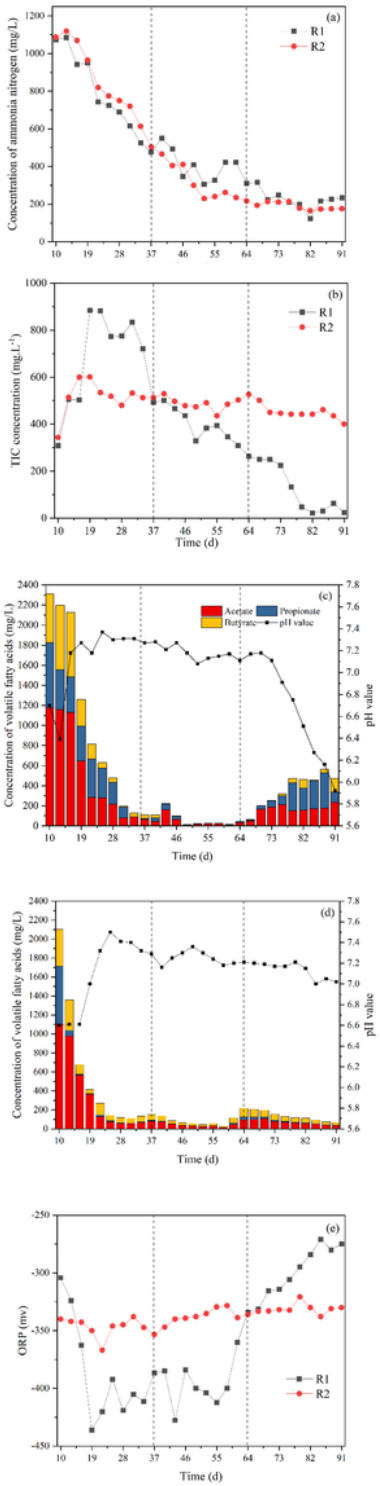

Figure 2

Concentration of ammonia nitrogen (a); concentration of total inorganic carbon (b); concentration of volatile fatty acids and pH value of R1(c) and R2(d); ORP (e);in Phase Ⅰ–Phase Ⅲ, respectively, had organic load rates of 2, 3, and 4 gTS/(Lreactor·d).

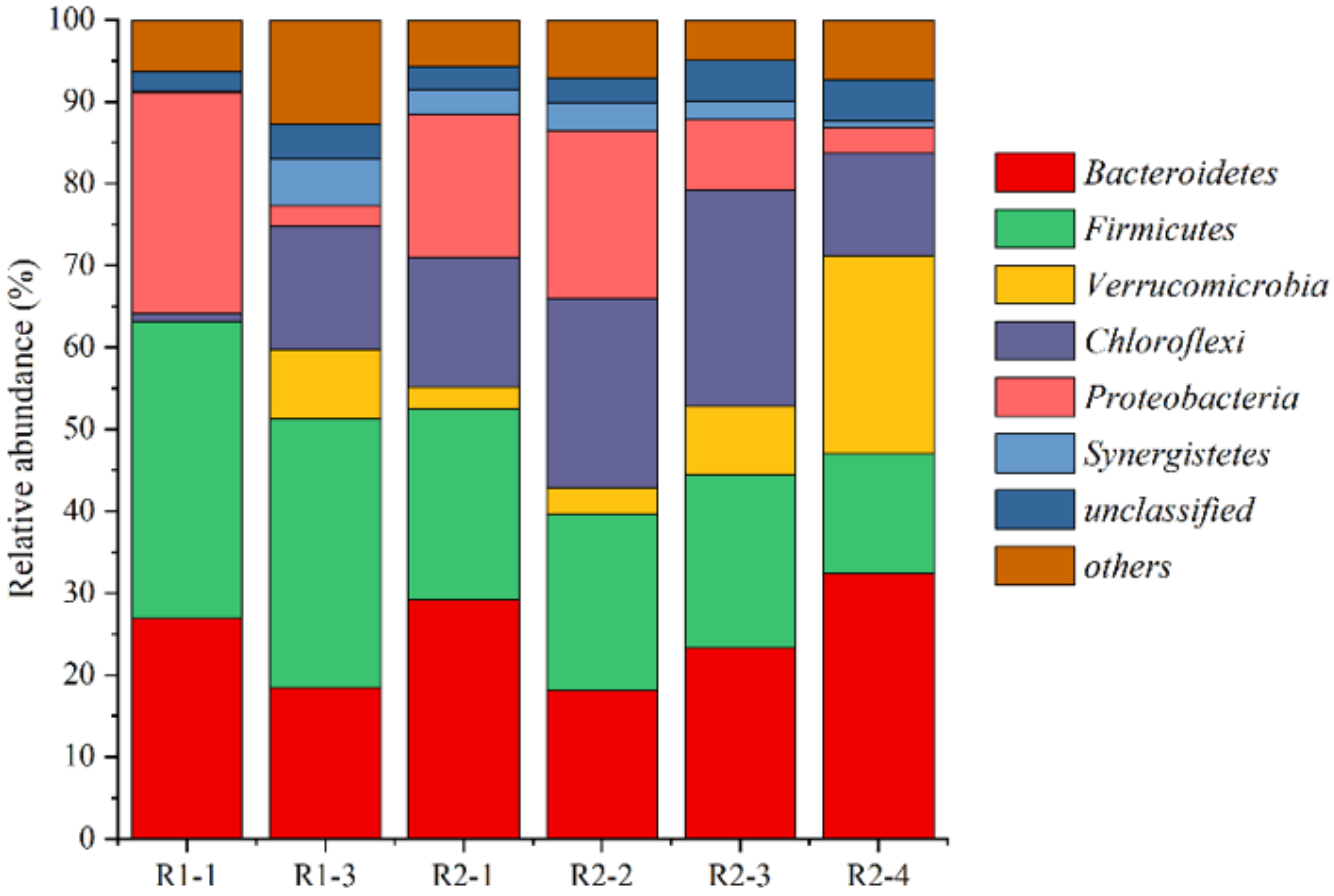

Figure 3

Bacterial sequence distributions at phylum level

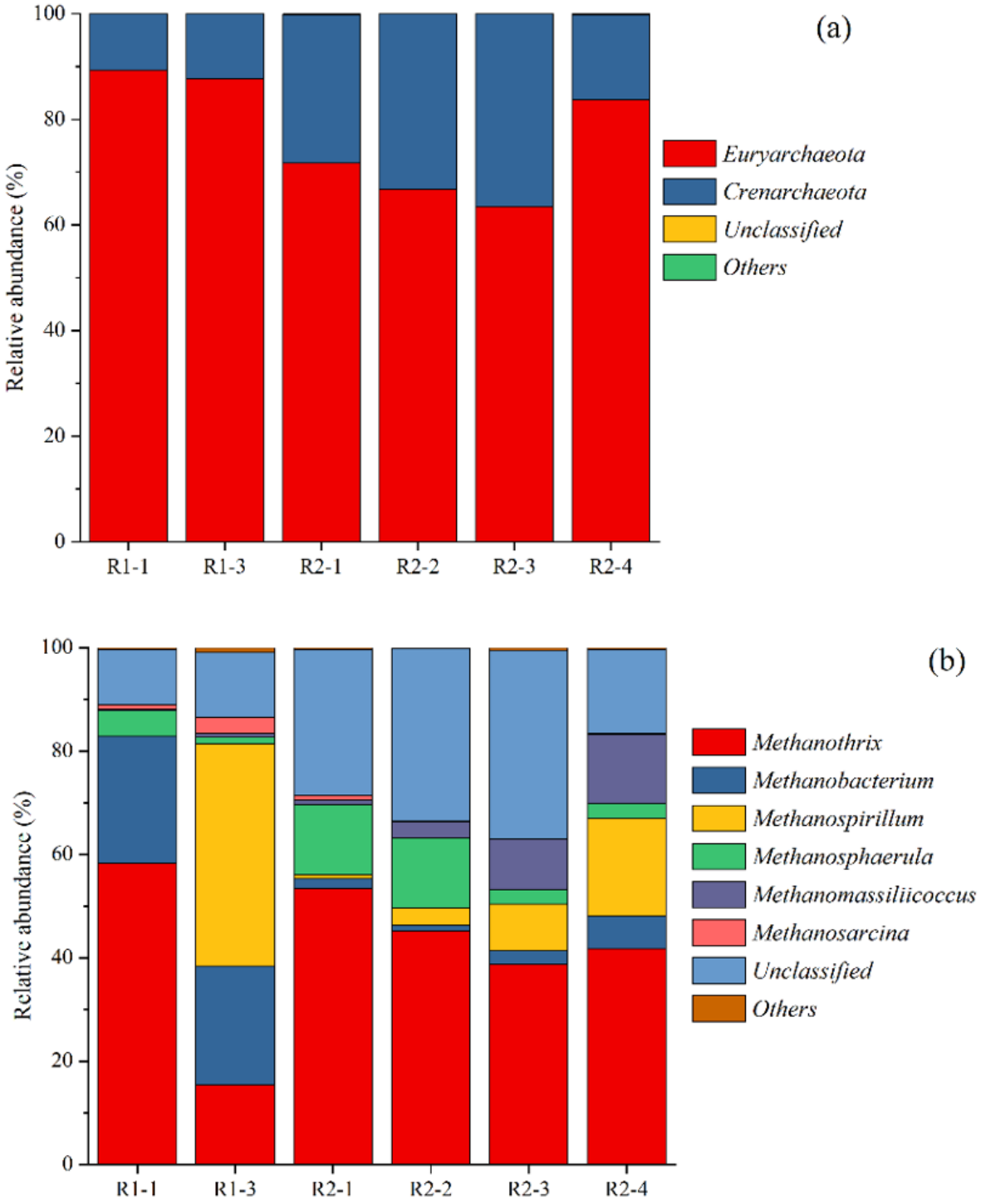

Figure 4

Variation of archaeal communities structure; a)Percent of archaeal community abundance on phylum level;b) Percent of archaeal community abundance on genus level

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Microbialrawdata.xlsx