Microbial Community in Ethanol-methane Coupling Fermentation Treating Food Waste

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The microbial communities in ethanol-methane coupling fermentation reactors were studied. The community structure variation was monitored at the genus and phylum levels using PowerSoil® DNA Isolation Kit with 16S ribosomal RNA (16S rRNA). The distribution of microbial communities in the ethanol reactor was higher than the methane reactor at the phylum and genus level, indicating the influence of coupling fermentation. *Clostridia* (hydrogen producer) was the dominant species throughout the process at genus and phylum levels. This result indicates the efficient degradation of organic acids. In addition, Archaea methanogen species (aceticlastic methanogens) utilize both acetate and hydrogen to produce methane. The dominance of *Methanosaeta* rather than *Methanosarsina* in the anaerobic digestion reactor (R1) of coupling fermentation added further valuable information on food waste treatment. Moreover, lactic acid bacteria species (*Lactococcus*) was dominant in the ethanol reactor (R2), suggesting the efficient conversion of food waste to lactic acid, which could continue its conversion to ethanol. Interestingly, the high amount of ammonia, salts, and volatile fatty acids (VFAs) (including high acetate) could promote the SAO pathway in the coupling fermentation system.

Keywords: Microbial community; PowerSoil® DNA isolation kit; SAO pathway; Food waste; Stillage

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INTRODUCTION

Biofuel production from biomass waste has recently gained substantial attention as a substitution for fossil fuel (Zhang *et al.* 2014). Ethanol fermentation coupled with anaerobic digestion (AD) has been proposed as a promising technology to develop sustainable food waste management while enhancing energy recovery. The efficient and stability of anaerobic digestion process mainly depends on exchanged and syntrophic interactions of functions of microorganisms that govern this process (Croce *et al*. 2016; Ren *et al.* 2018). Although there is a wide range of anaerobic digestion processes, it faces many challenges due to the limited amount of information that has been published about the relationship between process performance and dynamics of microbial communities (Franke-Whittle *et al*. 2014). Understanding the dynamic and structure of microbial community could help in optimization of AD parameters (Guo *et al*. 2014).

Methane production is performed in four steps: 1) hydrolysis, during which bacteria converts organic raw material to amino acids, sugar, and fatty acids; 2) acidogenesis, in which acidogenic bacteria further degrade the first step components into organic acids, alcohol, CO_2, and H_2; 3) acetogenic bacteria step utilize fatty acids and alcohol to produce acetate and more CO_2 and H_2; and 4) methanogenic bacteria convert

these products to biogas (Kim *et al*. 2014). However, the microbial community diversity might be affected by various factors such as pH, VFA concentration, ammonium-nitrogen concentration, temperature, and hydraulic retention time (HRT) (Kim *et al.* 2014). For example, propionic acid concentration less than 30 mmol/L increased ethanol production in coupling fermentation of cassava mash, but at propionic acid concentration greater than 53.2 mmol/L and lower pH values totally inhibited ethanol production. This was because of the growth of contaminated bacteria such as acidogenic and lactogenic in ethanol fermentation reactor (Zhang *et al.* 2012). Furthermore, ammonium and protein-rich feedstock such as organic waste sewers of animal farms and ethanol fermentation plants (stillage) could affect microbial composition in AD process. In this case, syntrophic acetate oxidation bacteria (SAOB) occupy a unique niche and major role in methane production, which is known as the SAO pathway (Westerholm *et al.* 2016).

Generally, the stillage from the ethanol reactor used as substrate for methane production contains a high amount of volatile fatty acids (VFAs). Accordingly, the methane reactor receives a product containing a high acetate concentration, compared with the natural process, which comprises two steps (hydrolysis, fermentation) before obtaining acetate to form methane. There are two mechanisms for methane production from high acetate feedstock. The aceticlastic pathway is accomplished by either *Methanosaeta* or *Methanosarsina.* The second mechanism can be carried out in two steps, where acetate is oxidized to CO_2 and H_2 by acetate-oxidizing bacteria (mainly *Clostridia* species) and then hydrogenotrophic methanogens convert these products to methane (Karakashev *et al*. 2006). Higher acids concentration causes system acidification, which leads to system collapse, but renewable stability is accomplished by syntrophic acetate oxidation (SAO). Syntrophic acetate oxidation is the better mechanism for acetate degradation particularly in the existence of ammonia and volatile fatty acids. To date, the microbial community structure of ethanol-methane coupling fermentation from food waste has not been investigated.

This study investigated the microbial community structure in two-stage reactors to reveal the bioethanol and biomethane pathway. A goal was to enhance the process stability and productivity of coupling fermentation. Moreover, the structure of the bacterial and archaeal communities was evaluated at the genus and phylum level to detect the effect of coupling fermentation on both reactors.

EXPERIMENTAL

Characterization of Raw Materials and Process Description

In order to investigate the microbial community in ethanol-methane coupling fermentation from food waste, the following methods were used: Food waste was collected from a canteen at the University of Science and Technology, Beijing (USTB), China and pretreated. Then the characteristics of food waste and sewage sludge were determined and recorded in Table 1. The stillage eluted of ethanol fermentation was utilized as substrate for methane production. Before usage for methane fermentation, the characteristics of the stillage were detected and listed in Table 1.

As displayed in Fig.1, firstly, food waste after pretreatment was used as substrate for ethanol fermentation under the optimum conditions of pH 5, temperature 30 °C, and fermentation time of 40 h, following the procedure reported by (Ma *et al.* 2008). Secondly, the stillage eluted of ethanol distillation was used as substrate for methane

fermentation by anaerobic digestion process (AD), under optimum condition of pH 7, temperature 37 °C, operated at a stirring speed of 120 rpm, for a retention time of 30 days (Angelidaki *et al*. 2009). Effluent of AD was recycled in the next batch of ethanol fermentation as dilution water instead of tap water.

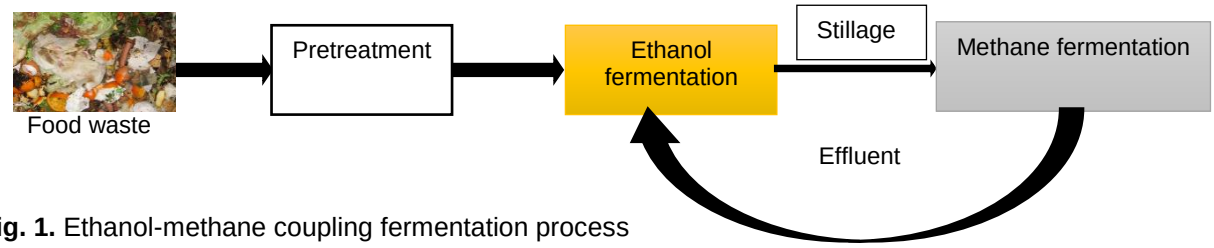

Fig. 1. Ethanol-methane coupling fermentation process

Table 1. Composition of Substrates and Inoculum for Coupling Fermentation

Parameter		FW			Stillage			Inoculum		
	unit	Average	SD±	N	Average	SD±	n	Average	SD±	n
TS[a]	%	20.76	1.6	4	9.4	1.2	3	11.49	0.8	3
VS[a]	%	9.6	1.2	4	7.47	0.6	3	7.01	1.2	3
SS	%	5.3	0.6	4	/	/	/	3.6	0.6	3
VSS	%	4.5	0.7	4	/	/	/	2.2	0.7	3
TCOD	g/L	161	24.1	4	48.5	6.5	3	77.2	8	3
SCOD	g/L	73	13.5	4	31.4	2.5	3	11.6	7.5	3
PH	NA	5.48	1.5	4	3.88	0.6	3	7.48	3.2	3
C [aa]	%	53.68	3.7	4	47.1	0.9	3	26.71	1.4	3
N [aa]	%	2.54	2.6	4	5.3	0.6	3	2.88	0.6	3
C/N	%	21.13	5.4	4	8.8	1.5	3	9.27	2	3
NH+4-N	mg/L	48.2	8.5	4	249.71	8	3	1985	8.6	3
TVFA	mg/L	1620.2	10.5	4	10897	15.2	3	/	/	/
Reducing sugar [aa]	%	13.65	5.4	4	/	/	/	/	/	/
total sugar[aa]	%	60.23	4.7	4	/	/	/	/	/	/
starch[aa]	%	46.12	6	4	/	/	/	/	/	/
protein[aa]	%	15.56	3.2	4	/	/	/	/	/	/
fat	%	18.06	4.5	4	/	/	/	/	/	/

[a] = based on wet material; [aa]= based on dry material; /= not detected, NA= not applicable

Analytical Techniques

Chemical oxygen demand (COD), soluble COD, Ammonia-Nitrogen (CH_4^+-N), total solids (TS), and volatile solids (VS) were analyzed following the standard methods of the American Public Health Association (APHA 2005). The pH was measured using a pH meter (Shanghai Bailun Company, China). Food waste elemental compositions

including C, H, O, N, and S were measured with an elemental analyzer (Vario EL III CHNS). Total volatile fatty acids (TVFA) were measured using gas chromatography (GC).

DNA Extraction and Amplification

Biomass samples were collected at different intervals of designated time from two reactors. One reactor converts stillage to methane (AD), and the other one converts food waste (FW) to ethanol using methane effluent as diluting water) for bacteria and archaea community analysis. Each sample was tested in duplicate. These samples were incubated at 50 °C for 90 min. They were transferred into mirocentrifuge tubes with purifying solution. After gently mixing the sample with solution by vortex, the tubes were immediately placed in ice (-80 °C) for 10 min. The samples were centrifuged at 10,000 rpm for 10 min, and the pellet was used for DNA extraction. The PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) was used to extract total genomic DNA from each sample. The 16S ribosomal RNA (rRNA) genes segments were amplified using primer pairs with the barcodes of 27F (5'-AGAGTTTGATC-CTGGCTCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') for bacteria and 344F (5'-ACGGGGYGCAGCAGGCGCGA-3') and 915R (5'-GTGCTCCCCCGCCA-ATTCCT-3') for archaea. Polymerase chain reaction (PCR) was implemented in 25 µL with 0.5 µM of forward and reverse primers, 1 µL of template, and 12.5 µL of primeSTAR Max Premix (Takala, Dalian, China). The thermal cycling consisted of initial denaturation at 98 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 15 s, with a final extension at 72 °C for 5 min.

Power Soil DNA Isolation Kit Protocol

To extract total genomic DNA from each sample, 0.25 g portions of samples were added to power bead tubes and gently mixed by vortex. Then 60 µL of solution C1 was added to each sample, mixed at maximum vortex speed for 10 min. followed by centrifugation for 30 seconds. Supernatant was transferred to clean 2 mL tubes. Then 250 µL of solution C2 was added and mixed for 5 s, incubated at 4°C for 5 min, followed by centrifugation for 30 s. Up to 600 µL of supernatant was transferred to a clean 2 mL tube, avoiding transfer of the pellet. Then 200 µL of solution C3 was added and briefly mixed, then incubated at 4 °C for 5 min followed by centrifugation for 30 s. Up to 750 µL supernatant was transferred into a clean 2 mL tube, then 1.2 mL solution C4 was added and mixed for 5 s. Next, 675 µL was loaded onto a spin filter and centrifuged for 60 s. The flow was discarded, and the loading was repeated twice more. 500 µL of solution C5 was added and the mixture centrifuged for 30 s. The fluid phase was discarded, then the solids were centrifuged for 60 s and placed spin filter in a clean 2 mL tube; 100 µL of solution C6 was added and placed in the center of the filter membrane and centrifuged for 30 minute. Finally, the spin filter was discarded, and the DNA in the tube was ready for the subsequent application

Pyrosequencing and Sequencing Analysis

The PCR products were purified and assessed quantitatively using the QIA quick gel extraction kit (Qiagen, Seoul, South Korea), then clustered at equal concentrations. The PCR products were sequenced on a Roche GS FLX 454 pyrosequencing platform in

the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The initial sequences were trimmed to reduce the impact of random sequencing errors, and then the data were pooled to operational taxonomic units (OTUs), as previously described (Yang *et al*. 2012). The initial removal of sequences was intended at those less than 200 bp and quality degree less than 25. A total of 73415 and 38024 high-quality 16S rRNA gene sequences were achieved from bacteria and archaea, respectively, with an average length of 482 bp and 533 bp. For any individual library, greater than 10,000 sequence reads were obtained from bacteria and 5000 sequences from archaea. Chao1 (non-parametric method or species estimator) was used to estimate the species abundance as described by (Chao 1987). The ACE and the Shannon index (diversity index) were computed using MOTHUR (software package used in the analysis of DNA). Principal coordinate analysis (PCoA) based on the weighted uni-Frac distance assessed the divergences in community composition. Canonical correspondence analysis (CCA) was conducted using Conoco for windows (version 4.5, Wageningen, Netherlands).

RESULTS AND DISCUSSION

Microbial Community Analysis in the Coupling Fermentation at Genus Level

As shown in Fig. 2, there are three intervals for the first stage (R1), which converted stillage of ethanol distillation to methane (AD) 10, 20, and 30 days, coupled with second stage (R2), which converted FW to ethanol used recycled water.

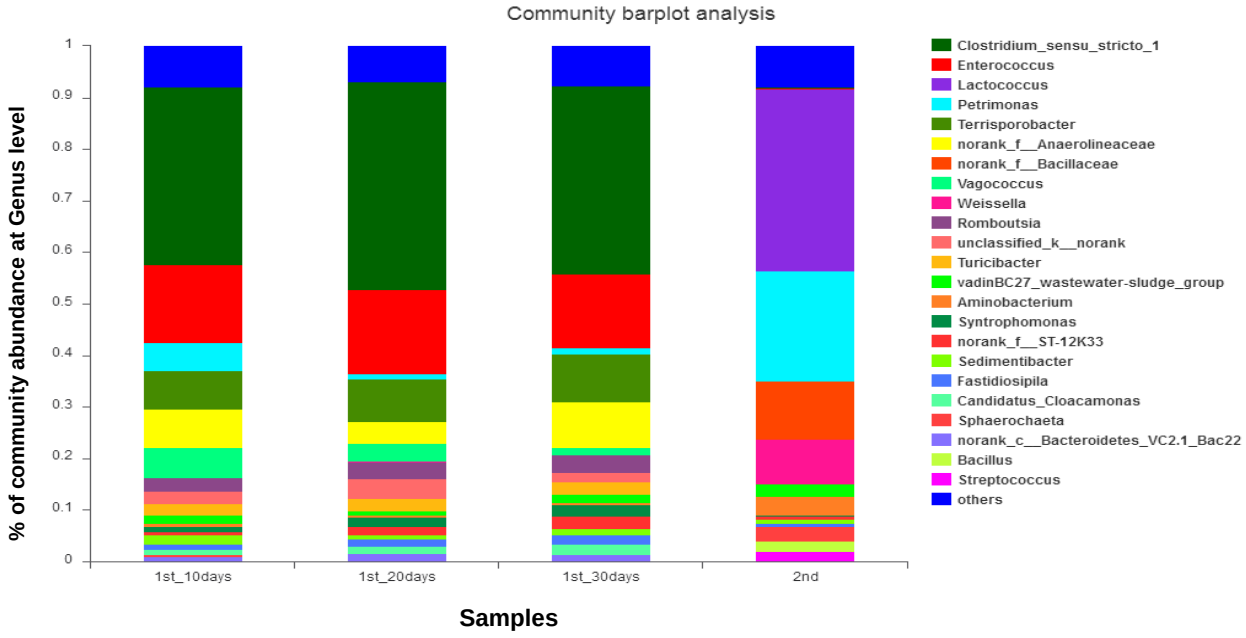

Fig. 2. Bacterial community structure at the genus level in the two stages coupling fermentation

The bacterial community analysis revealed that the dominant genus in the three intervals of (R1) was *Clostridium-sensu-strito-1*. *Clostridium* species is a major diverse category of anaerobic bacteria (Gram-positive rods), constituted of hydrolyzing and fermentative bacteria (syntrophic acetate-oxidizing bacteria (SAOB)) (Hagen *et al*.

2014). Thus, the *Clostridium* spp. showed a considerable proportion during all stages of anaerobic digestion, as tested on days 10, 20, 30, with amounts of 34.58%, 40.39%, and 36.57%, respectively. Interestingly, *Clostridium* could facilitate the syntrophic acetate oxidation and have capability for cooperation with hydrogenotrophic methanogens. The low existence of *Clostridium* genus in (R2), which are facultative anaerobes, demonstrated the unique characteristics of this genus that air acts as poison for their growth. Although there were no noticeable changes in the bacterial compositions during the overall period of the first stage, considerable discrimination was detected in a relative abundance of each genus. The proportion of *Enterococcus* observed as the second high abundance genus in first stage (R1) in this order (15.11, 16.25, and 14.23%) during the various intervals. *Enterococcus* is a facultative anaerobic bacterium that represent a dominant genus of lactic acid bacteria (LAB) (Gram-positive, and often occur in pairs). They are subgroup of streptococcus species, tolerant of a wide domain of environmental conditions including temperature of (10 to 45 °C), pH (4.5 to 10.0), and high contents of sodium chloride (Fisher and Phillips 2009). Regarding the second stage (R2), the dominant genus observed was *Lactococcus. Lactococcus* is a genus of LAB, which was previously classified as one genus of the *Streptococcus* group. They are homofermentive bacteria that convert glucose to lactic acid. Lactic acid is further converted to ethanol. Furthermore, 21% of *Petrimonas* was detected; members of this genus ferment carbohydrates as well as organic acids. In addition, there are low relative abundance of other bacterial genus.

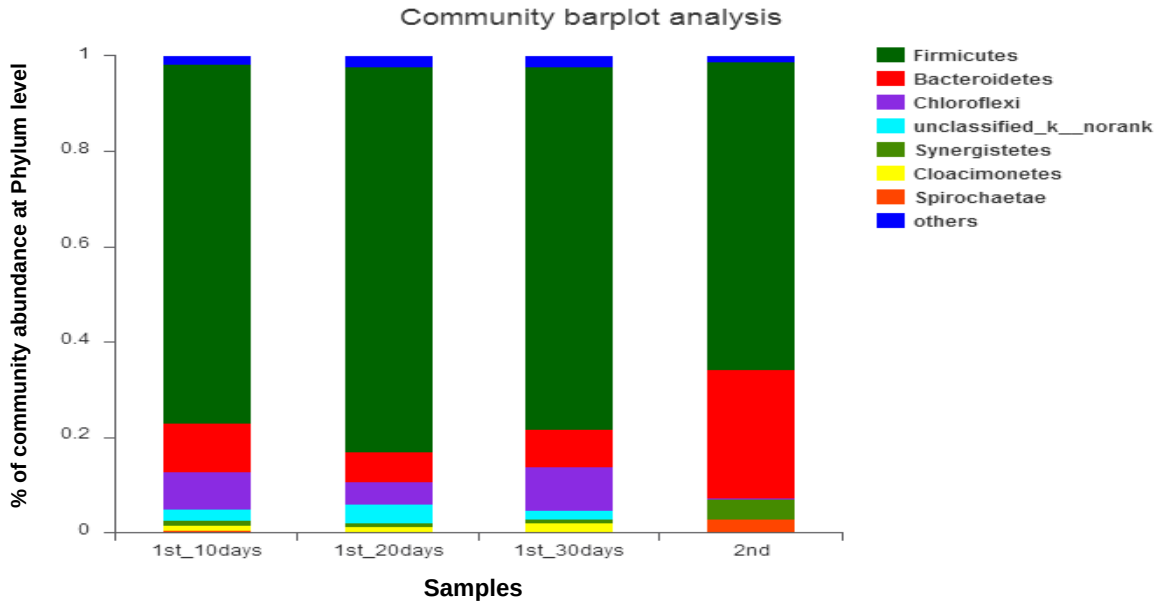

Fig. 3. Bacterial community structure at the phylum level in the two stages coupling system

The archaeal community analysis at the genus level for coupling fermentation showed lower diversity than bacteria. Figure 4 shows that more than 92% methanogens species (aceticlastic methanogens) had been the dominant species detected in methane reactor (R1) during fermentation period, to be composed of 80% *Methanosaeta* and 12% *Methanosarsina*. Although the volatile fatty acids was reduced within the period of 10 to 20 days that is favored by methanosaeta species, increased of total ammonia nitrogen (TAN) may inhibit their metabolism. Previous study showed that total ammonia

concentration of greater than 1700 mg/L could cause inhibition to *Methanosaeta* (Franke-Whittle *et al*. 2014). However, the proportion of *Methanosaeta* rose to 88% in spite of the rise in TAN to more than 2000 mg/L, indicating that TAN did not greatly affect the microbial community. *Methanosaeta* form multicellular clusters, which resist the inhibition effect due to accumulation of VFAs and TAN (Lin *et al*. 2012). In the period of 20 to 30 days the amount of *Methanosaeta* again decreased to 80%, while *Methanosarsina* relative abundance rose to 13%. This result demonstrates its faster adaptation to changes in conditions and its capability to utilize acetate, hydrogen, and ethanol as energy sources (Franke-Whittle *et al*. 2014). Notably, methanogen species convert VFAs and ethanol into acetic acid, which promotes the growth of aceticlastic methanogens.

As reported in previous research, methane reactors showed a dominance of *Methanosarsina* rather than *Methanosaeta* at the higher concentration of acetate (> 70 mg/L) because of the lower affinity of *Methanosarsina* for acetate than *Methanosaeta* (Kim *et al*. 2014). However, this study revealed the opposite pathway. This may be related to other factors that affected the competition between *Methanosaeta* and *Methanosarsina* such as hydraulic retention time, inoculate resources, and operating conditions. The optimal condition for aceticlastic methanogens (*Methanosaeta* and *Methanosarsina*) is pH 7.0 to 7.5 and temperature of 37 to 45 °C (Kim *et al*. 2015); the conditions in this study supported their growth. Regarding ethanol reactor, methanogens showed low abundance in the second stage (R2) with a relatively high abundance of *Lactococcus* (LAB genus).

Microbial Community Analysis in the Coupling Fermentation at the Phylum Level

The differences in microbial communities during the various process stages were examined at the phylum level. As shown in Fig. 3, the bacterial community at the phylum level showed relatively lower variation than genus level. The *Firmicutes* were dominant throughout the coupling fermentation period in both R1 and R2 reactors. *Firmicutes* are often classified to *Clostridia* (SAO) and *Bacilli* (Hagen *et al.* 2014). *Clostridia* are efficient in the production of hydrogen, which is then used by hydrogenotrophic methanogens. Hydrogenotrophic methanogens utilize hydrogen and carbon dioxides as carbon and energy sources. *Clostridia* can make a syntrophic association with hydrogenotrophic methanogens and promote organic acids degradation (Kim *et al*. 2014). In addition, there were relatively low abundance of *Bacteroidetes*, which increased drastically when *Firmicutes* decreased during all fermentation periods in both reactors. These findings were consistent with a previous report on anaerobic digestion (Hagen *et al.* 2014).

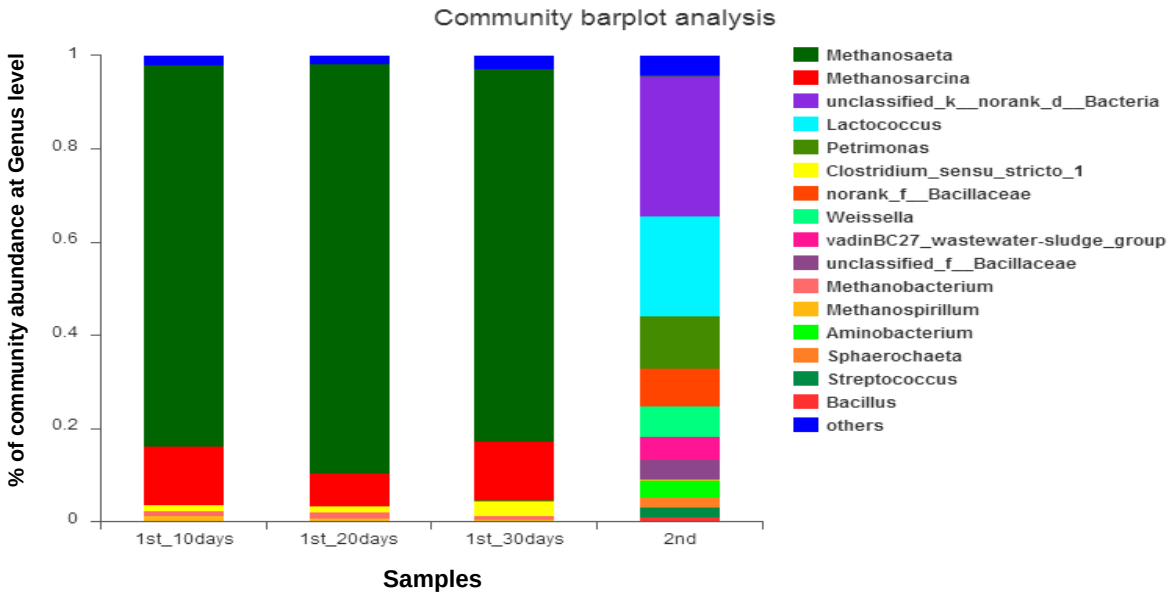

Fig. 4. Archaeal community structure at the genus level in the two stages coupling system

The distribution of the archaea sequences at the phylum level in each sample is displayed in Fig. 5. *Euryarchaeota*, *Firmicutes*, *Bacteroidetes*, *Synergistetes*, and *Spirochaetae* were the common species detected in the whole coupling fermentation process. This classification is comparable with those previously reported in food waste anaerobic digestion, *i.e.* 10 phyla with relative abundance of higher than 0.5% in any event one sample. Among them *Firmicutes* was the dominant phyla in the whole process (Guo *et al*. 2014). In the current study, *Euryarchaeota* showed high relative abundance with more than 90% throughout the first stage intervals. The proportion of *Firmicutes* increased inversely with the proportion of *Euryarchaeota* in all samples of first stage (R1). In the second stage (R2), *Euryarchaeota* appeared with a very low amount (0.4%), and *Firmicutes* appeared as the predominant phyla. This stage showed more phyla classification than the first stage. In sum, the phyla *Euryarchaeota* was dominant in the first stage samples, while *Firmicutes* was dominant in the second stage. These results indicated clear differences in the archaeal community in the two stages of coupling fermentation. However, there were some species transmitted from one reactor to the other.

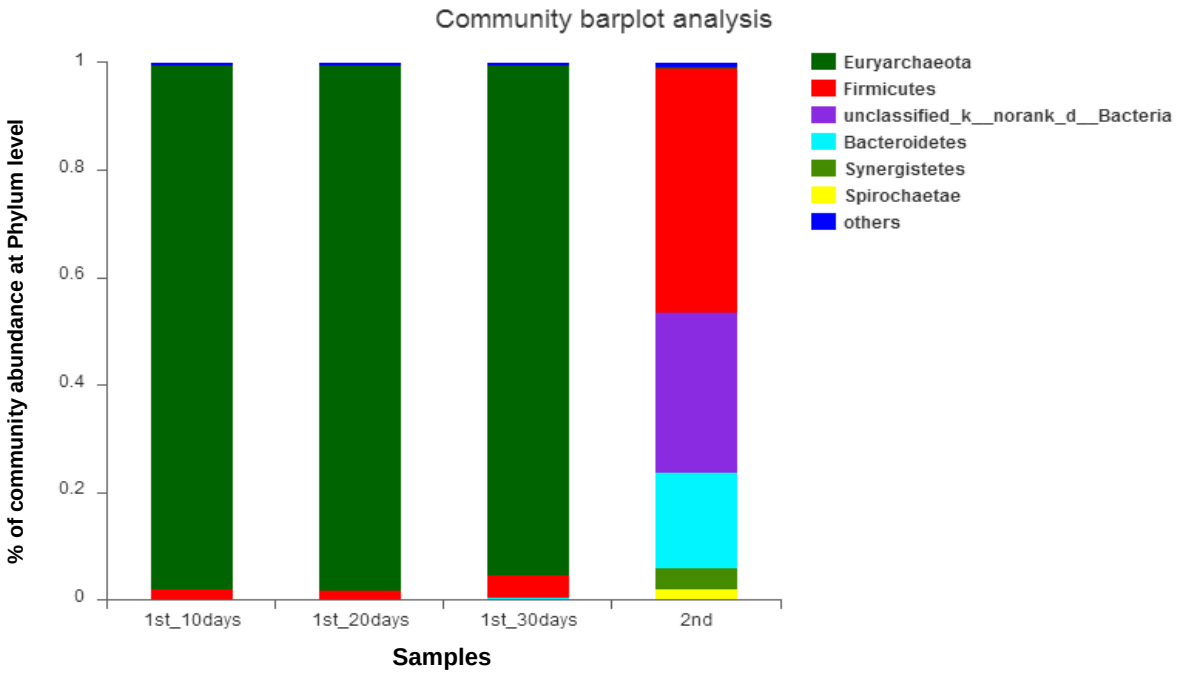

Fig. 5. Archaeal community structure at the phylum level in the two stages coupling system

CONCLUSIONS

1. Ethanol-methane coupling fermentation from food waste is an attractive technology for biofuel generation. This technology involves various microbial processes for biodegradable of organic components, which is governed by different kinds of bacteria. The observation revealed that the diversity of bacterial community structure at genus level was higher than that of phylum level in both reactors.
2. The diversity of archaeal community structure at the genus level was also higher than at the phylum level. However, the diversity of archaeal community structure in the second reactor was more than the first reactor. This clearly showed the effect of methane effluent on the ethanol reactor microbial species.
3. The existence of more than 92% of methanogens species in a methane reactor could facilitate methane production.
4. *Euryarchaeota* is a phylum of archaea that is predominant in the methane reactor with more than 90%, while it appeared with less than 0.4% in the ethanol reactor. Hence, *Euryarchaeota* is the methanogens type that can survive under extreme concentrations of salt. That indicated efficient utilization of stillage.
5. It must be considered for further studies, the effect of operational factors, such as hydraulic retention time and inoculate resources on the microbial community in ethanol-methane coupling fermentation process.

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