RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2019, **9**, 29570

Impact of physical structure of granular sludge on methanogenesis and methanogenic community structure

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Physical structures of sludge are critical factors determining the performance of the anaerobic digestion process, especially for the rate-limiting step, methanogenesis. Thus, to evaluate the effect of granular physical structure on methanogenesis and methanogenic community variation, intact and disintegrated granules were applied as inocula with formate, hydrogen and acetate as sole substrates in batch reactors. Kinetics results revealed that the physical structure of sludge had little impact on methane yield potential from three substrates, while a significantly different impact on methanogenesis rates of formate, hydrogen and acetate. The methanogenesis rate of formate in disintegrated granules was higher than that in the intact granular system, the methanogenesis rate of H_2/CO_2 in the intact granular system was higher than that in the disintegrated granules and the methanogenesis rate of acetate was similar with the in intact and disintegrated granular systems. Besides, in both intact and disintegrated granular systems, methanogenesis rates of formate were the highest, then followed the H_2/CO_2 and acetate was the lowest, indicating formate consumption has an advantage over hydrogen in the studied system. A microbial assay indicated that Methanobacteriales, Methanosarcinales and Methanomicrobiales are dominant methanogens on the order level, and the physical structure of granular sludge has little influence on methanogenic communities on the order level but showed significant influence on the species level. It enlightens us that the physical structure of sludge could be considered for regulating the anaerobic digestion *via* influencing the methanogenesis rates.

Received 6th June 2019
Accepted 5th September 2019

DOI: 10.1039/c9ra04257a

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Introduction

Anaerobic biological treatment technology is widely known as an important strategy for simultaneously achieving pollutant removal and energy generation.[1,2] However, the traditional anaerobic digestion (AD) process still has the challenge of low treatment efficiency, which is limited by the slow syntrophic metabolism of fermentation intermediates (alcohol and volatile fatty acids (VFAs)).[3–5] Many researchers have reported that syntrophic VFAs oxidization contributes to too much carbon flux in anaerobic methanogenic environments, which would directly lead to VFA accumulation.[6] VFAs (mainly propionate and butyrate) are metabolized by syntrophic communities of fatty acid-oxidizing bacteria and methanogens in an anaerobic methanogenic environment. The thermodynamically unfavourable reactions of VFA degradation required the rapid consumption of intermediates as well as methanogenic precursors, such as formate, acetate and H_2/CO_2, to make VFA degradation feasible.[7] Thus, in some cases, methanogenesis is considered as the rate-limiting pathway, since methanogens are relatively slow-growing microorganisms and have a limited range of available substrates,[8] in addition, these substrates' removal determined the efficiency of syntrophic VFAs degradation. Therefore, the high efficiency of the methanogenesis process was essential for the performance of AD process.

Methanogenesis process is dependent on several process factors of which the most important are nutrients, temperature, pH and inhibitors.[2,9] These factors influence the microbial activities and change the methanogens community structure to affect the efficiency of methanogenesis. In addition to biological structure, the physical structure of sludge was also viewed as a significant factor affecting the process of methanogenesis, because the diffusion distance between the syntrophic bacteria and methanogens may have a large impact on the rate of VFAs conversion as well as the efficiency of methanogenesis. High conversion rates of syntrophic substrates in methanogenic bioreactors are due to the high cell densities and short

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interbacterial distance (<10 μm) between syntrophic–methanogenic associations in granular sludge system.[10] Granular sludge, therefore, was supposed to a suitable system for anaerobic methanogenesis, since its structure could enhance the efficiency of interspecies transfer of formate, hydrogen and acetate,[9] and further improve the efficiency of the anaerobic process. Previous studies about the physical structure of granules mainly focused on the diffusion distance and granular layer structure,[10–12] while it is still unclear that whether the physical structure of granules can affect the microbial community structure shift.

Besides, formate, hydrogen and acetate, as main methanogenic precursors, are intermediate products of syntrophic metabolism, and the methanogenesis rates from these substrates determined the efficiency of syntrophic VFAs degradation. Some researchers found that the physical structure of sludge influenced the relative importance of hydrogen/formate transfer, and it supported that hydrogen transfer dominates in granular system, and formate dominates in flocs sludge system.[9] However, our previous study[13] revealed that formate presented rapid consumption rate. And this result was not corresponded to the expectation that H_2/CO_2 consumption by methanogens in the granular system was faster than formate and acetate according to Angelidaki and Batstone (2010).[9] Therefore, it is necessary to re-evaluate the effect of aggregated/flocculent structure of sludge on methanogenesis from the main intermediates during the AD process.

Comprehensive research is required to evaluate the impact of the physical structure of granular sludge on the shift of methanogens community structure and the methanogenesis rates from formate, acetate and H_2/CO_2. It could provide a better understanding of methanogenesis in anaerobic systems and further provide an efficient approach to improve the performance of AD. Therefore, batch reactors inoculating intact granules or disintegrated-granules with a different substrate (formate, acetate, or H_2/CO_2) were incubated for several days under mesophilic condition. Methane production from each reactor is measured along with the digestion time. Kinetic parameters of methanations from formate, acetate and H_2 were simulated utilizing a modified Gompertz model. Microbial communities associated with various reactors were analyzed using Illumina 16S rRNA gene amplicon sequencing.

Results and discussion

Physical structure of granular sludge affects methanogenesis kinetics

Under the assumption that methane production is paralleled with microbial growth in batch reactors, a modified Gompertz model was used to predict the kinetics parameters of methanogenesis from formate, acetate and H_2/CO_2 in both intact granular and disintegrated-granular systems. The predicted curves for specific CH_4 yield along with digestion time fit well with observed values in each system, with $R^2 > 0.98$ (Fig. 1, Table 1).

In the first generation, all of the batch reactors were inoculated with the same amount of sludge, the only difference was the physical structure of the sludge, consisting of intact granular sludge and disintegrated-granular sludge. Assuming that the inoculating error was within an acceptable range, the difference in methanogenesis from a specific substrate in a granular system and a homogeneous-granular system could completely be attributed to the difference in the physical structure of the sludge.

In general, the cumulative production of CH_4 increased along with digestion time in both granular and homogeneous-granular systems. CH_4 reached a stable value when the substrate was completely consumed (Fig. 1). Initially, formate was metabolized immediately in the granular system with a short lag time, while a little longer lag time (0.41 d) occurred in the disintegrated-granular system. This could be resulted from the high microbial densities in the granules, which would minimize formate transfer resistance.[9] The maximum CH_4 production rate from formate in the granular system was higher than that in the disintegrated-granular system ($U_{G\_formate} > U_{HG\_formate}$). The methane yield potential in the two systems seemed to have no significant differences, and the values were about 450–471 N mL_{CH_4} g_{COD}^{-1}. This indicated that the physical structure of granular sludge had an impact on methanation rate rather than the methane production potential. In addition, these values of methane production potential were significantly larger than that in digested manure (85–105 NmL_{CH_4} g_{COD}^{-1}) and sewage sludge (57.74 N mL_{CH_4} g_{COD}^{-1}) systems examined in a previous study.[13] This might due to the varying quantities and activities of methanogens in these systems.

The observed trend of specific methane production from H_2/CO_2 along with incubation time was quite similar to it in the formate added system. In addition, methane production also required a longer lag time in granular system than that in disintegrated-granular system (Table 1). However, the maximum CH_4 production rate from H_2/CO_2 in disintegrated-granular system was higher than that in granular system during the two-generation incubation ($U_{HG\_H_2/CO_2} > U_{G\_H_2/CO_2}$), which is contrary to the results obtained from formate added system. The physical structure of granule had a significantly different impact on methane production rates from formate and H_2/CO_2. This could be explained by the previous finding that more hydrogen-consuming methanogens would be expected to be freely suspended in the medium after the disintegration of granules.[14]

Generally, acetate metabolism by methanogens required a longer adaption time than formate and H_2/CO_2, and the lag time in the disintegrated-granular system can reach approximate one day. The values of methane production potential were quite close to both the granular and disintegrated-granular systems. Moreover, the maximum CH_4 production rate in the granular system was also similar to the rate in the disintegrated-granular system ($U_{HG\_acetate} \approx U_{G\_acetate}$), which means the physical structure of a granule had little influence on methanogenesis from acetate (Table 1).

In conclusion, the consumption of three substrates in disintegrated-granular systems required longer lag time. The physical structure of granular sludge has little impact on methane production potential in different systems, while influenced the maximum methane production rates.

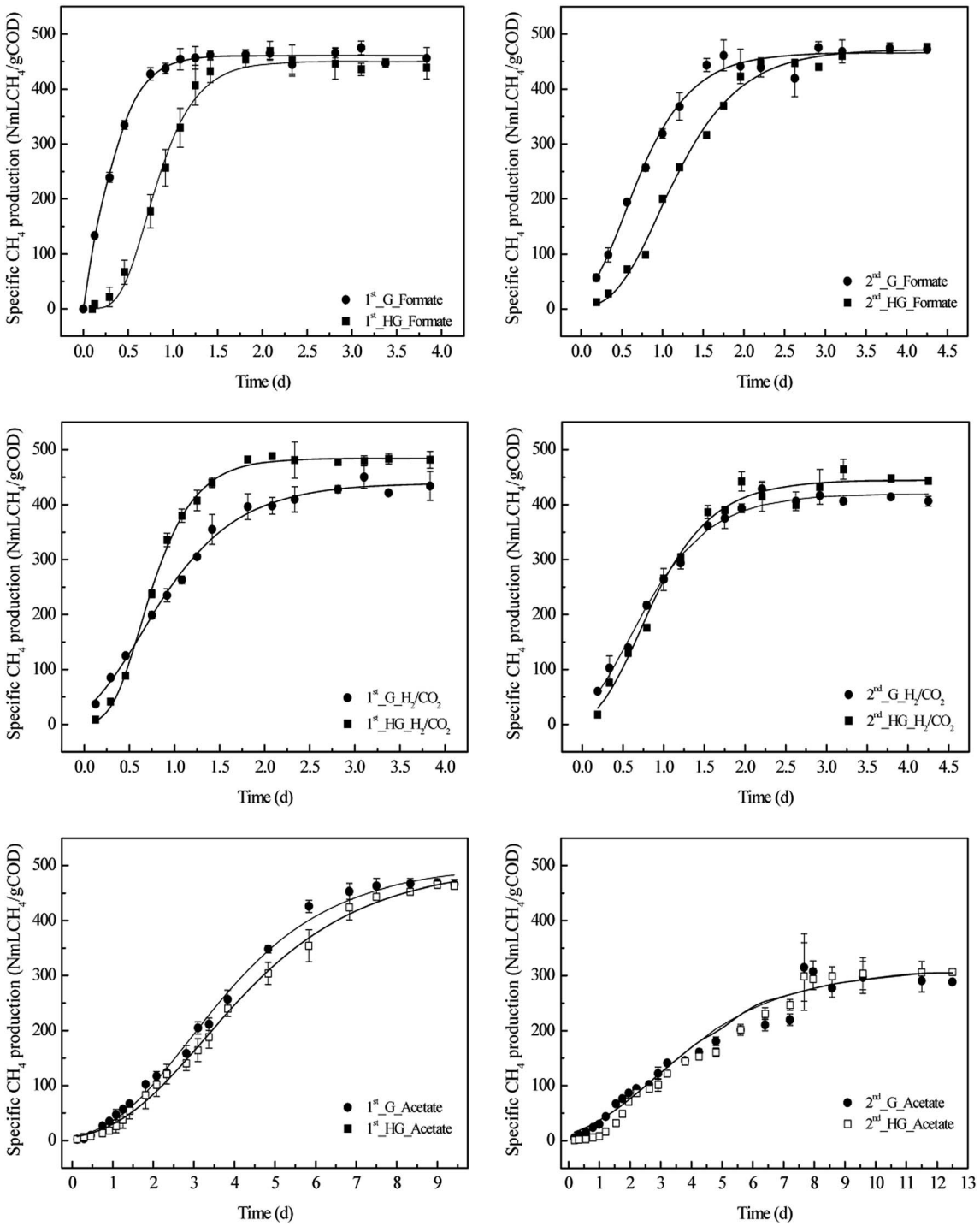

Fig. 1 Comparison between experimental data and prediction using modified Gompertz model in granular (G) and homogeneous-granular (HG) systems. Points are experimental data while line is model prediction. 1st stands for first generation incubation and 2nd for second generation incubation.

Comparison of methanogenesis rates from different substrates

In an intact granular system during second-generation incubation, the order of maximum CH_4 production rates from three substrates was as follows: $U_{G\_formate} > U_{G\_H_2/CO_2} > U_{G\_acetate}$. This finding is supported by previous research that formate was consumed faster than H_2/CO_2 in a granular system derived from an IC reactor.[13] Although part of the reason for the lower H_2/CO_2 degradation rate in the granular system was that H_2/CO_2 was supplemented as an external carbon source in a gas phase. The hydrogen has to transfer from the gas phase to the bulk liquid phase. This process could increase the mass transfer resistance

Table 1 The kinetic parameters simulated by modified Gompertz model[a]

		Parameters							
		A (N mL_{CH_4} g_{COD}^{-1})		U (N mL_{CH_4} g_{COD}^{-1} d^{-1})		λ (d^{-1})		R^2	
Generations		1st	2nd	1st	2nd	1st	2nd	1st	2nd
Formate	Granule	460.80 ± 5.62	465.76 ± 6.98	710.40 ± 11.35	381.70 ± 9.35	0.05 ± 0.01	0.08 ± 0.01	0.994	0.987
	H-Granule	450.21 ± 7.83	471.66 ± 8.96	556.14 ± 10.63	319.05 ± 8.61	0.41 ± 0.03	0.41 ± 0.02	0.989	0.994
H_2/CO_2	Granule	439.69 ± 8.36	419.69 ± 10.36	271.55 ± 6.96	242.05 ± 11.32	0.02 ± 0.01	0.02 ± 0.01	0.995	0.992
	H-Granule	484.31 ± 9.32	444.85 ± 5.63	540.33 ± 10.30	317.19 ± 10.23	0.29 ± 0.01	0.19 ± 0.03	0.998	0.986
Acetate	Granule	499.79 ± 8.24	309.79 ± 7.96	94.38 ± 7.63	48.02 ± 2.67	0.95 ± 0.10	0.37 ± 0.01	0.996	0.972
	H-Granule	497.97 ± 10.21	316.91 ± 10.36	84.90 ± 4.36	52.29 ± 3.26	1.09 ± 0.09	0.99 ± 0.04	0.998	0.985

[a] H-Granule stands for disintegrated granules and n value is 3.

compared to a real anaerobic granular system in which H_2/CO_2 is formed by syntrophic fatty acid bacteria closed to the methanogens.[9,13] Although there is no study to show the direct relation between methanation rate from formate/H_2 and the relative importance of interspecies formate/H_2 transfer, the rate of CH_4 conversion from methanogenic substrates has a positive correlation with the efficiency of interspecies electron transfer.[15] Since interspecies electron transfer during the anaerobic methanogenic process is generated using an electron-donating partner and carried by formate, acetate and hydrogen, and eventually sinks into methane. Dong and Stams (1995)[15] revealed that only formate-lux could result in a fast methane production rate. It concluded that formate was the main interspecies electron carrier in mesophilic suspended co-cultures, which indicated that formate consumption and formate transfer has close relations. Therefore, the high consumption of formate in the studied granular system revealed that formate might play an important role in the studied granular systems as an intermediate product. The lowest methanation rate from acetate was reasonable, since acetate is more complex than the other two substrates, which had been proven in a previous study performed by Pan *et al.* (2016).[13] In addition, research focusing on layered structure and juxtaposition of syntrophs and methanogens in the consortia revealed that the inner layer is mostly comprised of acetoclastic methanogens and the outer layer consists of fermentative bacteria.[11,16]

Similarly, the order in a homogeneous-granular system during second-generation incubation was also $U_{HG\_formate} > U_{HG\_H_2/CO_2} > U_{HG\_acetate}$. But actually, the variation in methanation rates between formate and H_2 was quite small. As mentioned above, the physical structure of sludge had opposite effect on formate and H_2/CO_2 methanation, with the disrupted granules decreasing the methanation rate from formate and increasing rate from H_2/CO_2. A higher methanation rate than observed may be obtained by deducting the increased mass transfer resistance of H_2/CO_2. Thus, it is difficult to define whether formate or H_2/CO_2 consumed faster in the studied homogeneous-granular system.

Therefore, the results revealed that either in the granular system or disintegrated-granular system, formate had a rapid conversion. Although there was no direct evidence to prove that formate was the dominant interspecies electron carrier in the studied granular system, it can be deduced that the role of formate played in a granular according to the result of rapid formate consumption. Thus, more attention should be paid to formate conversion when regulating and controlling of anaerobic digesters.

Microbial community variation

Alpha diversity analysis. The diversities of methanogens in each batch reactor were analyzed based on the 16S rRNA gene. There was no significant difference between each batch reactor in the numbers of orders and families determined, but differences occurred at the species level (Table 2). The numbers of species in the experimental samples were less than in the original granular sample, while sequence reads in the formate and H_2/CO_2 supplemented systems were slightly more than in the original granular sludge. This indicated that specific substrate (formate, acetate, or H_2/CO_2) incubation could have resulted in a reduction of methanogen diversity and an increase in the abundance of functional species.

Diversity and relative abundance of methanogens. In original granular system (G0), the relative abundances of detected methanogens accounted for 97.22% of the total archaea. The methanogens consisted of *Methanobacteriales* (48.43%), *Methanosarcinales* (36.97%) and *Methanomicrobiales* (11.82%) at the order level (Fig. 2a). *Methanobacteriales* mainly consisted of the species of *Methanobacterium beijingense*, an H_2/formate-utilizing methanogen,[17] and *Methanobacterium formicicum* (1.53%), which was found in culture with formate as the sole energy source in a pH-stat fermenter.[18] *Methanosaeta concilii* was the predominant species of *Methanosarcinales* in the original granular system, known as a critical organism in granulation.[19] *Methanomicrobiales*, an H_2/formate-utilizing methanogen of *unclassified_Methanolinea*, was the main species found in the original granular system.

For experimental systems, the relative abundance of *Methanosarcinales* increased compared to the abundance in the original granular sludge, and *Methanosarcinales* dominated in both intact granular and disintegrated-granular systems after two generations of incubation. *Methanomicrobiales* also showed a significant increase in both systems as well, with a relative

Table 2 Numbers of methanogens detected at different levels and alpha diversity index[a]

Levels	G0	G1	HG1	G2	HG2	G3	HG3
Orders	5	5	5	5	5	5	5
Families	15	14	15	15	15	13	14
Species	28	21	24	23	26	23	26
Sequence reads	46 342	46 961	46 736	41 544	42 648	48 109	56 489
Chao	37.00	26.00	25.33	27.60	33.25	24.00	33.00
Ace	37.43	29.66	26.38	29.38	34.41	24.45	36.28
Shannon	1.51	1.56	1.22	1.24	1.72	1.47	1.47
Simpson	0.68	0.70	0.54	0.59	0.73	0.71	0.61
Shannoneven	0.42	0.50	0.38	0.38	0.49	0.46	0.43

[a] G1 and HG2 were the samples from formate added granular and homogeneous-granular system; G2 and HG2 were the samples from acetate supplemented granular and homogeneous-granular system; G3 and HG3 stand for the samples from H_2/CO_2 injected granular and disintegrated-granular system.

abundance of 22.06% in the G1 reactor (formate added granular system) and 19.86% in the HG1 reactor (formate added disintegrated-granular system). However, the relative abundance of Methanobacteriales presented an obvious reduction, and the values dwindled to 8.01% and 4.17% in the G1 and HG1 reactors, respectively (Fig. 2a). Therefore, the G1

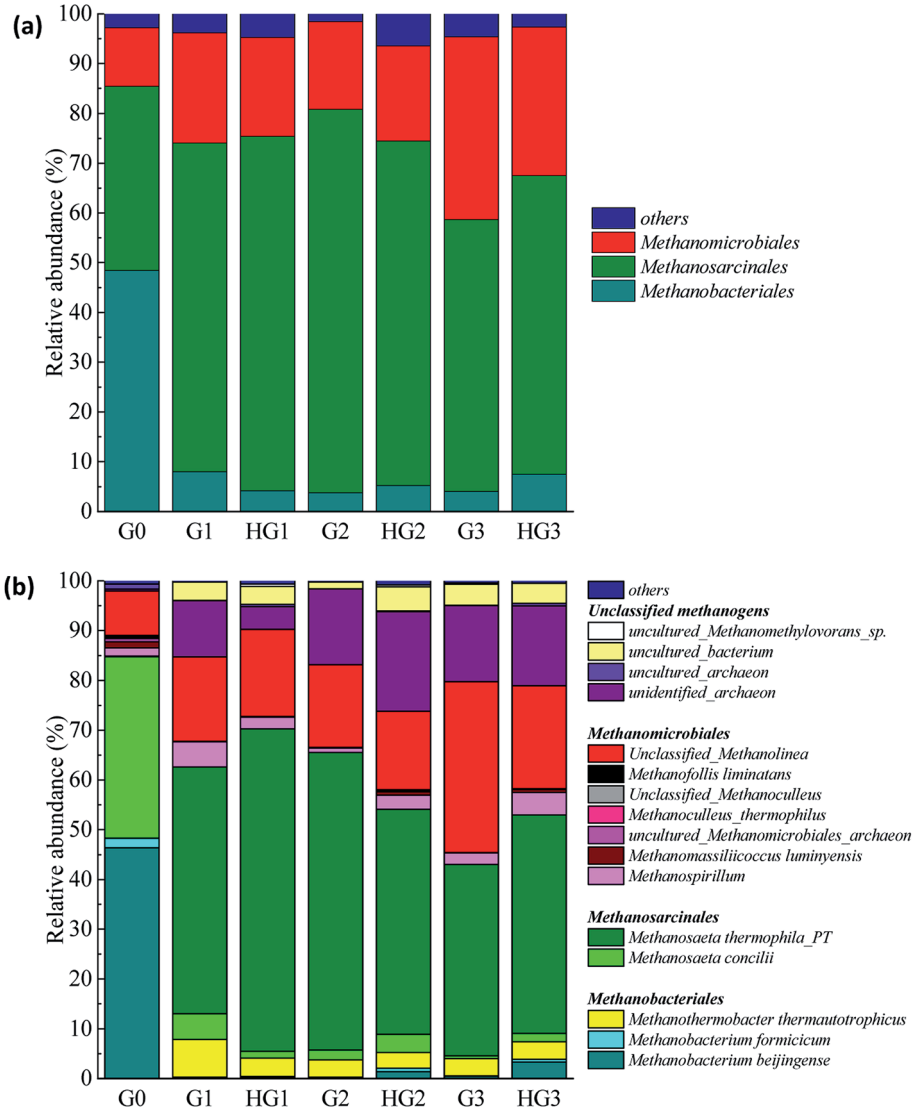

Fig. 2 Microbial community abundances in each batch reactors at order (a) and species (b) levels.

reactor had more hydrogenotrophic methanogens (30.07%) than the HG1 reactor (24.03%).

A small variation occurred between H_2/CO_2 that served as substrate reactors (G3 and HG3) at the order level (Fig. 2a). This means the physical structure of sludge has little effect on the microbial community shift on order level. In the G3 reactor (H_2/CO_2 added granular system), *Methanosarcinales* (54.60%), *Methanomicrobiales* (36.69%) and *Methanobacteriales* (4.04%) were detected, and *Methanosarcinales* (60.12%) was also the predominant methanogenic group in HG3 (H_2/CO_2 added the disintegrated-granular system), followed by *Methanomicrobiales* (29.83%) and *Methanobacteriales* (7.50%). At the species level, *Methanothermobacter thermautotrophicus* accounted for large percentages (43.85%) in the HG3 reactor. In addition, *Methanobacterium beijingense* (3.32%) and *Methanobacterium formicicum* were also detected in HG3. While the dominant specie of *Methanobacteriales* in the G3 system was *Methanothermobacter thermautotrophicus* (accounting for 3.53%). As for *Methanomicrobiales*, the HG3 reactor had a higher diversity than the G3, in which five species (relative abundance > 0.5%) were detected. While the G3 reactor contained fewer species, it had a higher relative abundance of *unclassified_Methanolinea* (20.72%) and *Methanospirillum* (4.52%).

Compared to the original granules, the *Methanosaeta concilii* outcompeted *Methanosaeta thermophila_PT* in both G2 (acetate added granular system) and HG2 (acetate added disintegrated-granular system) reactors. Surprisingly, no *Methanosarcina* spp. was observed in the studied system. However, comparing Fig. 2a and b, it was obvious that approximately 15% and 20% unclassified Methanosarcinales existed in the G2 and HG2 reactors, respectively. The uncultured methanogens might mostly consist of *Methanosarcina* spp. It has been reported that both *Methanosaeta* spp. (formerly *Methanothrix*) and *Methanosarcina* spp. were identified as important acetoclastic methanogens in granular sludge.[20] The similar microbial community structure and a similar amount of abundance of acetoclastic methanogens in the G2 and HG2 reactors could explain the similar values of U_{max} in the acetate added anaerobic batch systems. Theoretically, the structure of the sludge would have an impact on the rate of substrate consumption by microorganisms and thus influence the activity of functional microbes. This will finally cause a variation in microbial diversity and abundance, which would influence substrate degradation in turn. Therefore, it can be concluded that the physical structure of sludge had no significant effect on acetate methanation.

The impact of physical structure of granules on microbial community. The comparison of methanogenic community relative abundances between an original system, a formate added granular system, and a formate supplemented homogeneous-granular system revealed that the structure of microbial communities varied in the intact granular and homogeneous-granular system after two incubation generations with adding same substrate of formate. Similarly, the observed long-distance between point G3 and HG3 in Fig. 3 means a significant difference ($P < 0.05$) in microbial communities occurred between H_2/CO_2 added granular system and H_2/CO_2 added homogeneous-granular system. This result means that physical structure (aggregated or flocculent) could affect the methanogens community shift in anaerobic systems. But in the acetate added system, the variation between G2 and HG2 was quite small, and this supported the results for similar kinetics parameters predicted in reactors G2 and HG2. This means that microbial variation was related to the supplemented substrate. This statement is supported by previous studies that microbial composition and structure were influenced by adapting different substrate incubations in granular and suspended sludge systems.[21,22] Considering both the kinetic results and microbial communities detected in the experimental reactors, there was a higher abundance of formate and hydrogen-utilizing methanogens, *Methanothermobacter thermautotrophicus*, containing eight isolates, six of which were formate-utilizing ones[23] and *unclassified_Methanolinea*, in G1 than in HG1. This supports the results that the maximum CH_4 production in G1 was higher than in HG1. Similarly, the higher CH_4 production rate in the HG3 reactor during the second-generation incubation than in G3 could be attributed to the higher relative abundance of hydrogenotrophic methanogens, such as *Methanobacterium beijingense* and *Methanospirillum*.

Effect of substrates on microbial community shifts. Microbial community structures are possibly determined by substrate kinetics in anaerobic biomasses.[24] Generally, after two incubation generations, the microbial communities in intact granular and homogeneous-granular sludge systems showed significant difference from those in original granular sludge. Among them, the locations of G1, G2, and G3 were quite close according to a PCA plot (Fig. 3), which means the structure of the microbial communities in the granular systems supplemented with formate, acetate and H_2/CO_2, were similar. It indicated that substrates had no significant influence on the microbial community shift in granular sludge. The relative abundance of

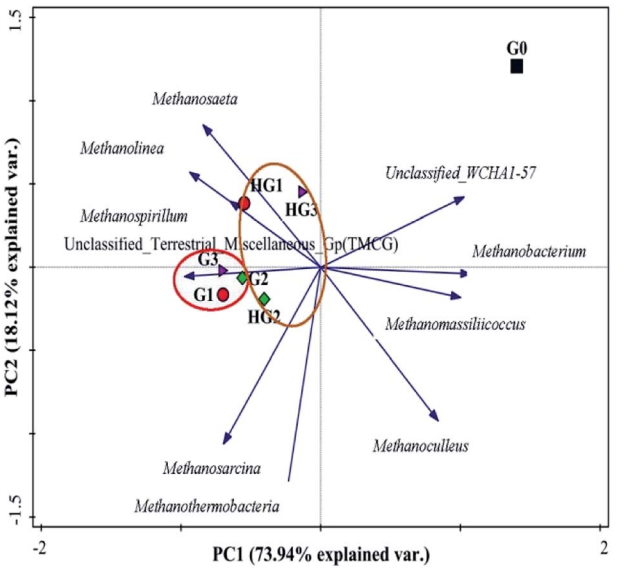

Fig. 3 The principal component analysis (PCA) of the microbial communities at family level between different batch reactors.

Methanobacteriales (*Methanothermobacter thermautotrophicus*) in G1 (formate added system) was higher than that in the G3 (acetate added system) reactor, while the relative abundance of *Methanomicrobiales* (*unclassified_Methanolinea*) in G3 was higher than that in G1. Both dominant species could utilize formate and H_2/CO_2, and the relative abundance of hydrogenotrophic methanogens in the G1 reactor (22.41%) was lower than in the G3 reactor (37.27%). These results could explain the decrease of the gap between $U_{formate}$ and U_{H_2/CO_2} in the second generation, with the values of the difference (N mL_{CH_4} g_{COD}^{-1} d^{-1}) reduced from 154.26 to 62.65.

In the disintegrated-granular system, the microbial communities structure in HG1, HG2 and HG3 showed a difference (Fig. 3), which indicated that different substrates had a different effect on methanogens community variation with inoculating disrupted granular sludge. These results agree with the previous statement that microbial community structures were related to substrates.[24] In addition, the similar amount of relative abundance of hydrogenotrophic methanogens in HG3 and HG1 supports the kinetics results that U_{H_2/CO_2} was quite close to $U_{formate}$ in the second generation.

Considering the small change in the methanogens community structure in the two systems and the kinetics results in the second generation, it can be concluded that the physical structure affects the methane production rate, and substrate instead of the physical structure of granular sludge contributed to the methanogens variation in different systems. Substrate conversion rate reflect the types and numbers of methanogens involved in substrate degradation.[22,25]

Experimental

Batch reactor experiments

Inoculum and substrates. Granular sludge was obtained from a mesophilic up-flow anaerobic sludge blanket (UASB) digester (pH = 7.4 ± 0.2) for alcohol wastewater AD from the Green Environmental Technology Company, Xuzhou. The mean diameter of granules is 1.5–2.0 mm, which was measured using a particle size analyzer (ZetaPLAS). Disintegrated granular sludge was obtained by disintegrating the initial granules with a tissue homogenizer under N_2 gas (Thomas Scientific). Methanogenic activity tests of both granules were conducted, and the detailed method is described in Soto *et al.* (1993).[26] The results revealed that the treatment of granules did not cause cell lysis and loss of microbial activity with the potential methanogenic activity of 5.5 and 5.7 μmol_{CH_4} g_{COD}^{-1} min^{-1} intact granular system and disintegrated granular sludge, respectively. The volatile solids (VS) and total solids (TS) were measured using the standard method,[27] with VS of 57.76 g L^{-1} and VS/TS of 0.75.

The substrates of formate and acetate were supplied by HCOONa and CH_3COONa (Sigma Chemicals). Stock solutions of 0.8 M HCOONa and 0.2 M CH_3COONa were prepared with distilled water. Thus, a 5 mL stock solution complementation could theoretically result in a 1 mmol CH_4 product (Table 3). A mixture gas of hydrogen and carbon dioxide with a ratio (v/v) of 4 : 1 was prepared using pure H_2 and CO_2 with a purity of 99.999%. The determination of the ratio of 4 : 1 was based on the stoichiometric coefficient of methanogenesis of H_2/CO_2 (Table 3).

Experimental setup. Batch experiments were conducted in 310 ± 1.5 mL serum bottles with working volumes of 50 mL. Three milliliters of granular sludge (or disintegrated granules), 15 mL of basic anaerobic medium (pH = 7.0), 0.5 mL $Na_2S{\cdot}9H_2O$ (2.5%), and 26.5 mL of distilled water were added to serum bottles and flushed with 99.99% N_2 for 15 minutes. The preparation of the basic anaerobic medium followed the procedures described in Angelidaki and Sanders (2004).[28] The serum bottles were sealed with rubber stoppers and aluminum caps to maintain an anaerobic condition. All these batch reactors were incubated in a thermostatic shaker at 37 ± 1.0 °C and 160 rpm after substrate addition. The amount of substrate addition was determined according to the stoichiometry in Table 3 to obtain the same amount of theoretical methane production (1 mmol CH_4). Thus, a 5 mL stock solution of formate (0.8 M) and acetate (0.2 M) was injected using a syringe (maximum span of 5 mL) with a final mass of 4 mmol and 1 mmol of formate and acetate, respectively. An H_2/CO_2 (4 : 1, v/v) mixture of gas was added to the systems, and 5 mmol of mixture gas was injected into serum bottles using a syringe (maximum span of 50 mL). The actual injected volume was calculated and modified using laboratory temperature and pressure methods according to the ideal gas law ($PV = nRT$), and 5 mL distilled water, instead of a substrate, was supplemented to make the same working volume of 50 mL. In addition, 5 mL of distilled water, instead of a substrate, was added in the control experiments, following the same procedures as found in experimental batch reactors. To reduce heterogeneity from the inoculum, all the performances were conducted in triplicate.

The first-generation incubation was finished when the substrates were consumed completely by methanogens in the batch reactors. To evaluate the variation of methanogens communities and the methanogenesis rates in the cultivated sludge using a sole substrate, a second-generation incubation was conducted. 5 mL of supernatant liquid was extracted from each batch reactor by a syringe. The rest of the slurry mixture was used for second-generation incubation. Then, substrates of formate, acetate, and H_2/CO_2 were added separately, and the subsequent procedures were the same as the first generation.

Methane production monitoring

Methane production was assayed in closed serum bottles with a headspace of 260 mL. Blank experiments were conducted where 5 mL distilled water was used for replacing the substrate solution as described above. The methane production from the three substrates was calculated by deducting the methane produced from the blank reactors.

The CH_4 production determination was conducted using gas chromatography FULI GC9790II with a column of TDX-01 (2 m long and a 3 mm inner diameter) and thermal conductivity detector (TCD). Helium served as the carrier gas. The temperatures of the packed column, detector, and injection port were set to 120 °C, 160 °C, and 160 °C, respectively.

Table 3 Free energies and typical microorganisms of methanogenesis reactions

Reactions	Stoichiometric coefficient of methanogenesis	ΔG^0 (kJ $mol_{CH_4}^{-1}$)	Microorganisms
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	4/1	−135	Hydrogenotrophic methanogens and some *Methanosarcina*
$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	4/1	−130	Many hydrogenotrophic methanogens
$CH_3COOH \rightarrow CH_4 + CO_2$	1/1	−33	*Methanosarcina* and *Methanosaeta*

The cumulative volume of CH_4 generated in a serum bottle was calculated by multiplying the headspace volume (260 mL) by the CH_4 percentage (mL of CH_4 per mL) in the headspace as determined by GC analysis. It must be noted that gas samples taken from batch reactors should equilibrate in batch reactors, which means the pressure in batch reactors have to be taken into consideration. In addition, the obtained value of cumulative CH_4 production was normalized in standard temperature and pressure (STP) conditions (0 °C and 1 atm) according to the ideal gas law ($PV = nRT$). The methane production assay was referenced to sample mass or chemical oxygen demand (N mL_{CH_4} g_{VS}^{-1} or N mL_{CH_4} g_{COD}^{-1}, N: normalized in STP). In this study, to ensure the unity of units, the mass of the substrate was converted to g COD, and the conversion factors used (COD/VS, g g^{-1}) for the formate, acetate, and H_2/CO_2 were 0.35, 1.07, and 8.00, respectively.

The modified Gompertz model

Recently, many studies have used the modified Gompertz model to predict biogas (CH_4 and CO_2) production potential by assuming that the rate of gas production is proportional to the microbial activity in anaerobic digesters.[29–31] The modified Gompertz equation is shown as follows:

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

where P – cumulative methane production, N mL g_{VS}^{-1} at any digestion time t; A – methane yield potential, N mL g_{VS}^{-1}; U – maximum rate of methane production, N mL $(g_{VS}\ d)^{-1}$; λ – lag phase period to produce methane, days; t – digestion time at which cumulative methane production P is measured, days; e – mathematical constant (2.718282).

The kinetic parameters of A, U, and λ were simulated for each batch reactor using non-linear regression with the help of SPSS software. These parameters were determined for best fit with high R^2 (>0.95).

DNA extraction and Illumina 16S rRNA gene amplicon sequencing

At the end of second generation incubation, the DNA from 500 mg of the sludge samples from each reactor was extracted after centrifuging 5 mL sludge for 20 minutes at 12 000 rpm. The DNA extraction protocol followed the manufacturer's instructions from the Fast DNA Spin Kit for Soil (MP bio, USA). The extracted DNA was subjected to electrophoresis in agarose gels (1.0% w/v) and visualized under a blue-ray. The extracted DNA was stored at −20 °C until further diversity and abundance analyses. The archaea 16S rRNA gene was amplified using the barcoded primer pair ARC-787f (5′-ATTAGATACCCSBGTAGTCC-3′) and ARC-1059r (5′-GCCATGCACCWCCTCT-3′).[32] Sequencing of archaeal amplicons was performed on Illumina MiSeq at Genenergy, Shanghai, China. The Illumina sequencing raw data were deposited in the NCBI Sequence Read Archive database (accession no. PRJNA491806).

Statistical analysis

Principal components analysis (PCA) was used to characterize the structure and relative abundance of microbial communities in the experimental batch reactors. The dominant microorganisms at the family level were displayed. The software, CANOCO 5.0, was used for data analysis.[33] Besides, Origin 8.0 was used for the production of graphics.

Conclusions

The physical structure of granular sludge had significantly different effects on formate and H_2/CO_2 conversion, whereas it had little impact on acetate conversion. And the physical structure of granules showed no significant effect on the methanogen composition shift in three systems on order level, while the substrate affects the succession of methanogens composition. Formate had a significantly rapid consumption rate in the granular system, which meant the role of formate in the anaerobic system might be undervalued. This study enlightened that enhancing formate transfer might be an effective approach for improving the performance of the AD process in the studied granular systems. Applying this approach to industrial methane production process could reach higher waste (water) treatment efficiency and energy product (methane) production.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors would like to thank the National Key Research and Development Program of China (Contract No. 2018YFD0500202-4), the National Natural Science Foundation of China (Contract No. 51678553, 21876167, 21477122, 51808525), the Project of the Natural Science Foundation of

Fujian Province (Contract No. 2017J05092), the IUE CAS Young Talents Frontier Project (Contract No. IUEQN201501), the Xiamen Science and Technology Project (Contract No. 3502Z20182003), the FY2015 Japanese-China Research Cooperative Program (Contract No. 2016YFE0118000), the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDA23030301) and Innovation Fund Project (2019000011) for their supports for this study. We would like to thank LetPub (http://www.letpub.com) for providing linguistic assistance during the preparation of this manuscript. We are grateful to Genenergy for help with Illumina sequencing raw data processing.

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