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Full Length Article

Phylogenetic Diversity of Methanogenic Archaea and Kinetics of Methane Production at Slightly Acidic Conditions of an Anaerobic Sludge

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Abstract

The methanogenesis at slightly acidic conditions is desired to enhance the efficiency and stability of an anaerobic digester. The composition of methanogens in an anaerobic sludge with methanogenic phenomemon at pH 6.0, was firstly investigated in current study. The results demonstrated a variety of methanogens existed in the sludge, including some acid-tolerance hydrogenotrophic methanogens. Then methanogenesis of the anaerobic sludge at slightly acidic conditions was measured by batch tests. When H_2/CO_2 was used as substrate, the accumulative methane yield at pH 5.5, 6.0 and 6.5 after 132 h cultivation were decreased by 17.3%, 12.9% and 0.03%, respectively compared with the control (pH 7.0), while the accumulative methane yield at pH 6.0 and 6.5 using acetate as substrate were significantly dropped to 4.8 and 18.6 mL from 23.9 mL at pH 7.0, respectively and no methane production was noticed at pH 5.5 during the whole cultivation. In addition, the methane production at pH 6.0 and 6.5 were hardly lagged when H_2/CO_2 was used as substrate, while the methane production from acetate at pH 6.5 was postponed by about 25 h compared with control. These results showed that H_2/CO_2 but not acetate could be converted efficiently into methane at slightly acidic conditions.

Keywords: Anaerobic sludge; Methanogens; Phylogenetic diversity; Methane production kinetics

Introduction

As an ideal cost-effective biological means, anaerobic digestion technology is used for the removal of organic pollutants in solid waste and wastewater and simultaneously produces methane as an energy source (Angenent *et al*., 2004; Wang *et al*., 2004; Chae *et al*., 2008; Mahmood *et al*., 2011). The anaerobic digestion is a prominent bioenergy technology worldwide, which uses undefined microbial cultures to produce methane from organic substrates. The organic wastes were anaerobically converted to methane needs cooperation of at least three groups of microbes-fermenting bacteria, syntrophic acetogens, and methanogens (Yadvika *et al*., 2004; Kosaka *et al*., 2006; Jha *et al*., 2011). Methanogenesis is the last step and considered as one of the rate-limiting steps in anaerobic digestion. The ability of methanogens to produce methane allows them to occupy a physiologically unique niche in anaerobic digestion system (Liu and Whitman, 2008). They play a key role in the anaerobic digestion, driving anaerobic digestion process by removing excess H_2 and short chain fatty acids (formate and acatate).

However, methanogens are very sensitive to some environmental factors such as pH, temperature, volatile fatty acids (VFAs) and inhibitory chemicals (Hajarnis and Ranade, 1993; Dhaked *et al*., 2003). Among these environmental factors, the pH is a main parameter influencing the methanogenesis in anaerobic digestion. Most methanogens grow and produce methane under neutral or slightly alkaline conditions (pH 6.8–8.5), and the anaerobic digestion systems usually are controlled under neutral conditions (Chae *et al.,* 2008; Demirel and Scherer, 2008). However, some shocks such as temperature fluctuation, an increase in organic loading rates, presence of toxic matter, would result in increase of organic acids, decrease of the pH and then caused the failure of anaerobic digestion systems. Thus, the acid tolerant methanogens is desired to enhance stability of anaerobic digestion. In our research, methanogenic phenomenon was observed at pH 6.0 in an anaerobic baffled reactor (ABR) treating sugar refinery wastewater, indicating some acid tolerant methanogens existed in ABR. In order to know of whether acid tolerant methanogens existed in ABR, composition of methanogens was firstly analyzed by polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE). Then methanogenic ability of an anaerobic sludge at slightly acidic conditions (pH 6.5-5.5) was investigated.

Materials and Methods

Inoculum

The granular sludge from the second compartment (C2) of an anaerobic baffled reactor (ABR) with four compartments treating sugar refinery wastewater was utilized as inoculum.

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The pH was 6.0 in C2 and methane content was 37.0% with 22.5% chemical oxygen demand (COD) removal, indicating some acid tolerant methanogens might be present in C2.

DNA Extraction, PCR and DGGE Analysis

The inoculated sludge was prepared for methanogenic composition analysis. Genomic DNA was extracted using a DNA extraction Kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The universal primers used to perform PCR were: 344F (5′-ACGGGGYGCAGCAGGCGCGA-3′, with a GC clamp) and 915R (5′-GTGCTCCCCCGCCAATTCCT-3′). The PCR amplification and DGGE was conducted as previous described (Ban *et al.*, 2012). All obtained partial 16S rRNA gene sequences were analyzed using the BLAST program of GenBank. Phylogenetic tree was constructed by software MEGA 3.1.

Batch Culture Procedure

In order to investigate methane production kinetics under low pH conditions, batch experiments were conducted in 150 mL serum bottles. A 10 mL of above granular sludge (2.3 g/L) as mixed liquid volatile suspended solid (MLVSS) was inoculated in each vial. All the bottles contained 40 mL nutrient solution with H_2/CO_2 (4:1 V/V) or acetate (1000 mg/L) as the carbon source. The nutrient solution was prepared as described by Angelidaki and Sanders (2004). The pH was adjusted to 5.5, 6.0, 6.5, 7.0 (control), respectively, using 1 mol/L HCl or 1 mol/L NaOH and then put them in a constant temperature air bath shaker (Harbin Donglian Electronic Technology Development Co. Ltd., Model HZQ-C) at 37°C and 130 rpm. To keep a constant pH range for each batch, the pH was adjusted every 12 h. Each bottle was purged with nitrogen gas for 3 min and then was capped with rubber septum stoppers. Test for each experimental condition was carried out in triplicate.

Analysis of Biogas, VFAs, MLVSS and pH

The amount of evolved gas was measured by releasing the gas pressure in the vials using appropriately sized glass syringes, ranging from 10 to 50 mL (Owen *et al.*, 1979). The fraction of CH_4 was measured with a SP-6800A gas chromatograph (Shandong Lunan Instrument Factory, China) as previously described (Chang *et al.*, 2011). After measuring biogas, liquid samples of 1 mL were taken out from each batch-culture for determining volatile fatty acids (VFAs) and pH. The liquid samples were first centrifuged at 13 000 rpm for 5 min, followed by 6 M HCl acidification, and finally VFAs assayed. The VFAs was analyzed by another gas chromatograph (SP6890, Shandong Lunan Instrument Factory, China). MLVSS and pH were determined according to the procedures described in the standard methods (APHA, 1998).

Kinetics Analysis

Kinetics of methane production under low pH were analyzed based on Gompertz model as expressed as equation (1), where M (mL) is the cumulative methane production at the reaction time t (h); P_{max} (mL) is the methane production potential; R_{max} (mL/h) is the maximum methane production rate; and λ (h) is the lag time. The cumulative methane production profiles were fitted with equation (1) for calculating P_{max}, R_{max}, and λ using software Origin 8.0 (Origin Lab Corporation, USA) (Lay *et al.*, 1994).

$$M = P_{max} \times \exp\left\{-\exp\left[\frac{R_{max} \times e}{P_{max}}(\lambda - t)\right] + 1\right\} \qquad (1)$$

Statistical Analysis

Each treatment was carried out in triplicate (n=3) regardless of H_2/CO_2 or acetate as substrate. The multiple comparison of cumulative methane production using H_2/CO_2 or acetate as substrate at different pH conditions (pH 5.5, 6.0, 6.5 and 7.0) was performed by least significant difference (LSD) – t test (α=0.05).

Results

Phylogenetic Analysis of Methanogens in Anaerobic Sludge

A 37% methane in biogas was contained from C2 (pH 6.0) of an ABR, indicating that some acid tolerant methanogens might exist in C2. To explore the composition of methanogenic archaea in C2, PCR-DGGE based on 16S rRNA gene sequences was performed. Eight methanogenic archaea bands (band 1~8) were obtained from PCR-DGGE gel and were then were sequenced. The phylogenetic placement of these eight sequences and reference 16S rRNA gene sequences of methanogens from NCBI database are shown in Fig. 1. Four groups were formed in the phylogenetic tree. Two groups were related to the order Methanomicrobiales, one involved in the order Methanosarcinales and an unidentified cluster.

Band 1 showed 94~95% sequence similarity to uncultured anaerobic methanogenic archaeon ET1-10 (AJ244286) and anaerobic methanogenic archaeon ET1-8 (AJ244284) that had been found in stable cellulose-degrading enrichment cultures (Chin *et al.*, 1999). Bands 2, 3, 4, 7 and 8 were grouped into the order Methanomicrobiales. Among them, bands 3, 4, 7 and 8 showed above 96% sequence similarity to *Methanospirillum hungatei* JF-1, *Methanolinea tarda* NOBI-1, *Methanoregula formicicum* SMSP and *Methanosphaerula palustris* strain E1-9c, separately. In addition, the band 2 formed a unique branch with two uncultured methanogens from the order Methanomicrobiales. Two bands (band 5 and 6) were closely related to the genus *Methanosaeta* in the order

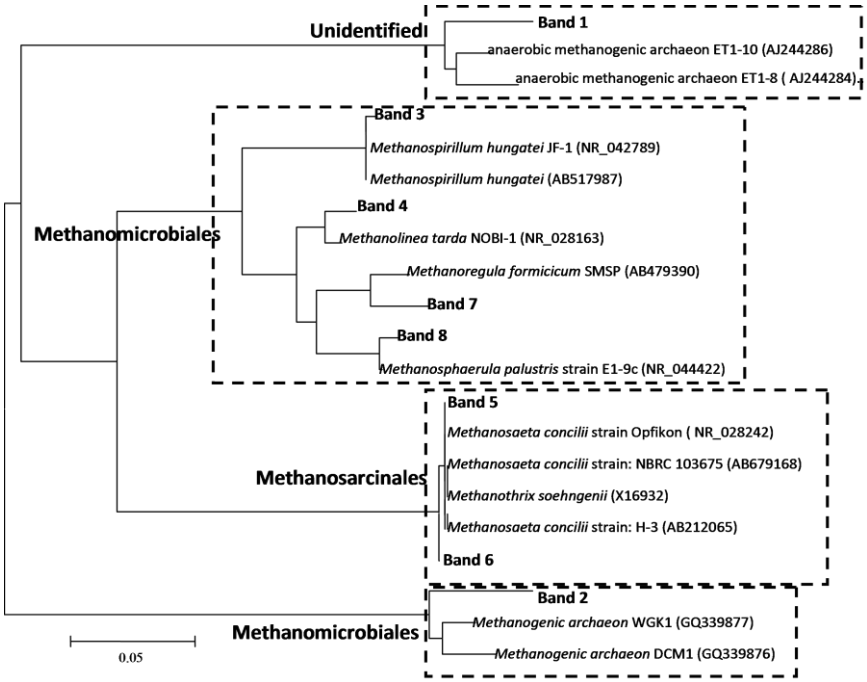

Fig. 1: Neighbour-joining tree of partial 16S rRNA gene sequences of methanogens Band 1~8: the clones of archaea from the inoculated sludge in this study

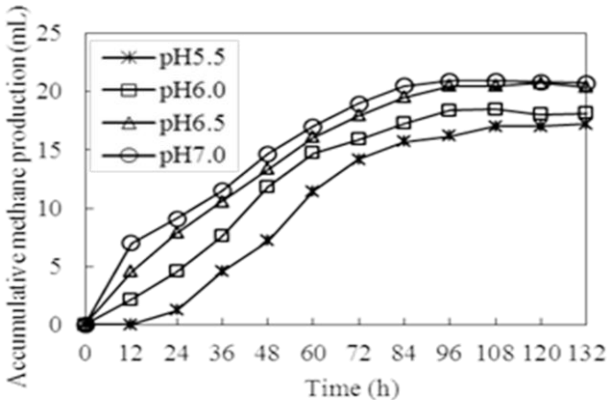

Fig. 2: Methane production using H_2/CO_2 as substrate at different pH conditions

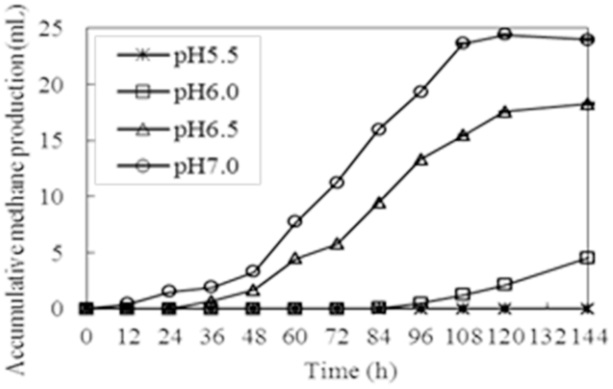

Fig. 3: Methane production using acetate as substrate at different pH conditions

Methanosarcinales.

Methane Production

To understand the methanogenic ability of the anaerobic sludge under slightly acidic conditions, the methanogenesis using H_2/CO_2 or acetate as substrate was evaluated, because they are the major intermediates and the main substrate for methanogens in the anaerobic digestion. As shown in Fig. 2, the anaerobic sludge exhibited high methanogenic activity under all experimental conditions when H_2/CO_2 was used as substrate although the methane rate was slightly decreased at acidic pH compared with the control (pH 7.0). The accumulative methane yield at pH 5.5, 6.0, 6.5 and 7.0 were 17.2, 18.1, 20.2 and 20.8 mL, respectively (Table 1).

Variations of accumulative methane yield with time using acetate as substrate under different pH conditions are depicted in Fig. 3. The methane production rate at slightly acidic conditions (pH 5.5, 6.0, 6.0) was obviously lower than the control (pH 7.0). Compared with the control, the accumulative methane yield at pH 6.5 and pH 6.0 after 144 h cultivation was decreased by 22.2% and 79.9%, respectively, while the methanogenesis could not take place at pH 5.5 (Table 1).

Kinetics Analysis

The kinetic parameters were estimated by Eq. 1. (Table 2).

The determination of coefficient (R^2) was over 0.99, indicating the Gompertz model was feasible to describe the progress of accumulative methane production in current study. The maximum methane production rate (R_{max}) using H_2/CO_2 as substrate at pH 7.0 was 0.3 mL/h. Compared with the control (pH 7.0), the R_{max} at pH 5.5, 6.0, 6.5 was reduced by 53.3, 20.0 and 10.0%, respectively and the maximum accumulative methane production (P_{max}) was decreased by 0.8 ~ 17.4%. The longest lag time (λ) was 14 h at pH 5.5 when H_2/CO_2 was used as substrate. For acetate as substrate, the methanogenesis was severely suppressed at pH 6.0 and pH 5.5, indicating that Gompertz model was not appropriate to reflect the progress of cumulative methane production. Even if the pH was decreased from 7.0 to 6.5, the λ was extended about 25 h as well as the P_{max} and R_{max} were reduced by 18 and 40%, respectively. The results of methane production kinetics confirmed that a high methanogenic activity of the anaerobic sludge was obtained using H_2/CO_2 as substrate, indicating the methanogenesis from hydrogenotrophic methanogens in C2 (pH 6.0) of the ABR.

Discussion

As the last step during anaerobic digestion, activity of methanogens was crucial for stable operation of a digester. In this study, methanogens in C2 of an ABR treating sugar refinery wastewater presented a high phylogenetic diversity (Fig. 1). Among them, *Methanospirillum hungatei* (band 3), *Methanolinea tarda* (band 4), *Methanoregula formicicum* (band 7) and *Methanosphaerula palustris* (band 8) were four hydrogenotrophic methanogens and all of them can use H_2/CO_2 and formate as substrate for growth (Ferry *et al.*, 1974; Imachi *et al.*, 2008; Cadillo-Quiroz *et al.*, 2009; Yashiro *et al.*, 2011). The first three hydrogenotrophic methanogens has an optimum pH of 6.6~8.0 for growth, while *M. palustris* is a slightly acidiphilic hydrogenotrophic methanogen with an optimum pH of 5.5 for growth (Ferry *et al.*, 1974; Imachi *et al.*, 2008; Cadillo-Quiroz *et al.*, 2009; Yashiro *et al.*, 2011). As we predicted, some acid tolerant methanogens were present in the inoculated sludge, making a high methanogenic activity at pH 5.5 when H_2/CO_2 as substrate. It was favorable for improving the performance and stability of an anaerobic digestion. However, only a few acid tolerant methanogens were identified and limited knowing of their physiological and ecological features (Savant *et al.*, 2002; Cadillo-Quiroz *et al.*, 2009). It was desired to isolate and identify more acid tolerant methanogens for further improving the performance and stability of anaerobic digestion systems. The H_2 removal at the slightly acidic conditions would decrease partial pressure of hydrogen and then improve the activity the syntrophic acetogens, which degrade short-chain fatty acids only under low hydrogen partial pressure conditions (Ariesyady *et al.*, 2007; Zhang *et al.*, 2012).

Acetate is not only a product of organic matter anaerobic conversion but also the primary substrate for methanogenesis under methanogenic conditions. It is estimated that approximately 70%-80% of methane is produced from acetate oxidation in anoxic environments (Lovley and Klug, 1982). In this study, two bands (bands 5 and 6) were closely related to aceticlastic methanogens (*Methanosaeta*). Species from the genus *Methanosaeta* use only acetate as substrate and they can use acetate at the pH range of 6.6~7.4 (Liu and Whitman, 2008; Demirel and Scherer, 2008). This study found that methanogenesis from aceticlastic methanogens were inhibited with pH decrease and no methane was produced at pH 5.5. The result is

Table 1: The characteristics of batch tests under different pH conditions

Substrate	Initial		Final		
	pH	H_2 (mL)	H_2 (mL)	pH	CH_4 yield (mL)*
H_2/CO_2	5.5±0.05	57.2±1.55	0	5.6±0.10	17.2±1.09[a]
	6.0±0.06	57.6±2.71	0	6.0±0.08	18.1±1.14[a]
	6.5±0.05	58.1±1.81	0	6.6±0.02	20.2±0.75[b]
	7.0±0.04	57.5±2.58	0	7.1±0.05	20.8±1.25[b]
	pH	Acetate (mg/L)	Acetate (mg/L)	pH	CH_4 yield (mL)*
Acetate	5.5±0.07	1135.5±20.51	1134.1±25.52	5.6±0.07	0[a]
	6.0±0.09	1115.4±50.62	570.8±15.54	6.1±0.06	4.8±0.02[b]
	6.5±0.05	1053.2±30.41	100.9±5.45	6.6±0.02	18.6±1.37[c]
	7.0±0.03	1196.7±80.63	22.4±3.66	7.1±0.02	23.9±1.64[d]

*There are no significant differences ($P > 0.05$) between the lines with the same characters and significant differences ($P < 0.05$) between lines with different characters

Table 2: Kinetic parameters of methane production under slightly acidic conditions

pH	H_2/CO_2 as substrate				Acetate as substrate			
	P_{max} (mL)	R_{max}(mL/h)	λ(h)	R^2	P_{max} (mL)	R_{max}(mL/h)	λ(h)	R^2
5.5	17.16	0.14	13.97	0.9924	—	—	—	—
6.0	18.23	0.24	1.25	0.9934	—	—	—	—
6.5	20.62	0.27	0.46	0.9958	20.94	0.27	28.95	0.9951
7.0	20.78	0.30	0.00	0.9922	25.70	0.45	3.5.5	0.9966

consistent with the previous report that indicated the pH range for aceticlastic methanogens was between 6.6 and 7.3 and they were inhibited strongly below 6.2 (Demirel and Scherer, 2008).

It can be known from Fig. 1 and 2 that the methanogenesis was inhibited at acidic conditions regardless of the H_2/CO_2 or acetate as substrate. However, the inhibition extent using H_2/CO_2 as substrate was significantly less than that of acetate as substrate, especially the anaerobic sludge had a higher utilization rate for H_2/CO_2 than acetate at pH 6.0 and 5.5. Similarly, Kim *et al.* (2004) reported that the hydrogen-utilizing methanogens were more tolerant to the acidic conditions than the other methanogens.

In conclusion, the phylogenetic diversity of methanogens was observed in C2 of the ABR. Especially, one band was similar to an identified acid tolerant methanogens, confirming some acid tolerant methanogens might exist in this ABR system. The methane production kinetics of anaerobic sludge from ABR revealed that the methanogenic activity was inhibited with the decrease of pH regardless of using H_2/CO_2 or acetate as substrate. However, the inhibited extent was higher using acetate as substrate than that of H_2/CO_2 as substrate.

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