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Microbial Community Composition and Response to Temperature Shock of a Mesophilic Propionate-degrading Methanogenic Consortium

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Abstract

In this study, a mesophilic propionate-degrading methanogenic community was obtained by subculture of anaerobic sludge from an anaerobic baffled reactor, using propionate as sole carbon source. The microbial community composition of the enriched culture was examined by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) based on 16S rRNA gene partial sequences. The result revealed that two major bands (B1 and B2) had a high similarity with the identified POB in genus *Syntrophobacter*. While *Methanobacterium* and *Methanothrix* were dominant methanogens in the enriched culture. They formed a great syntrophic relationship with specific COD_{pro} removal rate of sludge and specific methane production rate of sludge were 0.6 kg/(kg MLVSS d) and 280 L/(kg MLVSS d), respectively. In view of microbial metabolism would be significantly affected by temperature, response of the propionate enriched culture on temperature shocks was investigated. At the optimum temperature (35°C~40°C), propionate was degraded completely after 5-day cultivation. Temperature shocks, from 35°C to 30°C, 25°C, 20°C, resulted in propionate removal rate was decreased instantaneously. However, propionate removal rate was recovered to the value at 35°Cafter 2~3 days adaptation, indicating this propionate-degrading methanogenic community had a strong resistance to temperature fluctuation.

Keywords: Propionate; Propionate-oxidizing bacteria; Methanogens; Temperature

Introduction

Propionate is an intermediate product in anaerobic conversion of organic matters to methane and carbon dioxide. The degradation of propionate into acetate and H_2/CO_2 (and then to CH_4) accounts for 6% to 35% of the total methanogenesis (Koch *et al.*, 1983; Glissmann and Conrad, 2000). However, the oxidation of propionate is energetically unfavorable with a standard change in Gibbs free energy ($\Delta G^{o\prime}$) of +76 kJ per mol reaction, and its oxidation to acetate is the more difficult than the other the short chain volatile fatty acids and alcohol (Müller *et al.*, 2010; Amani *et al.*, 2011; Hanaki *et al.*, 1994). Therefore, it is usually to be accumulated when the anaerobic digesters are subjected to any shock such as an increase of organic load rate, temperature fluctuation and present of some toxins, even resulting in failure of the anaerobic process (Kaspar and Wuhrmann, 1978; Wong *et al.*, 2009; Shah *et al.*, 2009; Liu *et al.*, 2010). The toxic effects of propionate accumulation on anaerobic digestion process have been confirmed (Pullammanappallil *et al.*, 2001; Dhaked *et al.*, 2003; Gallert and Winter, 2008). Thus, degradation of propionate is very important for improving performance of anaerobic digestion process.

The biodegradation of propionate depends on cooperation between propionate-oxidizing bacteria (POB) and hydrogen-consumption bacteria such as sulfate reducing bacteria and hydrogenotrophic methanogens (Li *et al.*, 2012). POB supply substrate for methanogens through converting propionate in to acetate and H_2/CO_2, in return, methanogens drive propionate degradation process by eliminating the products of propionate metabolism. However, it has been reported that syntrophic acetogenic bacteria including POB are normally more susceptible to environmental change than anaerobic bacterial groups (Ariesyady *et al.*, 2007a). In addition, genomic information from *Pelotomaculum thermopropionicum*, a POB, suggested that the metabolic pathways are regulated by ecological factors and/or inclusive cellular conditions (Kosaka *et al.*, 2008).

As an important ecological factor, temperature plays a crucial role for microbial metabolism. Especially, the room temperature was below 20°C in winter in northern region of China. Therefore, a high efficient propionate-oxidizing bacterial community with a wide temperature range is beneficial to strengthen performance of an anaerobic digester in northern region of China. To obtain a high efficient propionate-oxidizing bacterial community, an

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anaerobic sludge from an anaerobic baffled reactor (ABR) was used as inoculum and propionate as the sole carbon for enriching POB and methanogens at mesophilic condition. Then the microbial composition was investigated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Syntrophic anaerobic degradation of propionate response to temperature shocks in the enriched culture was further investigated.

Materials and Methods

Enrichment

To obtain a high efficient propionate-degrading methanogenic consortium, an anaerobic granular sludge was sampled from the third compartment of an ABR with four compartments treating molasses wastewater (Li *et al.*, 2012). The 10 mL of the granular sludge with a mixed liquid volatile suspended solid (MLVSS) of 58.5 g/L was inoculated to a 250 mL serum bottle containing 130 mL nutrient solution with propionate (1500 mg/L) as the sole carbon. The nutrient solution was prepared as previous described (Angelidaki and Sanders, 2004). The initial pH in the culturing systems were adjusted to 7.5~8.0 by 3 M HCl or 3 M NaOH. The anaerobic environment was obtained in batch cultured system by replacing air with nitrogen gas for 3 min. The reaction system was carried out under the dark condition in a constant temperature air bath shaker (Harbin Donglian Electronic Technology Development Co., Ltd. Model HZQ-C) at 35°C and 130 rpm.

Concentration of volatile fatty acids (VFAs) and CH_4 content during the enrichment process were measured every 2 days. The samples were transferred into fresh liquid medium when the VFAs removals were over 95%.

DNA Extraction, PCR and DGGE Analysis

The samples of the original sludge and the enriched culture after subculture for 15 times were prepared for microbial composition analysis. Genomic DNA was extracted using a DNA extraction Kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instruction. The universal primers used to perform PCR are shown as following: 341F (5′-CCTACGGGAGGCAGCAG-3′, with a GC clamp) and 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) for eubacteria; 344F (5′-ACGGGGYGCAGCAGGCGCGA-3′, with a GC clamp) and 915R (5′-GTGCTCCCCCGCCAATTCCT-3′) for archaea. The PCR amplification and DGGE were conducted as described in the previous research (Wan et al., 2011). All obtained partial 16SrRNA gene sequences were analyzed using the BLAST program of GenBank.

Batch Culture Procedure

To understand the effect of temperature shock on the anaerobic degradation characteristics of propionate in the enriched culture, batch cultures were conducted in 150 mL serum bottles. Each bottle with 40 mL nutrient solution was inoculated with 10 mL of the propionate enriched culture (MLVSS 1.1 g/L) and the concentration of propionate in the mixture was about 400 mg/L. The nutrient solution was prepared as described in section Enrichment. 21 reaction systems were constructed and cultured at different temperatures (15, 20, 25, 30, 35 and 40°C) with the same initial pH 7.8. Each test was performed in triplicate.

Analysis

The evolved gas was computed as described in the previous study (Owen *et al.*, 1979). The quality of biogas was determined with a SP-6800A gas chromatograph (Shandong Lunan Instrument Factory, China) as previously described (Li *et al.*, 2009). After measuring biogas composition, liquid samples of 1 mL were taken from each batch for determining volatile fatty acids (VFAs) and pH.

The liquid samples were centrifuged at 13, 000 rpm for 3 min, followed by 6 M HCl acidification, and finally VFAs assayed. The VFAs was also analyzed by another gas chromatograph (SP6890, Shandong Lunan Instrument Factory, China) (Li *et al.*, 2009). MLVSS and pH were determined using the standard methods (APHA, 1998).

Results and Discussion

Microbial Community Analysis of the Enriched Culture

A high efficient propionate-degrading methanogenic consortium with specific COD_{pro} removal rate of anaerobic sludge of 0.6 kg/(kg MLVSS d) was enriched by subculture. During the enrichment process the initial granules were broken down gradually with continuous subculture and newly formed microorganisms were dispersed uniformly. The reason might be that batch culture is lack of hydraulic shear stress, unlike in the reactor (ABR). After 15 transfers, the microbial community composition in enrichment sample was investigated by PCR-DGGE based-16S rRNA gene. As Fig. 1a shown, the signal of some bands were enhanced obviously in enriched culture compared with the initial inoculated sludge, indicating some microbes were enriched when the propionate was used as sole carbon resource. All obtainable bands were excised from gels and then were sequenced. These 16S rRNA gene sequences were determined in NCBI database.

The bands B1 and B2 appeared as strong signal compared with initial inoculated sludge, indicating the occurrence of strong enrichment of these special bacteria. It can be known from Fig. 2 that they have a same code with identified POB in the genus *Syntrophobacter*, which seemed to be responsible for propionate degradation and acted as main functional bacteria community during the anaerobic digestion process (Shigematsu *et al.*, 2007; Ariesyady *et al.*, 2007b; Narihiro *et al.*, 2012). The band B1 exhibited 97% similarity to the *Syntrophobacter sulfatireducens* strain TB8106, while the band B2 had a common node with *S. wolinii* strain DB with 95% similarity. These two POB can

degrade propionate when they were co-culture with methanogens (Boone *et al.*, 1980; Chen and Dong, 2005a). *S. sulfatireducens* strain TB8106 has a temperature range of 20~48ºC for growth, but *S. wolinii*strain DB has not been detected. These results indicated that the *Syntrophobacter* was the major propionate-oxidizing functional community in the enriched culture.

Three bands (B5, B7 and B9) were identified as the phylum Firmicutes. They exhibited 99% sequence similarity with uncultured Firmicutes bacteria clones. Although the function of bacteria from phylum Firmicutes in current study are still unknown, some identified POB were belonged to phylum Firmicutes (Imachi *et al.*, 2002; 2007). The band B8 was matched by *Proteiniphilum acetatigenes* strain TB107, which was isolated from a propionate-enrichment culture and can accelerate the propionate degradation rate after adding to syntrophic propionate-degrading co-culture (Chen and Dong, 2005b). The enrichment of *P. acetatigenes* was favorable to strengthen the propionate degradation. Three obtainable major bands (B4, B6 and B8) had a common node with some bacteria within the phylum of Bacteroidetes, while the band B3 showed 89% similarity with uncultured candidate division OP11 bacteria.

Since the anaerobic oxidation of propionate could not be completed spontaneously, its degradation generally depends on the syntrophy between POB and methanogens under methanogenic environments (Schink and Stams, 2006; Tatara *et al.*, 2008). Therefore, methanogenic composition in the propionate enriched culture was crucial for propionate degradation. As shown in Fig. 1b and Fig. 2, bands A1 and A2 showed 99% similarity with acetotrophic methanogens in the genus *Methanosaeta*, in which species are specialists in utilizing acetate and they can use acetate at concentrations as low as 5-20 μM (Keyser *et al.*, 2006; Liu and Whitman, 2008). While bands A3 and A4 were related to hydrogenotrophic methanogens in the genus *Methanobacterium*, showing 98% and 100% sequence similarity with *M. aarhusense* strain H2-LR and *M. beijingense*strain M4, respectively. *M. beijingense* can use H_2/CO_2 and formate for growth and produced methane (Ma *et al.*, 2005).

Response of Syntrophic Propionate Degradation on Temperature Shocks

Temperature is the most important and principle operational parameter in anaerobic digestion process. When an anaerobic digestion system is suffered from temperature shocks, the activity of anaerobes containing POB would be inhibited. In order to understand the response of the propionate-degrading consortium on temperature, the impact of temperature shocks on syntrophic propionate degradation was evaluated for 6 days (Fig. 3). Temperature shock, from 35°Cto 40°C, led to an increase in the propionate degradation rate compared with the control.

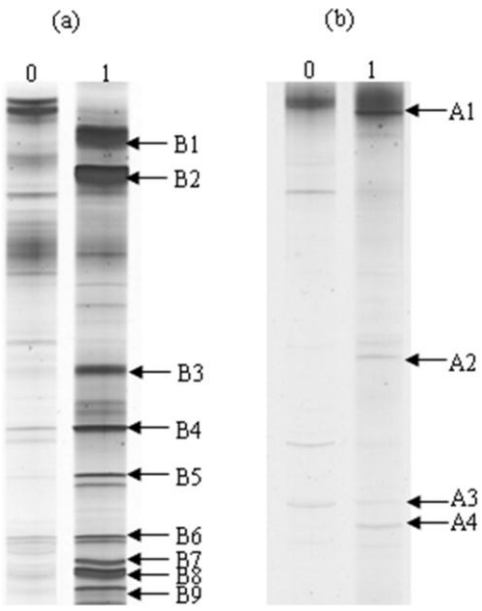

Fig. 1: DGGE analysis of PCR-amplified 16S rRNA gene fragments from propionate –degrading methanogenic consortium. (a) DGGE analysis of Eubacteria, (b) DGGE analysis of archaea Lane 0: Initial inoculated sludge (not be enriched); Lane 1: Propionate enriched culture

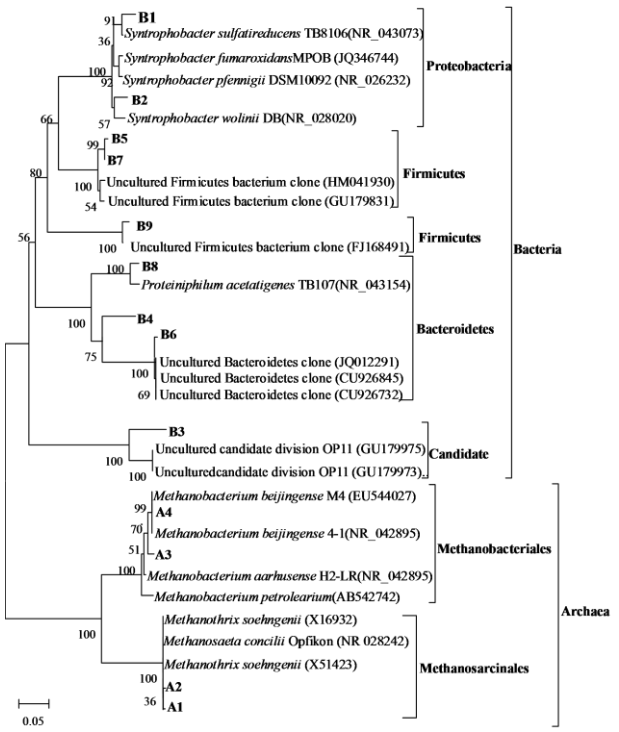

Fig. 2: The evolutionary distance of eubactera and archaea. The Neighbor-Joining tree was constructed using the MEGA 3.1 software. Accession numbers for reference sequences are shown in parentheses. The scale bar represents 5% sequence divergence

However, when temperature was suddenly decreased from 35°C to 25°C and 30°C, the propionate was oxidized gradually in the first 2 days after incubation and then rapidly. With the temperature shocks taken place from 35°C to 20°C and 15°C, propionate began to be degraded slowly after a lag phase and the propionate degradation rate at 20°C was resumed the value of the control (35°C) after 3 days adaptation.

Temperature fluctuation seemed not to be avoided for some ambient anaerobic digesters. In a previous research, the fastest growth of anaerobic propionate oxidizers occurred at 32~45°C. In addition, identified mesophilic syntrophic propionate-oxidizing bacteria in the genus *Syntrophobacter* can grow in temperature range of 20 to 48°C with optimum at 37°C (Harmsen *et al.*, 1998; Wallrabenstein *et al.*, 1995). In our study, all temperature shocks have not completely inhibited the activity of the POB, the propionate was still oxidized at 15°C although the rate was significantly reduced. For other temperature shocks, the propionate degradation rate was recovered the value of control (35°C) after adaptation, indicating the enriched culture has a wide temperature range for propionate oxidation.

Accumulative Methane Yield

The impacts of temperature shocks on methane production were similar to propionate degradation (Fig. 4). At normal temperature (35°C), a maximum accumulative methane yield (15.4 mL) was obtained at 5th day. The temperature shock, from 35 to 40°C, led to increased methane production and the maximum accumulative methane yield of 15.1 mL was obtained at fourth day (Table 1). However, when temperature was suddenly reduced from 35°C to 30°C, 25°C and 20°C, the methane production rate were rapidly decreased and the acetate but no hydrogen were accumulated temporarily, indicating the activity of aceticlastic methanogens was easily inhibited by these temperature shocks. When temperature was dropped to 15°C from 35°C, methanogenesis was significantly inhibited with acetate accumulation.

It can be known from Fig. 3~5 that temperature increase exhibited a positive response to propionate degradation and methane production, while temperature decrease would reduce the activity of both POB and aceticlastic methanogens. However, their metabolic activity would be recovered after 2~3 days at above 20°C.

Conclusion

Strengthening propionate conversion rate is an effective pathway to go further improving the stability of an anaerobic digestion process. In current study, a propionate-degrading methanogenic consortium with high propionate removal ability was successful enriched at mesophilic condition. In this enriched culture, two major microorganisms closely to identified POB in the genus *Syntrophobacter*, which might as the main functional community for propionate degradation. This enriched culture also contained hydrogenotrophic and acetotrophic methanogens from orders Methanobacteriales and Methanosarcinales. The POB and methanogens formed

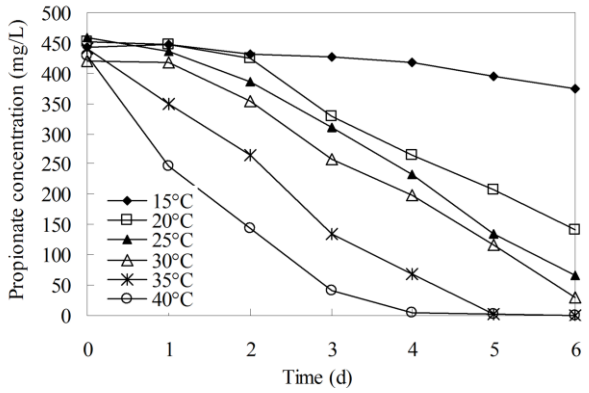

Fig. 3: Time course of propionate anaerobic degradation at different temperature conditions

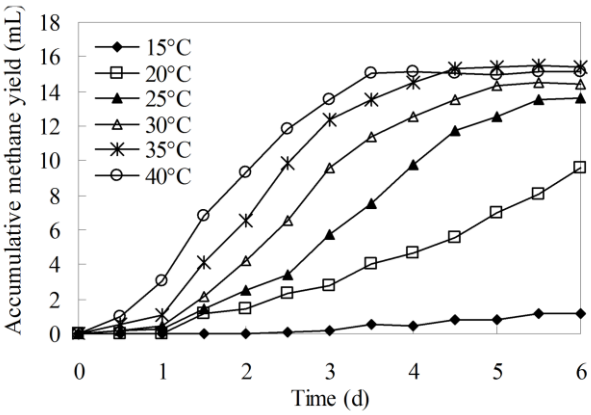

Fig. 4: Time course of methane production during propionate degradation process at different temperature conditions

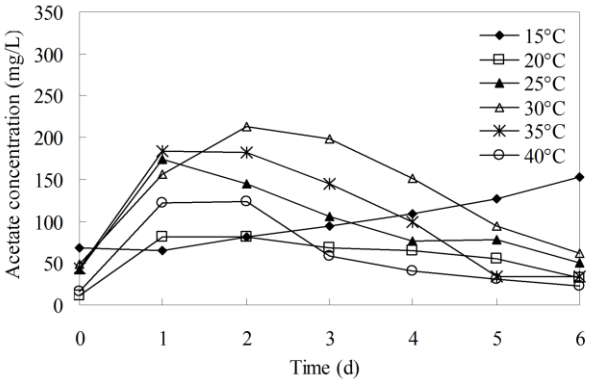

Fig. 5: Time course of acetate during propionate degradation process at different temperature conditions

Table 1: The characteristics of batch tests in different temperature conditions

Initial			Final			
Temperature (°C)	Propionic acid concentration (mg/L)	pH	Methane yield (mL)	Acetic acid concentration (mg/L)	Propionic acid concentration (mg/L)	Propionic acid removal (%)
15±0. 5	442.4±50.2	7.8±0.05	1.1±0.5	152.3±20.3	375.5±25.3	15.1±0.5
20±0.6	451.7±30.8	7.8±0.01	9.6±1.5	32.5±5.6	60.5±2.4	86.6±1.6
25±0.4	459.6±20.8	7.7±0.05	13.6±2.3	50.1±5.8	45.5±5.9	90.1±2.5
30±0.7	420.1±50.9	7.8±0.07	14.4±1.9	61.7±7.8	29.9±7.9	92.9±5.4
35±0.6	439.8±30.9	8.0±0.07	15.4±2.5	33.9±9.6	0.5±0.3	99.9±4.7
40±0.6	428.8±20.7	8.0±0.04	15.1±3.2	22.6±3.8	0.8±0.4	99.8±6.1

a good syntrophic relationship with specific propionate removal rate of anaerobic sludge of 0.6 kg/(kg MLVSS d). Temperature increase had a positive effect on propionate removal and methane production. However, temperature decrease resulted in that propionate degradation rate and methane production rate were reduced. After adaptation of 2~3 days, the propionate degradation rate and methane production rate were recovered to the value at 35°C. These results suggested that this propionate-degrading methanogenic consortium had great resistance ability to temperature shocks.

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