Long-term survival of methanogens of an anaerobic digestion sludge under starvation and temperature variation

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Introduction

Methanogens are methane producing microorganisms belonging to Phylum Euryarchaeota of Archaea. Methanogens have received extensive attention in biogeochemistry and biotechnology due to their ability to produce methane, an important renewable energy as well as a significant greenhouse gas (Cakir and Stenstrom, 2005; Demirel and Scherer, 2008). Use of regenerated methane as a source of energy is beneficial for energy, economy and environment (Demirel and Scherer, 2008). Methane is produced from a range of substrates, including carbon dioxide and H₂ (the electron donor), methyl compounds and acetic acid in anaerobic digestion (Garcia et al., 2000). Methanogens commonly encounter starvation conditions, where their substrates are not readily available. Thus, behavior of methanogen population responding to starvation have been studied. Hwang et al. (2010) reported that reduction of organic loading resulted in decrease in methane production due to population and activity changes of methanogens. Earlier studies have discontinued feeding to induce starvation conditions in batch systems (Hwang et al., 2010). However, the use of such non-continuous systems cause difficulty in determining the actual population and community dynamics of methanogens in response to starvation conditions, since a residual carbon substrate can remain for a long-time period.

Temperature is one of the most important factors of microbial activity and growth in anaerobic digestion (Krakat et al., 2010; Levén et al., 2007), and the activity and growth rate of microorganisms, including many methanogens, are greater under thermophilic conditions than mesophilic conditions (Kiyohara et al., 2000). In addition, thermophilic regimes are not suitable for the survival of pathogenic microorganisms in anaerobic digestion (Sahlström, 2003). Therefore, thermophilic regimes can be suitable for many forms of anaerobic digestion although it has some disadvantages, such as a higher energy requirement and greater process instability (De la Rubia et al., 2013). Methanogens presumably show differential responses to starvation under different temperature regimes. This information may provide an insight into anaerobic digester operation and management. The objective of this study was to investigate population and community dynamics of methanogens under

Abstract

To investigate starvation effect on methanogen community, two identical membrane reactors were continuously operated for 84 consecutive days, with a temperature change from 50°C to 20°C. Continuous feeding washed out 97% biomass from reactors during the experimental period. Quantitative PCR, using mcrA genes, indicated that the methanogen abundance decreased from 7.0×10⁷ to 1.2×10⁷ mcrA copies ml⁻¹ (volume basis) at 50 °C, and then increased to 4.4 ×10⁷ mcrA copies ml⁻¹ at 20 °C (p<0.05). Correspondence analysis indicated that methanogen communities were distinctly grouped by each temperature. Canonical correspondence analysis indicated that temperature showed a significant correlation with the methanogen community composition. These results suggest that methanogens can survive for a long time (at least more than 84 days) under starvation conditions, and that temperature could be a primary factor determining the density and community of methanogens.

Key words

Methanogens, Population dynamics, Starvation, Temperature
starvation stress at two different temperature regimes. In the present study, starvation condition of substrates was induced by using a continuous membrane system.

Materials and Methods

Inoculum preparation: A sludge sample was obtained from an anaerobic digester (45-50 °C) in Jungrang municipal wastewater treatment plant, Seoul, Korea in June 2010. Before use, the anaerobic sludge was washed three times with fresh basal medium, and was purged with 5-grade N gas (99.99% v/v, Dong-A gas, Seoul, Korea). The basal medium consisted of NH₄Cl (0.321g l⁻¹), KH₂PO₄ (0.489 g l⁻¹), MgSO₄·7H₂O (0.074 g l⁻¹), NaCl (0.018 g l⁻¹), CaCl₂·2H₂O (0.059 g l⁻¹), FeSO₄·7H₂O (0.005 g l⁻¹) and trace element solution (1 ml l⁻¹). The trace element solution contained Na₂MoO₄·2H₂O (0.036 g l⁻¹), CoCl₂·6H₂O (0.190 g l⁻¹), MnCl₂·4H₂O (0.500 g l⁻¹), CuCl·2H₂O (0.002 g l⁻¹), NH₄·6MoO₄·4H₂O (0.090 g l⁻¹), H₂BO₃ (0.030 g l⁻¹) and ZnCl₂ (0.140 g l⁻¹). The final pH of the medium was 7.2.

Membrane reactors: Two identical membrane bioreactors were set up. Fig. 1 shows a schematic diagram of the reactor system. Their height and inner diameter were 50 and 15 cm (approximately 8.8 l), respectively. Hollow-fiber type membranes (with a pore size of 0.04 μm, a diameter of 7.5 cm and height of 41.6 cm) (PHILOS, Shihung, Korea) were immerged in the reactors, with a pH controller. Five liters of washed sludge (anaerobic digester (45-50 °C)) in Jungrang municipal wastewater treatment plant, Seoul, Korea in June 2010. Before use, the anaerobic sludge was washed three times with fresh basal medium, and was purged with 5-grade N gas (99.99% v/v, Dong-A gas, Seoul, Korea). The basal medium consisted of NH₄Cl (0.321g l⁻¹), KH₂PO₄ (0.489 g l⁻¹), MgSO₄·7H₂O (0.074 g l⁻¹), NaCl (0.018 g l⁻¹), CaCl₂·2H₂O (0.059 g l⁻¹), FeSO₄·7H₂O (0.005 g l⁻¹) and trace element solution (1 ml l⁻¹). The trace element solution contained Na₂MoO₄·2H₂O (0.036 g l⁻¹), CoCl₂·6H₂O (0.190 g l⁻¹), MnCl₂·4H₂O (0.500 g l⁻¹), CuCl·2H₂O (0.002 g l⁻¹), NH₄·6MoO₄·4H₂O (0.090 g l⁻¹), H₂BO₃ (0.030 g l⁻¹) and ZnCl₂ (0.140 g l⁻¹). The final pH of the medium was 7.2.

Fig. 1: Schematic diagram of membrane reactors for inducing starvation

The present study employed continuous membrane system to physically remove residual carbon substrates. Experimental results were identical from both reactors, unless otherwise stated. Oxygen analysis, using GC and colorimetric determination using resazurin dye, confirmed that oxygen was free in both reactors during the entire experimental period. The membrane system with continuous feeding, effectively deprived organic compounds from the reactors during the 84 days experimental period. COD concentration decreased from 81 mg l⁻¹ at day 0 to 31 mg l⁻¹ at day 84, representing a reduction of around 60%. Cell dry weight was reduced from 1066.7 mg-DCW l at day 0 to 31 mg l at day 84, representing a reduction of around 99%. It is generally accepted that a substrate limiting condition reduces methanogen activity and population density (Morozova and Wagner, 2007). Consistently, methane production was observed for the first 11 days, and then ceased by continuous feeding at 50 °C (Fig. 2), in which the deprivation of organic substrates efficiently hindered the methanogen activity. However,
unexpectedly, methanogenesis appeared with an acclimation period of more than 10 days after temperature was reduced to 20 °C. The result indicates that low temperature allowed methanogens to remain active under starvation condition.

A total of 10 methanogen groups were quantified using quantitative PCR (Table 1 and Fig. 3). The continuous flow significantly lowered the methanogen abundance from $7.0 \times 10^7$ to $1.4 \times 10^7$ mcrA copies ml⁻¹ in reactor A (80% reduction) and $9.6 \times 10^6$ mcrA copies ml⁻¹ in reactor B (87% reduction) at 50 °C ($p<0.05$) on volume basis (Fig. 3a). However, the temperature shift from 50 to 20 °C significantly increased the methanogen abundance to $4.2 \times 10^7$ mcrA copies ml⁻¹ (335%) in reactor A and $4.5 \times 10^7$ mcrA copies ml⁻¹ (468%) in reactor B ($p<0.05$), although the flow was continued. These population results are consistent with the methanogen activity results, indicating that the medium flow could adversely affect the survival and activity of methanogens. These results also suggested that methanogens were more tolerant than expected under substrate-limiting conditions. On the basis of dry cell weight (DCW), starvation increased the methanogen density from $6.5 \times 10^7$ to $5.1 \times 10^8$ mcrA copies mg DCW⁻¹ in reactor A and $2.4 \times 10^8$ mcrA copies mg-DCW⁻¹ in reactor B at the end of 84 days period ($p < 0.05$) (Fig. 3b). It was highly interesting that starvation dramatically increased methanogen density by two orders of magnitude from both reactors, on the basis of dry cell weight (DCW). Methanogens could be highly competitive as compared to other co-habitants under substrate-limiting conditions. The presence of methane in head space might be beneficial for survival of methanogens under starvation conditions. Zhang et al. (2011) and Deusner et al. (2010) showed that number of methanogens did not decrease due to presence of methane in atmosphere. In addition, reverse methanogenesis may be possible by methanogens under starvation condition. It has been observed that methane is oxidized by methanogens without oxygen. This anaerobic oxidation of methane (AOM) is likely to proceed via a reversed methanogenic pathway by methyl-coenzyme M reductase, the key enzyme of methanogenesis (Shima and Thauer, 2005). Scheller et al. (2010) demonstrated that the enzyme catalyzes AOM by cleavage of strong C–H methane bond.

In general, mesophilic conditions guarantee a more diverse community than thermophilic conditions in anaerobic digesters (De la Rubia et al., 2013). For instance, Sekiguchi et al.
(1998) found less microbial diversity in the thermophilic anaerobic digester as compared to thermophilic digester. A total of 8 methanogen groups, such as mbac-mcrA, mcp, MCR-2a, MCR-2b, msar, msp, MCR-7 and msa, were observed from the reactors (Table 1). Initially, there were 7 groups including mbac-mcrA, mcp, MCR-2a, msar, msp, MCR-7 and msa. The number of observed groups reduced to four (mbac-mcrA, mcp, MCR-7 and msa) at 50 °C since MCR-2b, msp and msa disappeared. Population densities of all observed methanogen groups reduced at 50 °C. However, temperature reduction increased the number of observed methanogen groups to six. The msa group (representing the family Methanosaetaceae) appeared again at 20 °C, but MCR-2b and msp were not further detected under starvation condition. In addition, MCR-2a, which was not present in the initial sample and at 50°C, appeared at 20°C. The abundance of MCR-2a made up 2.3-2.5×10² mcrA copies ml⁻¹, accounting for 56.5% of the net methanogen population, resulting in an increase in the total methanogen population. It is likely that MCR-2a group is more active under low temperature and limited substrate conditions. The mbac-mcrA, mcp, MCR-7 and msar population levels were relatively stable during starvation period. These results indicate that methanogens do not easily die off under starvation condition but await favourable conditions for growth.

Table 1: Dynamics of methanogen populations in the membrane reactors A and B.

<table>
<thead>
<tr>
<th>Group</th>
<th>Reactor A (copies ml⁻¹)</th>
<th>Reactor B (copies ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial 50 °C 20 °C</td>
<td>Initial 50 °C 20 °C</td>
</tr>
<tr>
<td>mbac-mcrA</td>
<td>3.4×10⁷±1.3×10⁷ 1.3×10⁷</td>
<td>3.4×10⁴±1.3×10⁴ 1.3×10⁴</td>
</tr>
<tr>
<td>mrtA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>mcrA</td>
<td>7.2×10⁷±1.3×10⁷ 1.3×10⁷</td>
<td>7.2×10⁴±1.3×10⁴ 1.3×10⁴</td>
</tr>
<tr>
<td>mcp</td>
<td>6.5×10⁷±1.6×10⁷ 1.6×10⁷</td>
<td>6.5×10⁴±1.6×10⁴ 1.6×10⁴</td>
</tr>
<tr>
<td>MCR-7</td>
<td>2.1×10⁷±2.9×10⁷ 2.9×10⁷</td>
<td>2.1×10⁴±2.9×10⁴ 2.9×10⁴</td>
</tr>
<tr>
<td>MCR-2a</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>FEN</td>
<td>9.0×10⁷±2.5×10⁷ 2.5×10⁷</td>
<td>9.0×10⁴±2.5×10⁴ 2.5×10⁴</td>
</tr>
<tr>
<td>msar</td>
<td>2.1×10⁷±3.9×10⁷ 3.9×10⁷</td>
<td>2.1×10⁴±3.9×10⁴ 3.9×10⁴</td>
</tr>
<tr>
<td>msa</td>
<td>1.2×10⁷±6.3×10⁷ 6.3×10⁷</td>
<td>1.2×10⁴±6.3×10⁴ 6.3×10⁴</td>
</tr>
<tr>
<td>SUM</td>
<td>7.0×10⁷±1.4×10⁷ 1.4×10⁷</td>
<td>7.0×10⁴±1.4×10⁷ 1.4×10⁴</td>
</tr>
</tbody>
</table>

n.d., not detected

Fig. 4: Correspondence analysis (a) and canonical correspondence analysis (b) of methanogen communities of membrane reactors. Solid and blank indicate membrane reactors A and B, respectively, in (a). The symbol x indicates methanogen groups and ▪ indicates methanogen communities in (b).
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Methanogen communities were analyzed using CA and CCA after data normalization (Fig. 4). CA was applied for comparison of methanogen communities (Fig. 4a). The first two axes of CA plot explained 70.1% and 27.3% of the total variance, respectively. CA plot showed that methanogen communities were grouped according to temperature, and that communities were similar between reactors A and B under each condition. The initial community (located at -1 to -0.5 of the first axis) was closer to the community at 50 °C (-0.5 of the first axis) than that at 20 °C (0.8 to 1.5 of the first axis), indicating that the temperature shift substantially changed the methanogen community composition.

CCA was performed to analyze the relationship between community composition and temperature. The CCA result produced 2 axes accounting for 72.3 % and 27.7% of the variance of species-environmental relation (Fig. 4b). Monte Carlo permutation testing indicated that temperature showed a correlation with community composition (p<0.005). CCA indicated that the relative abundances of all methanogen groups, except for that of MCR-2b, were positively correlated with temperature. MCR-2a was negatively correlated with temperature, whose relative abundance peaked to 56.5% at 20°C. MCR2b, msp, msa and mcp were more abundant in the initial sample, while mbac-mcrA and mcr-7 were more abundant at 50°C. The relative abundance of msar did not change at 50°C, as compared to initial abundance. These community results indicated that temperature regime could strongly induce changes in the methanogen community composition as well as among individual populations under starvation conditions.

To sum up, continuous feeding ceased the activity and population growth of methanogens at 50 °C. After a temperature shift to 20 °C, methanogen activity appeared again and the population grew, although the feeding was continued. Community analysis indicated that temperature significantly affected community composition. Therefore, methanogens survived for a longer period (more than 84 days) and temperature was determined to be an important factor of their survival and activity.

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References


