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

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Abstract

To investigate starvation effect on methanogen community, two identical membrane reactors were continuously operated for 84 consecutive days, with a temperature change from 50ºC to 20ºC. Continuous feeding washed out 97% biomass from reactors during the experimental period. Quantitative PCR, using *mcrA* genes, indicated that the methanogen abundance decreased from 7.0×10^{7} to 1.2×10^{7} *mcrA* copies·ml^{-1} (volume basis) at 50 °C, and then increased to 4.4×10^{7} *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

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 H_2 (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

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_2 gas (99.99% v/v, Dong-A gas, Seoul, Korea). The basal medium consisted of NH_4Cl (0.321g l^{-1}), KH_2PO_4 (0.489 g l^{-1}), $MgSO_4$. $7H_2O$ (0.074 g l^{-1}), NaCl (0.018 g l^{-1}), $CaCl_2$. $2H_2O$ (0.059 g l^{-1}), $FeSO_4$. $7H_2O$ (0.005 g l^{-1}) and trace element solution (1 ml l^{-1}). The trace element solution contained Na_2MoO_4. $2H_2O$ (0.036 g l^{-1}), $CoCl_2$. $6H_2O$ (0.190 g l^{-1}), $MnCl_2$.$4H_2O$ (0.500 g l^{-1}), $CuCl_2.2H_2O$ (0.002 g l^{-1}), $NH_4.6Mo_7O_{24}$. $4H_2O$ (0.090 g l^{-1}), H_3BO_3 (0.030 g l^{-1}) and $ZnCl_2$ (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. Hallow-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 (an initial concentration of 1067 mg dry cell weight (DCW)·l^{-1}) were added to the reactors. The liquid and membrane volumes were 5 and 0.8 l, respectively, and the head space volume was 3 l. CH_4 gas (99.99% v/v, Dong-A gases, Seoul, Korea) was injected using a 50 ml gas tight syringe at a final concentration of 10% at the headspace. The membrane bioreactors were placed on a rotary shaker (Vision Scientific, Bucheon, Korea) and were agitated (80 rpm). The basal medium was used as an influent. Two identical reactors were continuously operated for 84 days with 5 hr hydraulic retention time (HRT). The temperature was changed from 50 to 20 °C at day 53. Resazurine, a redox indicator, was added to a final concentration of 1 ppm in order to monitor the conditions of aeration within the system (Karakashev *et al.,* 2003).

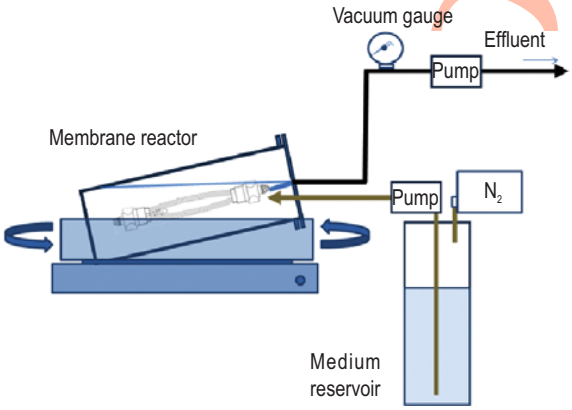

Fig. 1 : Schematic diagram of membrane reactors for inducing starvation

Analysis of methane and oxygen : CH_4 and O_2 concentrations were monitored using gas chromatography (GC), as previously described (Kim *et al.*, 2013a). Dissolved methane flowed out from the reactors due to continuous flow of the medium. An identical reactor was operated for 15 days at a HRT of 5 days, without the inoculation of methanogens. Methane concentration at the head space were monitored throughout the 15-day period. The dissolved methane that flowed out was quantified. Methane baselines were constructed by subtracting the amount of seeped methane from the highest methane concentration for each reactor (data not shown).

Analysis of methanogens : Samples were taken from the membrane reactors at days 0 (initial), 35 (50 °C) and 84 (20 °C). Samples were collected in triplicate. For quantitative PCR of 10 methanogen groups (Steinberg and Regan, 2009), DNA standards were prepared as mentioned previously (Kim *et al.,* 2013b). They were serially diluted 10-fold for standard curve construction, ranging from $1x10^3$ to $1x10^7$ gene copies. qPCR was then carried out as previously described (Kim *et al.,* 2013b). The abundance of each group was normalized as a percentage of the total methanogen abundance, and methanogen communities were then further analyzed using correspondence analysis (CA) and canonical correspondence analysis (CCA) with Canoco version 4.5 software (Microcomputer Power, Ithaca, USA). CA was performed to distinguish communities on the basis of their composition. CCA was performed to analyze the relationship between the community composition and an environmental variable, such as temperature.

Results and Discussion

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 70%. Cell dry weight was reduced from 1066.7 mg-DCW l^{-1} at day 0 to 13.7 mg-DCW l^{-1} at day 84, representing a reduction of about 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 reducted 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 7.0×10^7 to 1.4×10^7 *mcrA* copies·ml^{-1} in reactor A (80% reduction) and 9.6×10^6 *mcrA* copies·ml^{-1} in reactor B (87% reduction) at 50 ºC ($p<0.05$) on volume basis (Fig. 3a). However, the temperature shift from 50 to 20 °C significantly increased the methanogen abundance to 4.2×10^7 *mcrA* copies ml^{-1} (335%) in reactor A and 4.5×10^7 *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 6.5×10^7 to 5.1×10^9 *mcrA* copies mg DCW^{-1} in reactor A and 2.4×10^9 *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.*

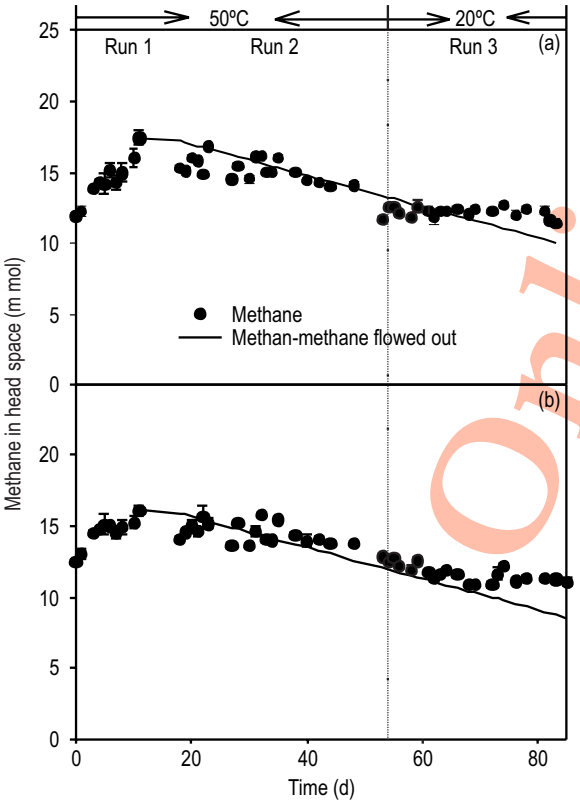

Fig. 2 : Methane concentrations of membrane reactors A (a) and B (b). Solid lines are the methane base lines, constructed by subtracting dissolved methane that flowed out

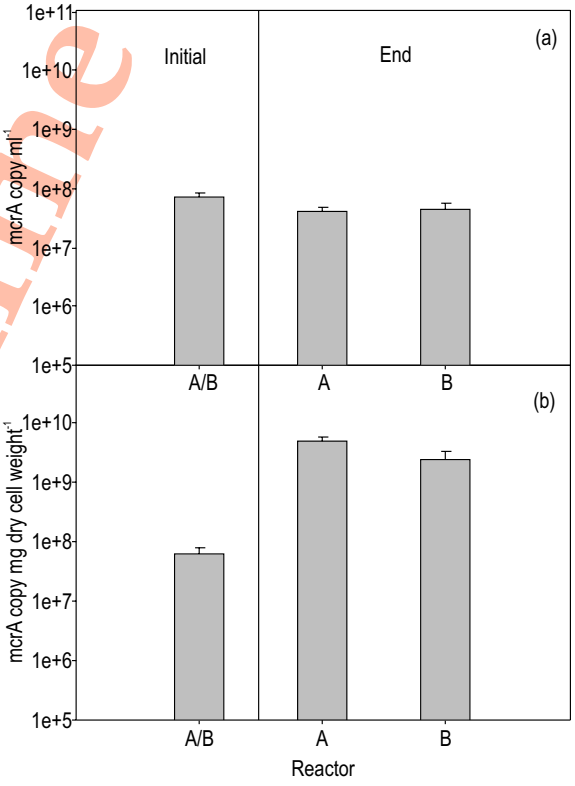

Fig. 3 : Total mcrA copy numbers per unit volume (a) vs. unit biomass (b) in membrane reactors A and B at the initial and end of the 84-d starvation period

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

Group	Reactor A (copies ml^{-1})			Reactor B (copies ml^{-1})		
	Initial	50 °C	20 °C	Initial	50 °C	20 °C
mbac-mcrA	$3.4\times10^{5}\pm1.3\times10^{5}$	$3.2\times10^{5}\pm9.4\times10^{4}$	$2.0\times10^{5}\pm9.5\times10^{4}$	$3.4\times10^{5}\pm1.3\times10^{5}$	$2.1\times10^{5}\pm5.3\times10^{4}$	$3.0\times10^{5}\pm1.5\times10^{5}$
mrtA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mcp	$7.2\times10^{6}\pm1.3\times10^{6}$	$3.6\times10^{5}\pm1.8\times10^{5}$	$3.0\times10^{5}\pm1.5\times10^{5}$	$7.2\times10^{6}\pm1.3\times10^{6}$	$3.4\times10^{5}\pm2.0\times10^{5}$	$4.0\times10^{5}\pm2.6\times10^{5}$
msp	$6.5\times10^{6}\pm1.6\times10^{6}$	n.d.	n.d.	$6.5\times10^{6}\pm1.6\times10^{6}$	n.d.	n.d.
MCR-7	$2.1\times10^{7}\pm2.9\times10^{6}$	$9.9\times10^{6}\pm9.0\times10^{5}$	$1.1\times10^{7}\pm4.9\times10^{6}$	$2.1\times10^{7}\pm2.9\times10^{6}$	$6.0\times10^{6}\pm2.1\times10^{5}$	$1.5\times10^{7}\pm6.4\times10^{6}$
MCR-2a	n.d.	n.d.	$2.5\times10^{7}\pm1.4\times10^{5}$	n.d.	n.d.	$2.3\times10^{7}\pm4.9\times10^{6}$
MCR-2b	$9.0\times10^{5}\pm2.5\times10^{5}$	n.d.	n.d.	$9.0\times10^{5}\pm2.5\times10^{5}$	n.d.	n.d.
FEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
msar	$2.1\times10^{7}\pm3.9\times10^{6}$	$3.7\times10^{6}\pm1.5\times10^{6}$	$3.9\times10^{6}\pm4.0\times10^{6}$	$2.1\times10^{7}\pm3.9\times10^{6}$	$3.0\times10^{6}\pm4.8\times10^{5}$	$3.7\times10^{6}\pm2.9\times10^{6}$
msa	$1.2\times10^{7}\pm6.3\times10^{6}$	n.d.	$1.7\times10^{6}\pm1.2\times10^{6}$	$1.2\times10^{7}\pm6.3\times10^{6}$	n.d.	$2.1\times10^{6}\pm1.7\times10^{6}$
SUM	$7.0\times10^{7}\pm1.4\times10^{6}$	$1.4\times10^{7}\pm2.7\times10^{6}$	$4.2\times10^{7}\pm7.9\times10^{6}$	$7.7\times10^{7}\pm1.4\times10^{6}$	$9.6\times10^{6}\pm1.1\times10^{6}$	$4.5\times10^{7}\pm1.2\times10^{7}$

n.d., not detected

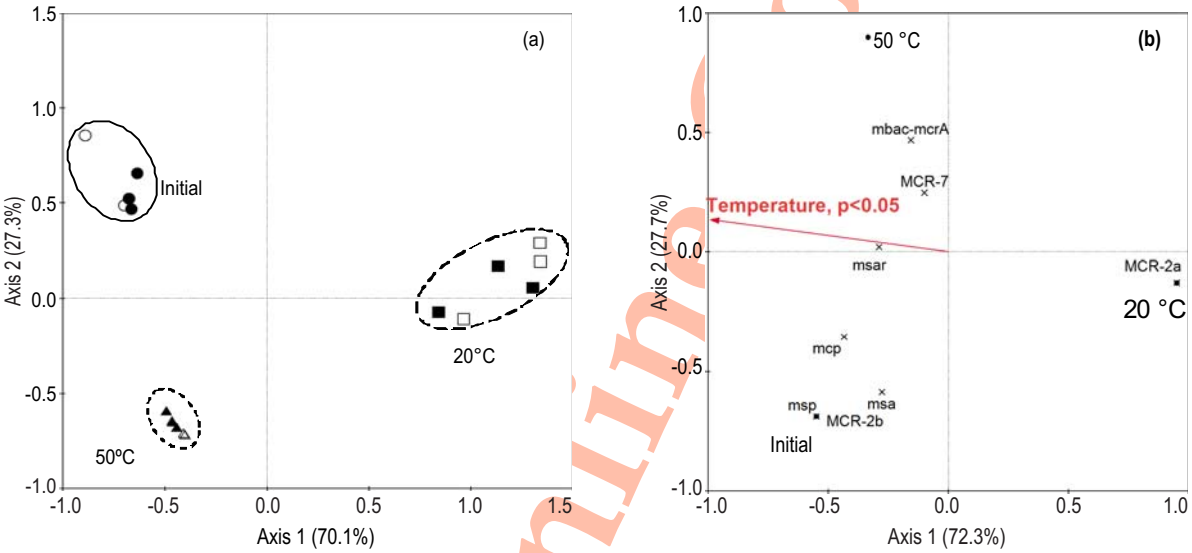

Fig. 4 : Correspondence analysis (a) and canonical correspondence analysis (b) of methanogen communities of membrane reactors. Solid and blank indicate membrane reactors A and B, respectively, in (a). The symbol × indicates methanogen groups and ● indicates methanogen communities in (b)

(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 *msar*) at 50 ºC since *MCR-2b*, *msp* and *msa* disappeared. Population densities of all observed methanogen groups reduced at 50 ºC. However, temperature reduction increased the number of observed methanogen groups to six. The *msa* group (representing the family Methanosaetaceae) appeared again at 20 ºC, 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 50ºC, appeared at 20ºC. The abundance of *MCR-2a* made up 2.3-2.5×10^{7} *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.

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 ºC (-0.5 of the first axis) than that at 20 ºC (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 20ºC. *MCR2b*, *msp*, *msa* and *mcp* were more abundant in the initial sample, while *mbac-mcrA* and *mcr*-7 were more abundant at 50 ºC. The relative abundance of *msar* did not change at 50ºC, 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 ºC. After a temperature shift to 20 ºC, 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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