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Analysis of Relationship Between Microbial and Methanogenic Biomass in Methane Fermentation

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Abstract To analyze the relationship between biomass of microorganisms and methane production, the total biomass of bacteria and archaea (BA) during methane fermentation was analyzed by the environmental DNA analysis method. In the case of using methanogenic sludge as a seed which is generally used for methane fermentation, the total BA biomass reached to 1.5×10^8 to 3.6×10^8 cells/ml when methane was produced. On the other hand, soil suspension was used as a seed; methane was not produced for 14-day cultivation. However, the total BA biomass reached to above 1.5×10^8 cells/ml. The methanogen biomass was counted by using a fluorescence microscope (coenzyme F_{420}), and the methanogen biomass and the ratio of methanogens in the total of BA were analyzed during methane fermentation. At the methane-producing phase, the methanogen biomass reached to 1.3×10^8 cells/ml, and the ratio of methanogens was above 70% of the total BA. When the ratio of methanogens in a seed was changed, the methane-producing phase was moved. However, the relationship between methanogens and other microorganisms at the methane-producing phase was almost similar.

Keywords Methane fermentation · Microbial biomass · Methanogen · Environmental DNA (eDNA) · Coenzyme F_{420}

Abbreviations

BA	Bacteria and archaea
eDNA	Environmental DNA
DAPI	4′,6-diamino-2-phenylindole

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Introduction

Methane fermentation is an anaerobic process that occurs in various kinds of microorganisms, mainly acidogens and methanogens [1]. In recent years, methane gas has been regarded as a good source of renewable energy from biomass and biomass waste. Methane production from various kinds of organic substrates has been investigated, and efficient fermentation equipment has been developed to enhance methane production [2–5].

Various types of microorganisms participate in methane production; therefore, it is important to analyze the types and ratios of microorganisms during this process to develop optimal systems for methane production. Methane fermentation is monitored by the measurement of gas production, pH, COD, etc. [6, 7]. Phylogenetic diversities of bacteria and archaea (BA) in several methanogenic cultures were determined by 16S rDNA analysis [8, 9]. Many species of methanogenic archaea, anaerobic bacteria, and unidentified microorganisms were found in a high-efficiency methane-producing reactor.

The microbial community in methanogenic reactors has been investigated by denaturing gradient gel electrophoresis, single-strand conformation polymorphisms, terminal-restriction fragment length polymorphisms, etc. [10–14]. The community structure dynamically changed during stable methane production. To improve the efficiency of methane production, quantitative monitoring and controlling of the total microbial biomass in the methanogenic culture is important.

To determine conditions for a stable high rate of methane production, the total biomass of bacteria and archaea was quantified using the environmental DNA (eDNA) method, and the relationship between biomass and gas production was analyzed. Furthermore, the methanogen biomass was quantified based on coenzyme F_{420}, and the ratio of methanogens in the total BA at the methane-producing phase was studied.

Materials and Methods

All chemicals were purchased from Wako Pure Chemicals (Tokyo, Japan) or Nacalai Tesque (Kyoto, Japan). Mesophilically and anaerobically digested sludge was collected from a cattle waste treatment plant (capacity of fermentor, 110 m^3; processing performance, 4.5 t/day) and used as the seed sludge. Sewage sludge, activated sludge, and food waste were collected from various industries and used as substrates.

A modified phosphate-buffered basal medium [15] was modified and used as the basal medium and contained the following (per liter): 3 g sodium acetate, 3 g sodium formate, 100 ml mineral solution I, 10 ml mineral solution II, 10 ml vitamin solution, 0.2 g yeast extract, 2 ml 0.1% resazurin solution, 3 g methanol, 6 g $NaHCO_3$, 0.5 g L-cysteine-HCl·H_2O, and 0.25 g $Na_2S·9H_2O$. Mineral solution I contained the following (per liter): 4 g KH_2PO_4, 4 g K_2HPO_4, 10 g NH_4Cl, and 1 g $MgCl_2·6H_2O$. Mineral solution II contained the following (per liter): 4.5 g nitrilotriacetic acid, 0.4 g $FeCl_2·4H_2O$, 0.12 g $CoCl_2·6H_2O$, 0.01 g $AlKSO_4$, 1 g NaCl, 0.02 g $CaCl_2$, 0.01 g $NaMoO_4·2H_2O$, 0.1 g $ZnCl_2$, 0.01 g H_2BO_3, 0.01 g $CuSO_4·5H_2O$, and 0.02 g $NiCl·6H_2O$. The vitamin solution contained the following (per liter): 2 mg biotin, 2 mg folic acid, 10 mg B_6 (pyridoxine)-HCl, 5 mg B_1 (thiamine)-HCl, 5 mg B_2 (riboflavin), 5 mg nicotinic acid, 5 mg DL-pantothenic acid, 0.1 mg B_{12} (cyanocobalamin), 5 mg lipoic acid, and 5 mg *p*-aminobenzoic acid.

Methane fermentation was carried out in a 500-ml flask with 360 ml of basal medium or a fermentation substrate. The water content of each fermentation substrate was controlled at

92%. The basal medium and all substrates were autoclaved for 15 min at 121 °C. Seed sludge or soil suspension (40 ml) that was preserved at −80 °C with 20% (*v*/*v*) glycerol was inoculated into the basal medium or fermentation substrate. Cultivation was carried out over 48 h at 37 °C with gentle shaking (40 rpm), and the biogas was collected with 1 l gas bag (Tedlar Bag AA-1, DuPont, DE, USA). The amount of biogas was measured by a gas counter (MGC, Ritter, Bochum, Germany), and the composition of biogas was analyzed by a gas chromatograph (GC-4000, GL Sciences, Tokyo, Japan). The GC was equipped with a thermal conductivity detector (electric current adjusted to 100 mA), steel columns (1/8 in.× 2 m) packed with Porapak Q (80/100 mesh, Shinwa Chemical Industries, Kyoto, Japan). Helium was used as the carrier gas, and the flow rate was 20 ml/min. The injector, oven, and detector were maintained at 130 °C, 110 °C, and 130 °C, respectively. Quantification of methane was calculated by the amount and the composition of biogas.

The eDNA was extracted from 1 ml sample of methane fermentor by the slow-stirring method [16]. After electrophoresis on a 1.0% agarose gel, eDNA was quantified using Kodak 1D 3.6 imaging software (Kodak, NY, USA), and the total BA biomass was calculated using an experimentally determined calibration curve.

To quantify the total BA biomass by the 4′,6-diamino-2-phenylindole (DAPI) staining method, pretreatment and staining of the sample were carried out as described previously [16]. The sample was observed under 330–385 nm irradiation using an epifluorescence microscope (BX50 BX-FLA, OLYMPUS, Tokyo, Japan), and the cells with pale fluorescence were counted as BA (excitation wavelength of DAPI is 461 nm). Total BA biomass was calculated using the equation as described previously [16].

The methanogen biomass was quantified by coenzyme F_{420} analysis, which specifically detects methanogens [17, 18]. Samples were pretreated as described by Aoshima et al. [16]. The sample was observed under 400–440 nm irradiation using an epifluorescence microscope, and the blue-green fluorescent cells (excitation wavelength of coenzyme F_{420} is 472 nm) were counted as methanogens. Both *Methanobrevibacter arboriphilus* NBRC 101200 and *Escherichia coli* JM109 were observed under 330–385 nm irradiation (DAPI staining method); however, only *M. arboriphilus* NBRC 101200 was detected under 400–440 nm irradiation (coenzyme F_{420} analysis). Biomass was calculated as described by Aoshima et al. [16]. The ratio of methanogens to total BA was calculated by comparing the methanogen biomass determined from coenzyme F_{420} analysis to the BA biomass determined using the eDNA method.

Results and Discussion

Bacteria and Archaea Biomass in the Methane-Producing Reactor

In order to understand methane production, analysis of the total BA biomass during methane fermentation is important. To construct a calibration curve for quantification of the total BA biomass by the eDNA method, methanogenic samples from methane fermentor (26 samples) were analyzed using a slow-stirring and a DAPI staining methods. The slow-stirring method could extract the DNA from pure culture of various archaea (data not shown). Viable numbers of BA were counted using a fluorescence microscope after the DAPI staining, and the relationship between the amount of eDNA and the viable numbers of BA was analyzed (Fig. 1). The amount of eDNA (X; μg/ml) was strongly correlated with the BA biomass (Y; cells/ml). The equation of the calibration curve was $Y=1.23\times10^7\ X$

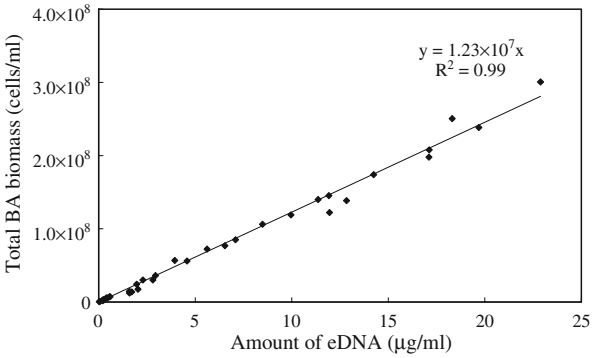

Fig. 1 Relationship between total BA biomass obtained using DAPI staining and eDNA in 26 methanogenic sludge samples. *Line* shows the correlation between total BA biomass

(cells/ml), and the correlation coefficient (R^2) was 0.99. The total BA biomass was deduced by the amount of eDNA in the methane-producing reactor, and the detection range was 1.2×10^7 to 3.1×10^8 cells/ml (Fig. 1).

Total BA Biomass at Methane-Producing Phase

The total BA biomass at the methane-producing phase was analyzed. The methanogenic sludge had an initial BA biomass of 1.5×10^7 cells/ml. This sludge, which is generally used as a seed for methane fermentation, was inoculated into medium or fermentation substrate, basal medium, sewage sludge, activated sludge 1, activated sludge 2, food waste, and rice. The eDNAs were extracted and BA biomass was calculated using the calibration curve. Methane production was observed in all media, and the BA biomass in each medium exceeded 1.5×10^8 cells/ml at the methane-producing phase (Table 1). Soil suspension was then used as a seed instead of the methanogenic sludge. The initial BA biomass was 8.5×10^7 cells/ml in the soil suspension. Using this material as a seed, the BA biomass exceeded 1.5×10^8 cells/ml at 24-h cultivation. However, methane production was not observed even after 14-day cultivation.

In order to compare cell growth between cultures seeded with methanogenic sludge or soil suspension, BA biomass in basal medium were analyzed for 48 h (Fig. 2). BA cell growth in each culture was almost the same, and the biomass in each culture exceeded 1.5×10^8 cells/ml at 24-h cultivation. Methane production was observed at 24-h cultivation when methanogenic sludge was used as a seed, while methane production was not detected when soil suspension was used as a seed. These results suggest that methanogens were either absent or present at very low biomass in the soil suspension. Therefore, monitoring of methanogens in the reactor is necessary to accurately analyze conditions for optimal methane production.

Methanogen Biomass at Methane-Producing Phase

To analyze the methanogen biomass at the methane-producing phase, methanogenic sludge was used as a seed to inoculate basal medium or sewage sludge medium, and the methanogen biomass was counted using a fluorescence microscope. The methanogen biomass exceeded 1.0×10^8 cells/ml in both media at the methane-producing phase (Table 2).

Table 1 Total BA biomass at the methane-producing phase.

Medium or substrate[a]	Seed	Total BA biomass[b]		Methane-producing time (days)
		Initial ($\times 10^7$ cells/ml)	Methane-producing phase ($\times 10^8$ cells/ml)	
Basal medium	Seed sludge 1	1.5±0.2	1.5±0.3	4
	Seed sludge 1	1.5±0.2	1.8±0.1	4
	Seed sludge 2	2.7±0.1	1.5±0.1	2
Sewage sludge	Seed sludge 1	1.5±0.2	1.5±0.1	7
	Seed sludge 1	1.5±0.2	1.5±0.2	7
Activated sludge 1	Seed sludge 1	1.5±0.2	2.3±0.1	3
	Seed sludge 1	1.5±0.2	2.2±0.1	3
Activated sludge 2	Seed sludge 1	1.5±0.2	3.4±0.1	3
	Seed sludge 1	1.5±0.2	3.6±0.1	3
Food waste	Seed sludge 1	1.5±0.2	1.5±0.3	2
	Seed sludge 1	1.5±0.2	1.8±0.1	2
Rice	Seed sludge 1	1.5±0.2	1.6±0.1	5
	Seed sludge 1	1.5±0.2	1.8±0.2	5
Basal medium	Soil suspension	8.5±0.1	–[c]	–[d]

[a] All substrates were autoclaved at 121 °C for 15 min

[b] Total BA biomass were quantified by environmental DNA analysis

[c] Maximum BA biomass was 3.5×10^8 cells/ml at day 2

[d] Methane production was not detected after 14-day cultivation

When soil suspension was used as a seed instead of the methanogenic sludge, methane was not produced in either medium during 14-day cultivation. Methanogens were not observed in either medium, indicating that the methanogens were either absent or present in very low biomass in the soil suspension. Various kinds of microorganisms can participate in methane production; therefore, simultaneous analysis of the BA and methanogen biomass is necessary to determine optimal conditions for methane production.

Methane production was initiated when the methanogen biomass reached 1.3×10^8 cells/ml in this study. The conversion of organic acids or hydrogen into methane is the rate-limiting reaction in methane fermentation; therefore, a high methanogen biomass appears to be a crucial factor for this reaction to occur efficiently. For the rate-limiting reaction to proceed smoothly in methane fermentation, reaching and maintaining a high methanogen biomass, for example 1.3×10^8 cells/ml, could be important.

Growth of BA and Methanogens During Methane Production

In order to investigate the relationship between biomass and methane production, growths of BA and methanogens using different seeds during methane production were analyzed (Fig. 3). There were very low methanogen biomass in the soil suspension; therefore, two types of seed were prepared by mixing the methanogenic sludge and soil suspension. Seed-H had an initial BA biomass of 5.6×10^7 cells/ml and contained approximately 70% methanogens (Fig. 3a). Seed-L had an initial BA biomass of 7.0×10^7 cells/ml and contained approximately 15% methanogens (Fig. 3b).

The cell growths of BA using Seed-H and Seed-L were almost the same (Fig. 3a, b). However, the methanogen biomass using Seed-L increased at a higher rate than that using

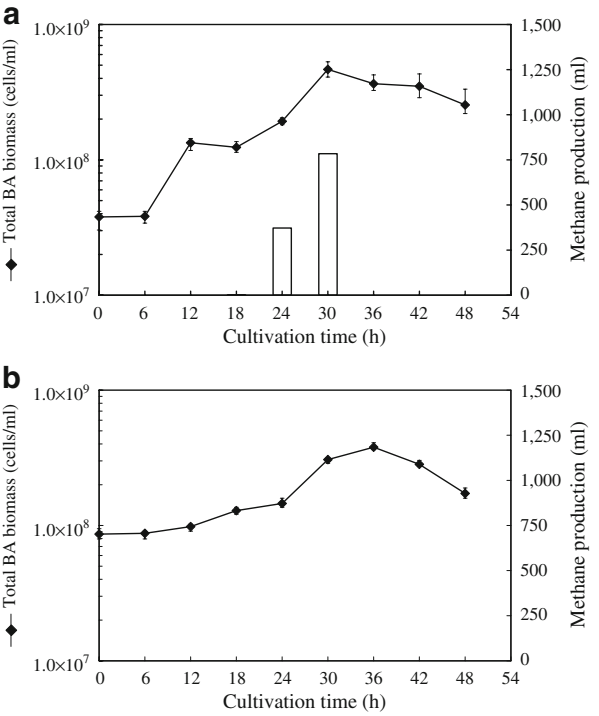

Fig. 2 Time course of total BA biomass analyzed by eDNA and methane production. Methanogenic sludge (**a**) or soil suspension (**b**) was inoculated as seed sludge. *Closed diamonds* and *bars* indicate total BA biomass and methane production, respectively

Seed-H during 0–6-h cultivation (Fig. 3b). Methane production was observed at 24- (Seed-H) or 30-h (Seed-L) cultivation, and the biomass of BA and methanogens in each culture at the methane-producing phase were almost the same (Table 3). The ratios of methanogens in the total BA biomass at the methane-producing phase in each culture were 72% and 87%, respectively. Reaching and retaining a critical biomass and controlling the ratio of methanogens in the total BA biomass appear to be important factors for stable and high methane production.

Table 2 Methanogen biomass at the methane-producing phase.

Medium or substrate[a]	Seed	Methanogen biomass[b]		Methane-producing time (day)
		Initial ($\times 10^7$ cells/ml)	Methane-producing phase ($\times 10^8$ cells/ml)	
Basal medium	Seed sludge 2	1.1±0.2	1.3±0.2	2
	Seed sludge 2	1.1±0.2	1.6±0.1	2
Sewage sludge	Seed sludge 2	1.1±0.2	1.5±0.1	7
	Seed sludge 2	1.1±0.2	1.7±0.3	7
Basal medium	Soil suspension	N. D.	–[c]	–[d]

N. D. not detected

[a] All substrates were autoclaved at 121 °C for 15 min

[b] Methanogen biomass were quantified by F_{420} analysis

[c] Methanogens were not detected at days 2, 7, and 14

[d] Methane production was not detected after 14-day cultivation

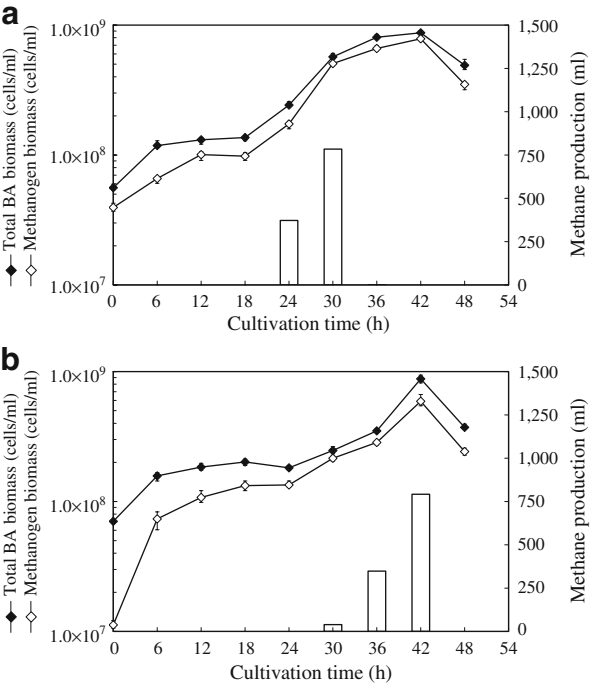

Fig. 3 Time course of total BA biomass, methanogen biomass, and methane production. Methanogenic sludge (**a**) or a mixture of methanogenic sludge and soil suspension (**b**) was inoculated as seed sludge. *Closed diamonds*, *open diamonds*, and *bars* indicate total BA biomass, methanogen biomass, and methane production, respectively

Various types of microorganisms, such as organic acids-, hydrogen-, and methane-producing strains, participate in methane fermentation. The cell growth of methanogens in pure culture is relatively slow; however, methanogen cell growth can be enhanced by hydrogen-producing bacteria [19, 20]. Propionate-oxidizing bacteria produce hydrogen and acetate from propionate, and the hydrogen produced in the methane fermentor is consumed by hydrogenotrophic methanogens [21–23]. This enhances the production of hydrogen and acetate because it avoids growth inhibition by accumulation of metabolites. Consequently, methane production by methanogens using hydrogen and acetate is increased. Methanogens comprised more than 70% of the total BA biomass at the methane-producing phase in this study; thus, methane production appears to be enhanced by methanogens that consume organic acids and hydrogen. On the other hand, the high ratio of methanogens in the fermentor may lead to direct production of organic acids and/or hydrogen from substrates. Either consumption or direct production of organic acids and hydrogen by methanogens may contribute to methane production; therefore, analyzing this effect is important to optimize methane production.

Table 3 Biomass of BA and methanogens at the methane-producing phase.

Seed	Biomass[a] ($\times 10^8$ cells/ml)		Ratio of methanogens (%)	Methane-producing time (h)
	BA	Methanogens		
Seed-H	2.4±0.2	1.8±0.1	71.6	24
Seed-L	2.5±0.2	2.2±0.1	87.2	30

[a] Biomass of BA and methanogens were quantified by environmental DNA analysis and F_{420} analysis, respectively

Conclusion

Quantification methods of BA and methanogen biomass were constructed using eDNA and coenzyme F_{420}. The range of BA and methanogen biomass were 1.5×10^8 to 3.6×10^8 and 1.3×10^8 to 2.2×10^8 cells/ml at methane-producing phase, respectively. Methanogen biomass became dominant microorganism in the methane-producing fermentor. Analysis and control of BA and methanogen biomass are important factors for the stable methane production.

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