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Establishment and Analysis of Microbial Communities Capable of Producing Methane from Grass Waste at Extremely High C/N Ratio

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***Abstract*— Acclimation of microbial communities, aiming to methane production from grass as a sole substrate at extremely high carbon-to-nitrogen (C/N) ratio, was conducted. In a series of experiments with various sizes of added grass, two microbial communities showing high methane production were obtained with powdered grass. In the two microbial communities designated NR and RP, Bacteroidia including genera *Bacteroides, Dysgonomonas, Proteiniphilum,* and *Alistipes* were detected as dominant members in eubacteria. It was also shown that Methanomicrobia and Methanobacteria including genera *Methanomassiliicoccus* and *Methanobacterium* were found as dominant members in methanogen. It is noteworthy that nitrogen fixation were observed both in NR and RP, suggesting that insufficiency of nitrogen sources would be complemented by uptake of nitrogen from gaseous phase in culture.**

***Index Terms*— grass waste, high C/N ratio, methane fermentation, nitrogen fixation.**

I. Introduction

Grass from public space, including roadside-verges and river-terraces, is usually cut and left (mulching), or incinerated as city waste with a cost. Annual biomass yield of grass in public space is reached 3-6 t dry matter/ha [1]. Considering a world-wide trend of environment-friendly society, energy recovery from the grass waste becomes important for management of maintenance cost in public space or even for energy supply in the society [2, 3]. In Japan, there is no datum on gross production of all available grass species, however, for a representative grass *Miscanthus sinensis*, 2 million t dry matter/year of nationwide production is estimated [4]. Therefore, approach for utilization of the huge amount of grass containing *Miscanthus* is required. Mowing grass is usually carried out within a confined area per day, transportation and accumulation of grass for waste treatment (eg. incineration) often become problematic because of consuming time, labor (energy) and cost. One of the possible solutions is installation of small methane or hydrogen fermentation system in each confined area and construction of network for dispersed generation of energy, mainly electricity. Benefits among utilization of distributed energy resources and their networking have long been discussed [5-7].

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Anaerobic digestion is a conventional method for biomass utilization [8]. Despite the fact that a great deal of research for methane production from grass has been conducted especially in recent years, almost processes need supply of abundant sludge source such as sewage and manure [9, 10]. Many studies indicated that the optimal carbon-to-nitrogen (C/N) ratio in methane fermentation were 25-30, and the C/N ratio higher than 40 are not generally suitable [11]. The C/N ration of raw grass shows a wide range; wild grassy weed is at relatively high (>59) while lawn grass is at low (<29) [12]. Therefore, it is difficult to conduct methane fermentation with wild grass-only, also being due to less degradability of lignocellulose [13]. To produce methane from grass, pretreatment of grass such as chemical/physical processing and ensiling is required to enhance substrate availability and methane productivity [14-16]. To construct a system utilizing distributed grass biomass on-site, it is preferable that methane fermentation is progressed in the vicinity of grass-mowing area, without addition of other sludge sources.

In this study, we succeeded to establish microbial communities capable of producing methane from grass as a sole substrate. In addition, the established microbial communities showed high methane production at extremely high C/N ratio.

II. Materials and Methods

A. Acclimation of Microbial Communities Producing Methane fro Grass

Japanese wild grass Gyougi-shiba (*Cynodon dactylon*, generally known as bermudagrass but Gyougi-shiba shows high C/N ratio around 200) was collected in University of Yamanashi and used as substrate grass in this study. The grass was completely dried and cut in 10-50 or 2-5 mm length, or ground to powder under 1 mm diameter for investigation of effect of substrate size.

Muds from Nigorigawa river (NR) and Yonbugawa river (YR) in Yamanashi prefecture, and a reservoir pond (RP) in University of Yamanashi were used as sources of microbial communities. Every 5 mL of source suspension were inoculated into 100 mL-volume glass bottles containing 95 mL media (0.1 M NaH_2PO_4-K_2HPO_4 buffer, pH 7.2, and 1% w/v grass at final concentration). Inoculated bottles were sealed with rubber stoppers, purged with nitrogen gas, and the cultivations were conducted at 35˚C statically. The microbial communities were subcultured every 90 days by transferring 5 mL of cultures to 95 mL of same fresh media and continuing incubation.

B. PCR-DGGE Analyses

Microbial cells were harvested by centrifuging 1 mL culture and removing supernatants, and then total genomic DNAs were extracted according to the method of Zhu *et al.* [17]. Obtained DNAs were resuspended with sterilized water (7 µg/mL), and the 0.5 µL aliquots were used for 50 µL-scale PCR with 25 µL of 2 x Ampdirect Plus (Shimadzu, Kyoto), 0.5 U of BioTaq HS DNA polymerase (Bioline Reagents, London), 2.5 pmol each of forward and reverse primers. For eubacteria detection, primers 357F-GC and 517R were used [18]. For methanogenic archaea detection, DNAs were amplified in 20 µL-scale with 1.0 pmol each of primers A1f and Met1340R [19], and then 0.5 µL aliquots of the amplified DNAs were used for following 20 µL-scale nested PCR with primers GC-clamp-ARC787F and ARC1059R [20]. The PCR were carried out at the condition described by Muyzer *et al.* [18].

Denatured gradient gel electrophoresis (DGGE) were performed by DCode system (Bio-Rad Laboratories, Hercules) using gradient gel with concentration from 6% polyacrylamide-1.4 M urea-8% deionized formamide to 12% polyacrylamide-4.2 M urea-24% deionized formamide under 0.5 x TAE buffer (pH 8.3). Five microliters of PCR-amplified samples were applied on the gel and were separated at 60˚C with 50 V for 30 min followed by 150 V for 6 h. Separated DNAs were visualized by SYBR Green I staining and UV radiation.

C. Cloning and Sequencing of PCR Products

PCR-amplified DNA fragments were ligated with pTAC-2 vector using DynaExpress TA PCR Cloning Kit (BioDynamics Laboratory, Tokyo). Ligated DNAs were transformed into *Escherichia coli* JM109, and then ampicillin-resistant and β-galactosidase-negative transformants were picked up. Colony-PCR with M13 forward and reverse primers (Primers M4 and RV, Takara Bio, Kusatsu) were carried out to select transformants harboring plasmids with insertion of DNA at predicted sizes. Plasmids from selected transformants were purified using FastGene Plasmid Mini Kit (Nippon Genetics, Tokyo) and were sequenced by outsource service (Greiner Japan, Tokyo). Analysis of determined nucleotide sequences was performed using GENETYX-MAC Network Version 14.0.11 (Genetyx, Tokyo). Phylogenetic analysis was performed by MEGA7 Version 7.0.18 [21].

D. Analytical Procedures

For analysis of methane production, gaseous phase with positive pressure in culture bottles were collected by syringe every 10 days. After sampling, the gaseous phase in culture bottles were refreshed by purging with nitrogen gas, and then cultivation was continued. The contents of methane in collected gas were determined by gas chromatography using Shimadzu GC2014 system equipped with Molecular Sieve 5A 60-80 column (3 mm ID x 3 m) and thermal conductivity detector. The analysis was carried out with column temperature at 40˚C for 15 min, then gaining temperature at rate of 20˚C/min, and finally at 200˚C for 20 min with nitrogen as carrier gas (30 mL/min).

After series of methane fermentation, contents of acetone-soluble fraction, holocellulose, α-cellulose in culture residues were determined by the method of Yokoyama *et al.* [22]. The hemicellulosic contents were calculated as differences between holocellulose and α-cellulose. Klason lignin contents in culture residues were determined according to the description of Browning [23].

Nitrogen fixation by acclimated microbial communities was confirmed with detection of ethylene yielded under acetylene atmosphere. Briefly, every 1 mL of subculture was inoculated into 33 mL-volume glass vials containing 19 mL media (0.1 M NaH_2PO_4-K_2HPO_4 buffer, pH 7.2, and 1% w/v powdered grass). Inoculated vials were sealed with rubber stoppers, purged with nitrogen gas containing 0.1% acetylene, and the cultivations were conducted at 35˚C statically. After 21 days, gaseous phase with positive pressure in culture vials were collected by syringe. The contents of ethylene in collected gas were determined by gas chromatography using Shimadzu GC2014 system equipped with Porapak N 80-100 column (3 mm ID x 2 m) and flame ionization detector. The column temperature on analysis was at 50˚C for 10 min with helium as carrier gas (60 mL/min).

III. Results and Discussion

A. Acclimation of Microbial Communities Producing Methane from Grass

Microbial communities NR, RP and YR were subcultured with different sizes (10-50 mm, 2-5 mm and <1 mm powder) of grass for 90 days three times, and the production of methane were investigated. The C/N ratio of grass used in this study was 217±101.6, as determined using elemental analyzer Flash EA 1112 (Thermo Fischer Scientific, Yokohama). Figure 1 shows changes of pH, methane and hydrogen production in subcultures. Data from first subcultures are not shown because it was considered that methane production from organic matters in inoculated muds would not be ignored.

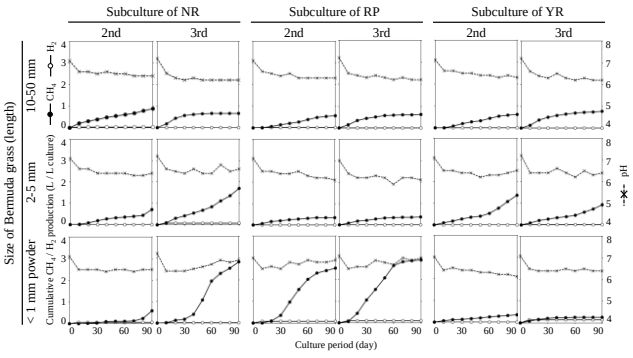

Fig. 1 Production of methane and hydrogen in subcultures derived from Nigorigawa river (NR), reservoir pond (RP) and Yonbugawa river (YR). Data from second and third subcultures are shown with changes of pH.

In all subcultures, pH decrease were observed in initial 10-day, and in the rest culture period pH were maintained at values between 6 and 7. It was also observed that all subcultures produced methane but little hydrogen. In comparison among added sizes of substrate grass, NR and RP

showed high production over 2 L-CH_4/L culture in third subculture with <1 mm powder size, however, YR showed lower production in all subculture (Fig. 1). Yu *et al.* described that powderization of grass did not affect yield of methane fermentation with anaerobic digester sludge [16]. In another side, Wall *et al.* reported that particle size of grass should be crucial for efficient continuous digester operation [24]. In subcultures of NR and RP with <1 mm powdered grass, it was strongly suggested that microbial communities with highly-adaptation to grass digestion were acclimated, possibly microorganisms in the communities became readily accessible to carbohydrate in grass. Thus, we focused on these subcultures, NR and RP both cultured with powdered grass. In the subsequent fourth subculturing with powdered grass, NR and RP maintained high production of methane indicating 2.75 L-CH_4/L culture and 3.22 L-CH_4/L culture in 90-day cultivation, respectively (Fig. 2A).

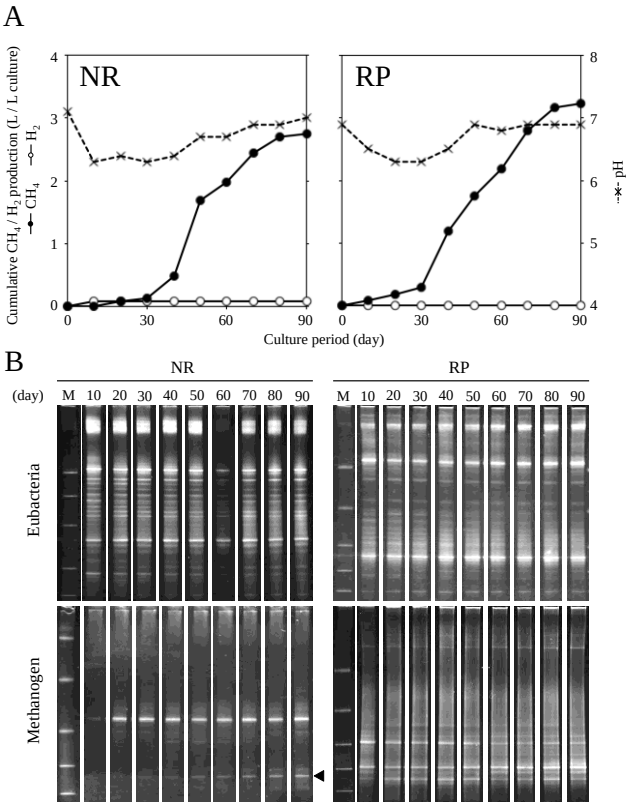

Fig. 2 Comparison of profiles in fourth subculture between NR and RP. (A) Production of methane, hydrogen, and changes of pH. (B) PCR-DGGE band patterns at every 10 days during culture. The lanes applied with 5 µL of DGGE Marker I (Wako Pure Chemicals, Osaka) are indicated by M. A position of band with apparent increase of intensity at late culture period is indicated by a closed triangle.

B. PCR-DGGE Analyses

Selected microbial communities NR and RP with powdered grass were stably produced methane well from 30 day-culture period onward. The culture profiles of fourth subculture and the chronological patterns of PCR-DGGE bands in NR and RP are shown in Fig. 2. In DGGE patterns, major 2 or 3 bands presumably derived from dominant members of communities were observed both for detection of eubacteria and methanogen in NR and RP (Fig. 2B). For detection of eubacteria, many minor bands were also observed, suggesting both NR and RP had intricate community structures. Unpredictably, almost DGGE bands did not show fluctuation in band intensity except one band that appeared at late culture period in NR (Fig. 2B). The observation of DGGE band patterns strongly suggested that the acclimated members of each microbial community made stable population for grass degradation and methane production, both in NR and RP.

C. Cloning and Sequencing of PCR Products

PCR-amplified DNA fragments from NR and RP were cloned with pTAC-2 vector and transformed into *E. coli* JM109. In constructed libraries of NR and RP, every 48 clones from eubacteria-targeted and methanogen-targeted amplification were sequenced using M13 forward primer.

Table 1 Variety of OTUs obtained with primers for eubacteria detection

Microbial community	OTU No.	Appearance frequency	Microorganism with highest 16S rDNA sequence similarity		
			Taxon	Accession No.	% Similarity
NR	NRb-OTU1	25/48	*Bacteroides* sp. 22C	AY554420	97-98
	NRb-OTU2	6/48	*Bacteroides* sp. 253c	AY082449	98-99
	NRb-OTU3	3/48	*Proteiniphilum* sp. S2	KP178480	99
	NRb-OTU4	2/48	*Alistipes finegoldii* DSM 17242	CP003274	98-100
	NRb-OTU5	2/48	*Cloacibacillus evryensis* strain LSPQ04216	KM881708	99-100
	NRb-OTU6	2/48	*Sedimentibacter hydroxybenzoicus* strain JW/Z-1	L11305	97-98
	NRb-OTU7	1/48	*Bacteroides* sp. 22C	AY554420	81
	NRb-OTU8	1/48	*Clostridium* sp. Marseille-P3122	LT598558	100
	NRb-OTU9	1/48	*Bacteroides cellulosilyticus*	LT223636	100
	NRb-OTU10	1/48	*Desulfovibrio marrakechensis* strain HAQ-7	KF536746	96
	NRb-OTU11	1/48	*Achromobacter xylosoxidans* strain F1-1-3	KX349991	100
	NRb-OTU12	1/48	*Syntrophomonas flectens*	AY290767	91
	NRb-OTU13	1/48	*Bacteroides ovatus*	AY652736	100
	NRb-OTU14	1/48	*Clostridium* sp. SW001	HM755724	98
RP	RPb-OTU1	18/43	*Dysgonomonas wimpennyi*	AY643492	98-100
	RPb-OTU2	13/43	*Bacteroides* sp. 22C	AY554420	98
	RPb-OTU3	2/43	*Acetanaerobacterium elongatum* strain Z7	AY487928	97-99
	RPb-OTU4	1/43	*Clostridium* sp. Marseille-P3122	LT598558	98
	RPb-OTU5	1/43	*Clostridium* sp. SW001	HM755724	99
	RPb-OTU6	1/43	*Dysgonomonas wimpennyi*	AY643492	97
	RPb-OTU7	1/43	*Syntrophomonas flectens*	AY290767	96
	RPb-OTU8	1/43	*Dysgonomonas wimpennyi*	AY643492	97
	RPb-OTU9	1/43	*Dysgonomonas wimpennyi*	AY643492	96
	RPb-OTU10	1/43	*Clostridium propionicum* DSM 1682	CP014223	100
	RPb-OTU11	1/43	*Bacteroides* sp. 22C	AY554420	98
	RPb-OTU12	1/43	*Dysgonomonas wimpennyi*	AY643492	93
	RPb-OTU13	1/43	*Dysgonomonas wimpennyi*	AY643492	93

Table 2 Variety of OTUs obtained with primers for methanogen detection

Microbial community	OTU No.	Appearance frequency	Microorganism with highest 16S rDNA sequence similarity		
			Taxon	Accession No.	% Similarity
NR	NRm-OTU1	28/46	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	98-100
	NRm-OTU2	7/46	*Methanobacterium bryantii*	Y18736	99
	NRm-OTU3	3/46	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	100
	NRm-OTU4	2/46	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	92-99
	NRm-OTU5	2/46	Candidatus *Nitrososphaera gargensis* clone SY117	KM041251	96
	NRm-OTU6	1/46	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	89
	NRm-OTU7	1/46	*Methanobacterium bryantii*	Y18736	84
	NRm-OTU8	1/46	*Methanobacterium bryantii*	Y18736	82
	NRm-OTU9	1/46	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	97
RP	RPm-OTU1	26/48	*Methanobacterium formicicum* DSM 3636	Z29436	97-100
	RPm-OTU2	7/48	*Methanosarcina barkeri* strain MS	JQ346756	99
	RPm-OTU3	6/48	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	99-100
	RPm-OTU4	3/48	*Methanobacterium formicicum* DSM 3636	Z29436	98-100
	RPm-OTU5	2/48	*Methanobacterium bryantii*	Y18736	99
	RPm-OTU6	1/48	*Methanosarcina barkeri* str. Fusaro	CP000099	74
	RPm-OTU7	1/48	*Methanobacterium subterraneum* strain PY-13	JQ268007	82
	RPm-OTU8	1/48	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	98
	RPm-OTU9	1/48	*Methanomassiliicoccus luminyensis* strain B10	HQ896499	92

Appearance frequencies and most similar 16S rDNA

sequences in DDBJ/EMBL/NCBI nucleotide sequence database searched by BLAST of determined operational taxonomic units (OTUs) were summarized in Table 1 and 2.

Overviewing variety of eubacteria in NR, members belong to Bacteroidia including *Bacteroides*, *Proteiniphilum* and *Alistipes* were dominant (81%), and members belong to Clostridia including *Clostridium* and *Syntrophomonas* were subdominant (10%). In RP, members belong to Bacteroidia including *Dysgonomonas* and *Bacteroides* were also dominant (86%), and members belong to Clostridia including *Clostridium* and *Acetanaerobacterium* were subdominant (14%). In varieties of methanogen, dominant and subdominant members in NR were Methanomicrobia related of *Methanomassiliicoccus* (76%) and Methanobacteria related of *Methanobacterium* (20%), respectively. By contrast, dominant and subdominant members in RP were Methanobacteria related of *Methanobacterium* (67%) and Methanomicrobia including *Methanosarcina* and *Methanomassiliicoccus* (33%), respectively. Related species listed in eubacteria detection (Table 1) were reported to utilize polysaccharides and to produce volatile fatty acids [25-30], suggestive to play major roles in degradation of grass carbohydrates and in pH decrease observed at initial periods of cultures. In addition, all related species listed in methanogen detection (Table 2) were known to utilize hydrogen and carbon dioxide for methane production mainly [31-33].

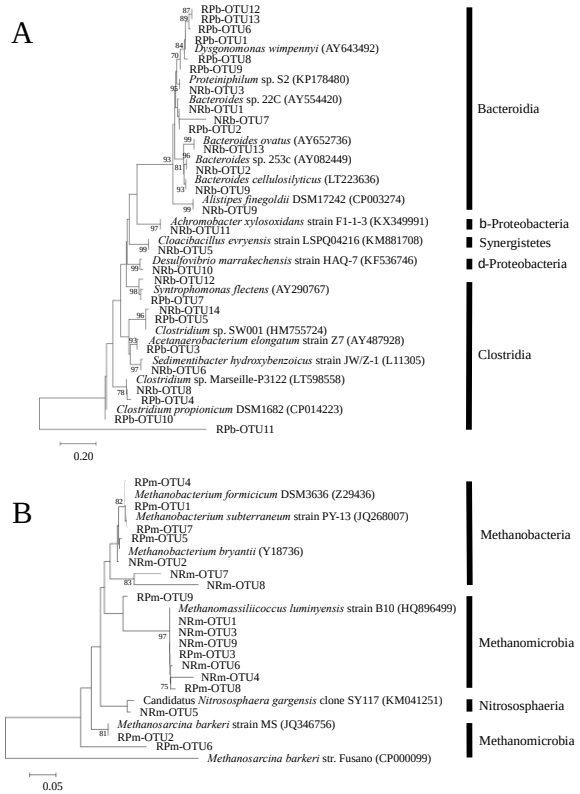

Fig. 3 Phylogenetic tree constructed with OTUs from NR and RP, and most similar sequences shown in Table 1 and 2, using Neighbor-Joining method [39]. The sums of branch length of the obtained optimal trees are 3.08879619 and 1.23343044 for 16S rDNA sequences of eubacteria (A) and methanogen (B), respectively. Bootstrap values (1000 replicate runs, shown as %) greater than 70% are indicated at branches. Bars indicate 20 and 5 substitutions per 100 nucleotide positions in (A) and (B), respectively. DDBJ/EMBL/NCBI accession numbers are shown in parentheses.

Therefore, it was surmised that Bacteroidia and Clostridia members would degrade grass carbohydrates to hydrogen and carbon dioxide via acid formation, and the Methanobacteria and Methanomicrobia members would convert hydrogen and carbon dioxide to methane, both in NR and RP.

Figure 3 shows phylogenetic relationship among OTUs obtained from NR and RP, and the related sequences available in nucleotide database. In phylogenetic dispersion both of eubacteria and methanogen, dominant OTUs from NR (named NRb and NRm) and RP (named RPb and RPm) were well mixed and clustered each other (Fig. 3A, clades of Bacteroidia and Clostridia; Fig. 3B, clades of Methanobacteria and Methanomicrobia). This result suggestively indicates that 'strict members adapted to methane production from grass at high C/N ratio' should be highly acclimated. In general co-digester with grass and manure, more diverse species have been detected besides the members of eubacteria and methanogen observed in NR and RP [34, 35].

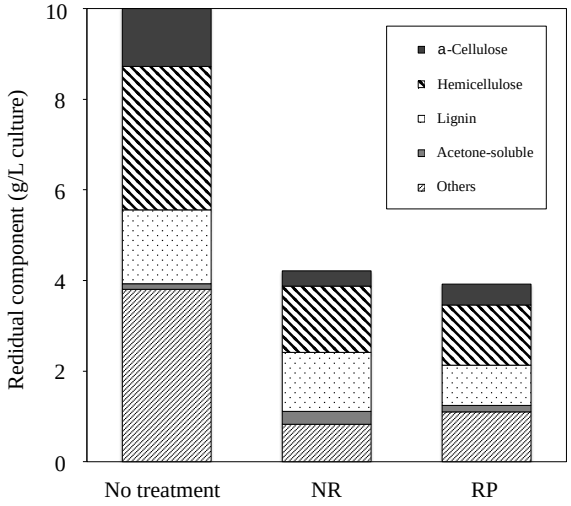

Fig. 4 Amounts of residual components in the substrate grass before (no treatment) and after (NR and RP) fourth subculture. Correspondences between column patterns and components are indicated in an inlet.

D. Decomposition of Grass Components

After fourth subculture of NR and RP, residual amounts of components in grass were determined, and the result is shown in Fig. 4. In a 1.0 g of Gyougi-shiba grass added to each culture, 0.13±0.04 g, 0.32±0.07 g and 0.16±0.01 g of α-cellulose, hemicellulose and lignin were contained, respectively. After 90 day-culture, 0.421 g in NR and 0.392 g in RP were remained as solid residues. Hence, approximate 60% of grass were degraded both in NR and RP. In NR, 75% of α-cellulose and 54% of hemicellulose were decreased in the substrate grass were decreased, while 65% of α-cellulose and 59% of hemicellulose were decreased in RP. Total production of methane in fourth subculture of NR and RP were 275.5 L-CH_4/kg grass and 322.7 L-CH_4/kg grass added, and final methane concentration in total biogas yielded in NR and RP reached 78% and 77%, respectively. Murphy and

Power estimated that methane potential from various grass has been 200-400 L-CH_4/kg volatile solid [36]. In this point, the methane production obtained in this study attained sufficient level. Conversely, approximate 30% of α-cellulose and 40% of hemicellulose in substrate grass were still remained to be utilized. Therefore, it is possible that enhancement of degradation ability for carbohydrates in grass would bring higher methane production. Further analysis for degradation manner in NR and RP is awaited.

E. Assay for Nitrogen Fixation

The substrate grass Gyougi-shiba used in this study was at extremely high C/N ratio around 200. In common sense on methane fermentation, adequate C/N ratio is regarded as 25-30. In respect to the variety of community members determined by clone sequencing (Table 1and 2), dominant members in NR and RP were not so varied from other methanogenic communities reported previously. In a valance of carbon and nitrogen, nitrogen was considered to be insufficient in NR and RP. A possibility descriptive to an insufficiency of nitrogen is that members in NR and RP require only trace amount of nitrogen sources. However, it was unlikely since 2.2±0.98 mg of nitrogen content in 1 g of added substrate grass (determined by elemental analysis) was considered to be too poor to nourish the community members. Another possibility is that nitrogen fixation from gaseous phase in culture occurred. To confirm the nitrogen fixation, we tested cultures purged with helium gas, instead of nitrogen gas. As a result, there was no production of methane in both cultures of NR and RP purged with helium. In addition, trial to detect nitrogenase activity by ethylene formation from acetylene succeeded to detect 6.4 mmol/L culture and 5.0 mmol/L culture of ethylene in 21-day cultures of NR and RP, respectively. In the 21-day cultures, 1.7 mg/L, 50.7 mg/L, 14.3 mg/L in NR and 5.0 mg/L, 65.7 mg/L, 17.7 mg/L in RP of ammonium, nitlite and nitrate ions were detected, respectively. The gross of determined contents of nitorogenous compounds obviously exceeded the available nitrogen amount in added substrate grass. Considering that possible nitrogen-fixing archaeon such as *Methanosarcina* and ammonia oxidizer such as *Nitrososphaera* were occasionally appeared in the community members of NR and RP (Table 2), nitrogen gas purged in culture should be fixed and utilized besides assimilation of organic nitrogen sources contained in substrate grass [37, 38].

IV. Conclusion

Two microbial communities producing methane at high yield were established by acclimation with subculturing method. Established communities NR and RP were able to degrade wild grass Gyougi-shiba at extremely high C/N ratio, and to produce biogas containing 78% and 77% of methane, respectively. Predominant members of NR and RP similarly consisted of Bacteroidia, Clostridia, Methanbacteria and Methanomicrobia relatives. Nitrogen fixation was confirmed both in communities NR and RP, indicating that insufficient nitrogen was supplied from gaseous phase in culture. It was considered that nitrogen fixation was significant key to adapt methanogenic communities to grass utilization at extremely high C/N ratio.

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