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Succession Culture-independent Methanogenic Archaeal from Manure Compost

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Ribotyping analysis combining with denaturing gradient gel electrophoresis (DGGE) have been successfully used and described the biodiversity of microbes especially archaea from cattle manure. DGGE profiles of the archaea methanogens based on culture-independent showed variation patterns from early mesophilic to maturation phases. During early mesophilic to thermophilic phases the DGGE bands at the upper part of the gel showed less variation. Meanwhile at the thermophilic phases, the upper bands were disappeared. While at the end of thermophilic phase the DGGE bands showed more variation compared to that in the early of mesophilic phase. However, in the maturation phase only four bands appeared on the middle and bottom of the gel. Detail analysis by comparing the sequence of 16S rRNA gene fragments to the GenBank showed that all the bands were closed to *Methanosaeta*, uncultured archaeon, and *Methanobrevibacter*, which belonged to the phylum of *Euryarchaeota*. Phylogenetic analysis of the 16S rRNA gene sequences showed that all the bands belonged to *Methanobacteriales* and *Methanosarcinales*. 18 out of 25 DGGE bands showed that the bands are forming a new cluster at *Euryarchaeota* based on phylogenetic analysis, where 14 bands were closed to *Methanobacteriales* and 4 bands are part of *Methanosarcinales*. Most of *Methanosarcinales* order were found at thermophilic phase and *Methanobacteriales* were found in all phases. In addition the organisms were dominant order in both mesophilic and maturation phases.

Key words: Manure, Compost, Methanogenic Archaeal, Culture-Independent, DGGE.

Archaea can thrive in various natural habitat and many of them grow in habitats at the extreme limits, such as temperature, pH, salinity, anaerobiosis, etc[1]. The archaeal domain composed of *Crenarchaeota* and *Euryarchaeota*. The *Euryarchaeota* contains methanogens, extreme halophilic, sulfate reducing, and extreme thermophilic sulfur metabolizing organisms. Five orders of archaea methanogens were identified as *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales* and *Methanosarcinales*. Methanogenic archaea are organisms that produce methane as a metabolic by product in anoxic conditions. Methanogenic activity during the composting process was demonstrated by the presence of methane in the air evolving from various compost materials[2, 3,4,5].

Composting of animal manure is one of the most effective techniques for an environmentally less burden technology due to of its recycling capability of organic waste. Composting is an aerobic process, where cattle manure is biologically degraded into stable material. During the composting process, complex microbial communities consisting of bacteria, fungi,

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and archaea conducted degradation of organic material from manure[6,7]. The understanding of microbial succession that play a role in the process is needed to effectively control of the composting process [9,10] . Although composting is considered to be an aerobic process, several studies report on the presence of methanogenic archaea during composting, indicating that anaerobic microenvironments were developed in compost[11]. During composting of cattle manure, archaea has been found to be important on ammonia oxidation called ammonia-oxidizing archaea (AOA) and shown as essential in nitrogen cycle[12,8]. Therefore, archaea is considered as an important component of the microbial community during composting process.

Culture-independent methods offer an alternative approach to study microbial diversity[13] and are usually more sensitive to detect the species compared to that culture-dependent approach[14]. Fingerprinting techniques, such as polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) have been successfully used and described biodiversity of microbes in compost environment [15,16,17] included bacteria, eukaryotes, and archaea[18,19]. Although archaea has been recognized as minor components on microbial community in compost, however the role is essential. Some reports showed considerable methane production from cattle manure compost[20]. The presence of methanogen from composting of organic waste was indicated by the presence of *Methanosarcina thermophila, Methanoculleus thermophilus,* and *Methanobacterium formicicum*[21]. In rice straw compost were found mainly *Methanomicrobiales*, uncultured *euryarchaeota* and *Methanosarcinales* [11], *Methanomicrobiales* and *Methanosarcinales*[22]. Archaeal community was found as *Methanomicrococcus* and *Methanosarcina* during cattle manure composting process in field-scale facility[8].

There are few information concerning the succession of methanogenic archaea in the composting process, especially from cattle manure compost. Thus, in this report we present the succession and phylogenetic profile of methanogenic archaeal communities during composting process based on PCR- DGGE analysis.

MATERIALS AND METHODS

Composting set-up

Composting process was conducted on Cigadung area at arround 3 km from laboratorium. Cattle manures and rice straws were mixed homogeneously at ratio of 3:1 (cattle manure:rice straw).

Isolation and Physicochemical analysis

Extract composts were prepared by mixing, 20 grams of the fresh sample in 180 mL of distilled water and then filtered. Supernatant of the extract were re-filtered through a 0.22-µm-pore-size cellulose membrane filter (Sartorius, Germany). The cells on membrane were resuspended in STE buffer (10 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 1 mM EDTA) and precipitated by centrifugation. Pellet containing microbial communities were stored at -20°C until used for DNA extraction. The pH of water extract supernatant was measured. The moisture content was obtained by drying the sample at 70 ^{0}C until reached constant weight.

DNA Extraction

Total community DNA from each phase of compost samples were extracted using Zhou method[23] with some modifications[24]. The pellet containing microbial cells were suspended in DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl), sea sand and proteinase K (10 mg/ml) in microcentrifuge tubes. The mixture was vortexed (Genie, G 560E, USA) at medium (half of maximum speed) for 15 min at room temperature. After vortexing, 20% SDS was added to the mixture, and the samples were incubated at 70°C for 2 h with gentle end-over-end inversions every 15 to 20 min. Supernatants was mixed with an equal volume of chloroform isoamylalcohol (24:1, v/v). The aqueous phase was recovered by centrifugation. The upper phase of the solution was transferred to new Eppendorf tube and the DNA was precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude DNA were obtained by centrifugation at 16.000 g for 20 min at room temperature, washed twice with cold 70% ethanol, and dissolved in sterile deionized water. The DNA was stored at -20°C until further used.

Amplification of 16S rRNA gene fragments

The 16S rRNA genes fragments were

amplified by PCR method using set of methanogenic archaeal primers as described by Watanabe et al [19]. One primer complements to region conserved among members of the Bacteria domain (*Escherichia coli* positions 333 to 348) incorporated with a 40-bp GC-clamp in order to increase separation of DNA bands in DGGE analysis: 5' CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGTCCAGGCCCTACGGG-3'. The other primer was constructed based on a universally conserved region (*E. coli* positions 707-691; 3' GGA TTA CAR GAT TTC AC-5'). These primers amplify a fragment at approximately 400 bp long. PCRs were performed by using Taq polymerase according to the instructions provided by manufacturer (Fermentas). A touchdown PCR program was implemented as follows: an initial denaturation step at 94°C for 5 min, followed by 30 cycles, where denaturation was performed at 94 °C for 1min, the annealing temperature was performed 1 min, and elongation was performed at 72 °C for 2 min. In the first 10 cycles, the annealing temperature was continually decreased by 1°C from 57 to 47°C every cycle. The final extension was for 10 min at 72°.

PCR product was determined by electrophoresis analysis of aliquots of PCR mixtures (0.5 µg ml^{-1}) ethidium bromide in 1.5% agarose and 1x TAE buffer (40 mM Tris HCl, 40 mM acetate, 1.0 mM EDTA) under UV light

DGGE and re-PCR of DGGE bands

DGGE was performed by loading 40 µl of the PCR products loaded into 8% (w/v) polyacrylamide-bisacrylamide (37.5:1) denaturing gels with gradients from 40 to 70% denaturant (100% of denaturant corresponded to 7 M urea and 40% [v/v] deionized formamide) [25]. Electrophoresis was performed with 1 x TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA) at 120 V and 60°C for 7 h by using D-code systems (Bio-Rad Laboratories). After electrophoresis, the gels were stained by silver staining methods[26]. The selected DGGE bands which are specific at each phase of composting were carefully excised and extracted with TE buffer (10mM Tris-Cl, 1 mM EDTA), in boiling water for 5 min then incubated overnight at 37°C. The extracted DNA was reamplified by using the same primers without addition of GC clamp. PCRs were carried out with the following conditions: initial denaturizing step at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, and final extension step of 10 min at 72°C. All PCR products were subjected to DNA sequencing, carried out by ABI PrismR 3100 Genetic Analyzer (Applied Biosystems) by the Macrogen Sequencing Service (Korea).

Phylogenetic analysis

The sequencing results were aligned to 16S rRNA gene sequences from GenBank database at NCBI (National Centre of Biotechnological Information) through web site http:// www.ncbi.nlm.nih.gov using BLAST program for screening of sequence similarity. Sequences alignments were performed by ClustalW program in MEGA 5.05. Phylogenetic reconstruction was accomplished with the phylogeny MEGA 5.05 inference package[27]. Phylogenetic trees were constructed from distance matrices by the neighbor-joining method[28], with substitution method Maximum Composite Likelihood[29]. The node reproducibility for tree topology was estimated by bootstrap analysis, which included 1000 replicate data sets.

RESULTS

16S-rRNA gene fragment

Total DNA from each phase of composting was successfully amplified. The DNA was used as template to amplify 16S rRNA gene fragment using primers as described in the methods. The amplification produced single band in agarose gel electrophoresis with size of about 400 bp as expected (Fig. 1).

DGGE profile of 16S rRNA Genes

The DGGE profiles of methanogenic archaeal communities in the composting process of cattle manure was shown in Fig 2. Some variation patterns were shown during the process. The number of DGGE band ranged from 13 to 15 were detected during early mesophilic phase, while from early thermophilic to thermophilic phases were detected at around 11-13 and 9-11 bands respectively (Fig. 3). During early mesophilic to thermophilic phases the numbers of the bands were decreased especially at the upper part of the gel. Meanwhile on the thermophilic phase, the upper bands were disappeared. At the end of thermophilic phase, the number of the bands appeared more compared to that on the early mesophilic phase

(20-22 bands) (Fig. 3). Most of intense bands were appeared at the upper and middle of gel, however in the maturation phase the number of the bands drastically decreased, only four bands in the middle and bottom of the gel. This indicated that the methanogenic archaeal community in the maturation phase was un-preferable.

Phylogenetic and Diversity of methanogenic archaea

The sequences of DGGE bands were compared to 16S rRNA gene sequences from GenBank database at NCBI (National Centre of Biotechnological Information) through web site http://www.ncbi.nlm.nih.gov using BLAST program for screening of sequence similarity to determine the closest relatives and phylogenetic affiliation. To assess the community based on 16S rRNA sequences of methanogenic archaeal genes, 25 bands were successfully sequenced and analyzed. The relative relationships among each other of DGGE bands were described on Table 1. All the bands obtained in this study belonged to the phylum *Euryarchaeota*. The majority of the sequences were closed to uncultured *Methanosaeta* (12 bands: 95-100%), uncultured archaeon (9 bands: 87-99%), and uncultured *Methanobrevibacter* (4 bands: 86-88%). Nucleotide sequences obtained in this study are available in the GenBank database NCBI under the accession numbers of KC410787 to KC410808.

Phylogenetic analysis of the methanogenic archaeal communities showed that all bands are consisted two orders of archaea methanogen, *Methanobacteriales* and *Methanosarcinales*. 18 out of 25 sequences formed a new cluster of *Euryarchaeota* (Fig. 4). 14 sequences were close to *Methanobacteriales* and the rest (4 sequences) were close to *Methanosarcinales.*

Table 1. Close relation of DGGE sequence of cattle manure compost with the GenBank data

Stage of composting	DGGE bands	Closest relatives			Similarity %
		Microorganisms	Phylogenetic affiliations	Accession number	
Early of mesophilic (28 ^{0}C)	AM1.1	Uncultured archaeon	*Euryarchaeota*	AB447777.1	90
	AM1.2	Uncultured archaeon	*Euryarchaeota*	HQ224860.1	97
	AM1.3	Uncultured archaeon	*Euryarchaeota*	HQ224859.1	91
	AM1.4	Uncultured archaeon	*Euryarchaeota*	AY426477.1	87
	AM1.5	*Methanogenic*	*Euryarchaeota*	DQ262559.1	94
	AM1.6	Uncultured archaeon	*Euryarchaeota*	FJ476903.1	94
	AM1.7	Uncultured archaeon	*Euryarchaeota*	FN993997.1	98
	AM1.8	*Methanosaeta*	*Methanosarcinales*	JQ282392.1	98
Early of thermophilic (50^{0}C)	AM2.1	*Methanosaeta sp*	*Methanosarcinales*	JN052771.1	99
	AM2.2	*Methanosarcinales*	*Methanosarcinales*	CU916209.1	98
	AM2.3	*Methanosaeta sp*	*Methanosarcinales*	JX576155.1	100
	AM2.4	*Methanobrevibacter sp*	*Methanobacteriales*	FJ938099.1	99
	AM2.5	*Methanosaeta sp*	*Methanosarcinales*	EU72225.1	99
thermophilic (60^{0}C)	AM3.1	*Methanosaeta* sp.	*Methanosarcinales*	JQ282391.1	100
	AM3.2	*Methanobrevibacter millerae*	*Methanobacteriales*	NR_042785.1	91
	AM3.3	*Methanosaeta*	*Methanosarcinales*	JN052768.1	94
	AM3.4	*Methanobrevibacter*	*Methanobacteriales*	JF807183.1	86
	AM3.5	*Methanobrevibacter*	*Methanobacteriales*	JF807183.1	88
end of thermophilic (50^{0}C)	AM4.1	*Methanosaeta sp*	*Methanosarcinales*	JQ087785.1	99
	AM4.2	*Methanosaeta sp. enrichment* culture	*Methanosarcinales*	HQ133139.1	96
	AM4.3	Uncultured *euryarchaeote*	*Euryarchaeota*	AF293505.1	90
	AM4.4	*Methanosaeta sp*	*Methanosarcinales*	EU722274.1	99
	AM4.5	*Methanosaeta sp*	*Methanosarcinales*	JN651998.1	99
maturation (35 ^{0}C)	AM5.1	*Methanosaeta sp*	*Methanosarcinales*	JF947136.1	92
	AM5.2	Uncultured archaeon	*Euryarchaeota*	DQ262560.1	89

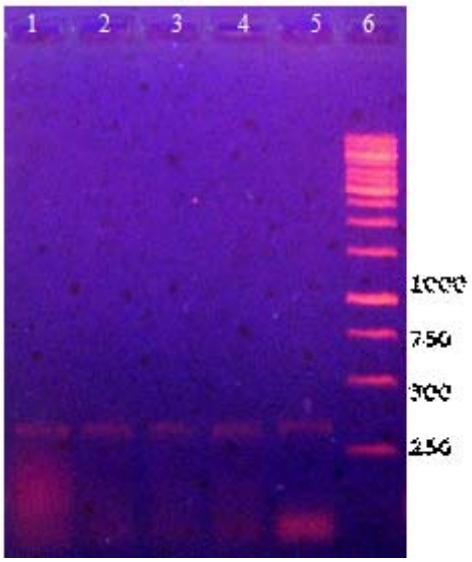

Fig. 1. Agarose gel electrophoresis of 16S rRNA gene fragments from methanogenic archaea during composting process. Band from: 1. early of mesophilic (28 ^{0}C); 2. early of thermophilic (50 ^{0}C); 3. thermophilic (60 ^{0}C); 4. end of thermophilic (50 ^{0}C); and 5. maturation phases (35 ^{0}C); 6. DNA marker

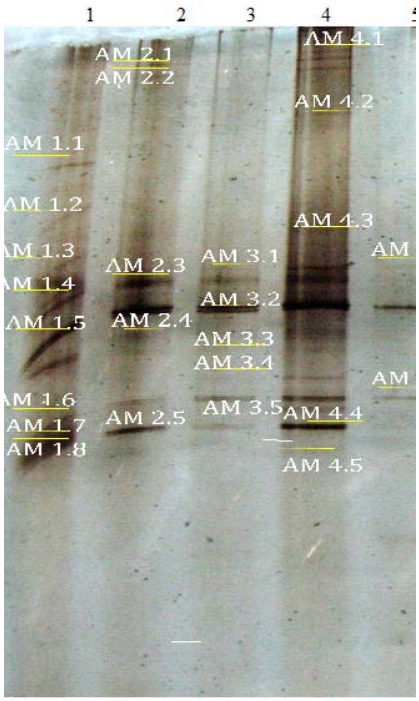

Fig. 2. DGGE profile of manure compost samples. Band from: early of mesophilic (1, temperature 28 ^{0}C); early of thermophilic (2, 50 ^{0}C); thermophilic (3, 60 ^{0}C); end of thermophilic (4, 50 ^{0}C) and maturation phases (5, 35 ^{0}C)

Based on sequence differences in the 16S rRNA gene fragment, nucleotide sequence differences between the samples indicates that the sequence of 16S rRNA gene fragments are not derived from the same organism. Some of archaeal methanogens obtained are also formed new clusters, particularly on the alleged orders *Methanobacteriales*. This data is typical from cattle manure compost.

DISCUSSION

The activity of microbe during composting process depends on environmental factors. The changes in physicochemical during the composting process is related with differences communities of microorganism, especially for methanogenic archaea. Community profile based on PCR-DGGE has been widely used to examine succession in microbial communities[5,16,17,30].

The archaea community is recently considered as an essential component of microbial community during composting process since the community produce methane as product of energy metabolism[31]. Some archaea which oxidizing ammonia also has a key role in the process since the end product of nitrification such as nitrate and give beneficial for plant growth when compost is applied to agricultural sector[7]. Ammonia-oxidizing archaea (AOA) are more dominant than the ammonia-oxidizing bacteria (AOB) in cattle manure of composting process[8].

In this study, the samples were collected from early mesophilic phase (temperature 28 ^{0}C), until maturation stage (35 ^{0}C). The pH was detected in range of 7.3-8.9, whereas the moisture content decreased from 88.32% to 43.46%. In the early stage, C/N ratio increased from 17.99 to 33.08 and then decreased gradually up to 22.3 maturation phase[32,33]. C-organic increased from 17.99 to 31.43 at early thermophilic phase and then decreased to 19.64 after maturation phase, whereas N total decreased from 1.05 to 0.89 at in maturation stage. All of these data suggested that the compost has matured and the composting method was sucessfull[10].

The data from the result showed that all archaea were close to uncultured methanogens of *Euryarchaeota* phylum. Although continuous aeration to maintain aerobic conditions during the

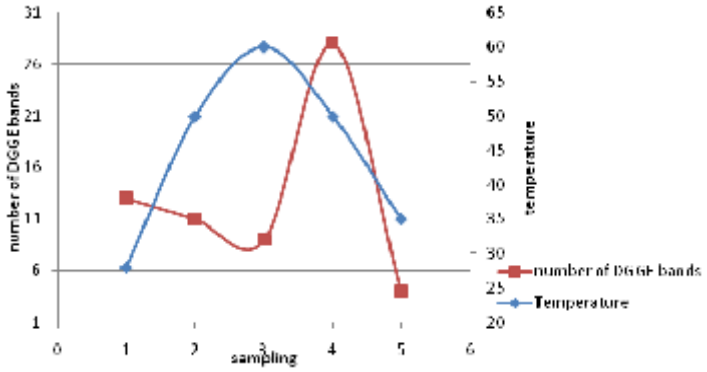

Fig. 3. Number the DGGE bands of the methanogenic archaeal communities.

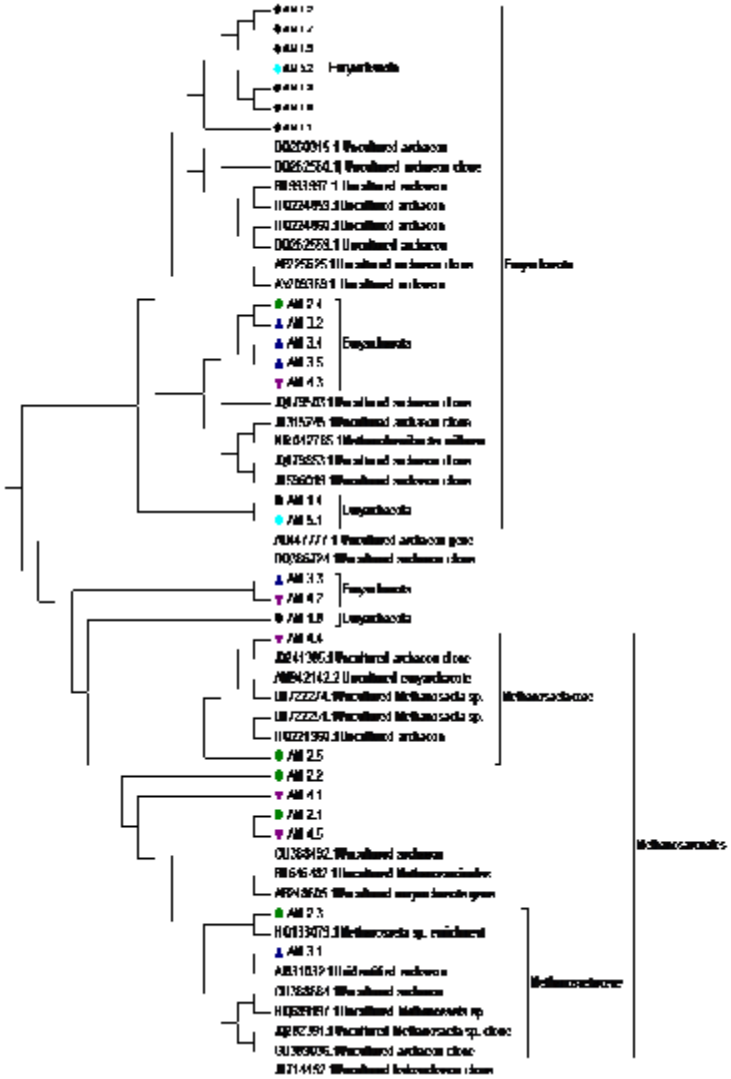

Fig. 4. Phylogenetic tree of the 16S rRNA gene sequences during composting process. The tree was constructed by using neighbor-joining method of the MEGA 5.05 software with 1,000 bootstrap replicates

process, the methanogenic communities were found in anaerobic conditions in the composting material. Anaerobic sites of organic compounds such as small clumps of manure are still exist[8]. Several new clusters of archaeal methanogens were found based on phylogenetic analysis (Fig. 4), particularly the *Methanobacteriales* order. These microorganisms are unique microorganisms that play typical role during the composting process and has not been reported previously. *Methanobacteriales* were found in all phases, but it was a dominant at mesophilic and maturation phases (28-35 ^{0}C), meanwhile *Methanosarcinales* were found at thermophilic phase (50-60 °C). These data showed that cattle manure with rice straw appeared differences of methanogenic archaea compared with other studies[8,11,22]. However, the methanogenic archaea could be derived from cattle manure in the early stages of the process. These results indicate that manure could sustain methanogenic community and serves as source of methane to the end of the thermophilic phase. While at the maturation phase methanogenic archaeal diversity found limited (Fig. 3). These suggested that at the maturation phase archaeal methanogens play less significant compared to the end of the thermophilic phase.

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