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Succession of Eukaryotic Community Microorganism from Cattle Manure during Composting Process

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The eukaryotic microorganism diversity from cattle manure during composting process was analyzed by ribotyping analysis combining with denaturing gradient gel electrophoresis (DGGE). DGGE profiles of the eukaryotic communities from culture-independent showed variation patterns among bands from early mesophilic to maturation phases. In the early mesophilic to thermophilic phases some bands showed strong intensity at the upper part of the gel, whereas at the end of the thermophilic to the maturation phases, the bands were gradually disappeared. Meanwhile on the bottom of the gel, the bands showed gradually decreased of the intensity up to maturation phase. Detail analysis by comparing the sequence of 18S rRNA gene fragments to the GenBank showed that at the early mesophilic phase, the compost inhabited mostly by Eimeriidae (Apicomplexa), Aspergillus, Penicillium and Paecilomyces (Ascomycota). Meanwhile the thermophilic phase were dominated by Eimeriidae, Gregarina (Apicomplexa), Aspergillus and Penicillium (Ascomycota). Whereas at the maturation phase the dominant organism were came from phylum Ascomycota.

Key words: Manure Compost, 18S rRNA, DGGE, Eukaryotic Diversity.

Composting is an aerobic process, where organic waste is degraded by microorganisms into stable material[1]. It consist three major phases: mesophilic, thermophilic and maturation phases. Quality and efficiency of composting process depends on composition of organic material, pH, moisture content and temperature[2]. In addition, the most important factor in composting process is the role of microorganisms and their activities[3]. The understanding of the microbial (such as eukarya) succession in the process is needed to effectively control of the process, especially during degradation of organic materials[4;5;6].

Culture-independent methods offer an alternative approach to study microbial diversity[7]. The method is usually more sensitive to detect the microbial species compared to that the culture-dependent[8]. Fingerprinting techniques, such as polymerase chain reaction (PCR) combining with denaturing gradient gel electrophoresis (DGGE) have been successfully used to probe biodiversity of microbes in compost environment[9;10;11,] including eukaryotes, e.g., fungi and protozoa[12]. Although the importance of eukaryotic organisms during composting process is considered to be minor in

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comparison to that of the bacteria, since self-heating occurred during composting is extreme (65 ^{0}C) for survival of eukaryotic organisms, but fungi have been recovered from all phases of composting process[13;14;15].

Fingerprinting approaches have been successfully used to profile and to characterize the eukaryotes community. The study of eukaryotic community structure in the composting process of rice straw using 18S rDNA PCR-DGGE analysis has been reported[16]. The result showed that this process affected composition of fungi, protozoa, algae, nematodes and *stramenopiles*. The eukaryotic communities of rice straw compost in paddy field soil has been reported to contain mainly of fungi (*Ascomycota* and *Chyridiomycota*) and protozoa (*Ciliophora* and *Euglyphida*)[12]. The succession of eukaryotic communities in composting process of domestic waste were dominantly inhabited by *Ascomycota, Stramenopiles*, *Basiodiomycota, Zygomycota Gymnamoeba, Chorophyta and Arthopods*[17].

However there is still limited information concerning the succession of eukaryotic microorganisms in the composting process, especially from cattle manure. Cattle manure contains microorganisms originating from faeces, feed residue and rice straw. In this report we present the succession profiles of eukaryotic organism during composting of cattle manure based on ribotyping (18S rRNA) and PCR-DGGE analysis.

MATERIALS AND METHODS

Composting set-up and Sampling

Composting process was conducted in Cigadung which is located at approximately 3 km from Lab in ITB. Cattle manures and rice straws were mixed homogeneously at a ratio of 3:1 (cattle manure: rice straw). A 1.2×1.0×1.0 m (length × width × height) composting bin was designed and built, which was covered to shield from sunshine and rain. Aeration was provided through open-ended air intake bamboo pipes inserted into the pile. Samples were collected every phases started from mesophilic until maturation phases. Samples were collected from each point at day 0, 11, 27, 38, and 88. Samples were immediately used for further analysis.

Isolation and Physicochemical analysis

Extract compost were prepared by shaking, 20 grams of the fresh sample in 180 mL of distilled water and then filtered. Supernatant of extract compost were re-filtered through a 0.22-µm-pore-size cellulose membrane filter (Sartorius, Germany). The cells on membrane were re-suspended 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 was measured in water extract supernatant. 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[18] with slight modifications[19]. 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 by vortexing (Genie, G 560E, USA) at medium vigorous (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 were 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 18S rRNA gene fragments

The 18S rRNA genes fragments were amplified by PCR method using a set of eukaryotic primers as described by Diez et al[20]. One primer lies on conserved region among members of the eukarya (*Saccharomyces cerevisiae* of positions 1423 to 1438) incorporated with a 40-bp GC-clamp in order to increase separation of DNA bands in DGGE analysis[21] (Table 1). The other primer is

universal primer (*Saccharomyces cerevisiae* positions 1641 to 1627). These primers amplify a fragment at approximately 200 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 30s, the annealing temperature was performed 1 min, and elongation was performed at 72 °C for 1 min. In the first 10 cycles, the annealing temperature was continually decreased by 1°C from 55 to 45°C every cycle. The final extension was for 10 min at 72°.

DGGE and re-PCR of DGGE bands

DGGE was performed by adding 40 µl of the PCR products loaded into a 8% (w/v) polyacrylamide-bisacrylamide (37.5:1) denaturing gels with gradients from 40 to 70% (100% of denaturant corresponded to 7 M urea and 40% [v/v] deionized formamide)[22]. 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[23]. 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 and then incubated overnight at 37°C. The extracted DNA was reamplified by using the same primers without addition of the GC clamp. PCRs were carried out with the following conditions: an 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 a final extension step of 10 min at 72°C. All of 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 compared to 18S 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[24]. Phylogenetic trees were constructed from distance matrices by the neighbor-joining method[25], with substitution method Maximum Composite Likelihood[26]. The node reproducibility for tree topology was estimated by bootstrap analysis, which included 1000 replicate data sets.

RESULTS

Physico-chemical characteristics

Composting process was conducted in Kelurahan Cigadung at around 3 km from the Lab. Cattle manure and rice straw were mixed homogeneously at a ratio of 3:1 (cattle manure:rice straw). Composting was carried out traditionally, self acting aeration systems and without using strater. A compost pile made with the size of length x width x height = 1.2×1.0×1.0 m. Composting pile was designed and built, which was covered to shield from sunshine and rain.

Physico-chemical properties during the composting such as changes in temperature, pH and moisture content were measured (Table 2). Compost samples were collected on day 0 (early mesophilic phase) with temperature at 28^0C, day-11 (early thermophilic phase) at 50^0C, day-27 (thermophilic phase) at 60^0C, day-47 (end thermophilic phase) at 50^0C and day-91 (maturation phase) at 35^0C (Fig. 1). In this composting process the highest temperature reached at 60^0C (day-27), then decreased gradually up to maturation phase on the day 91. During the process, the pH increased from 7.8 to 8.9 at thermophilic phase and then decreased to 7.3 after maturation phase, whereas the moisture content decreased gradually from 88.32% at mesophilic phase to 43.46% at the maturation phase. This result indicates that the compost has matured and the composting method was successful[27;6]. The high moisture content was one of the parameter for immature compost index. The concentration of organic materials in cattle manure with high immature compost supports the growth of microbial pathogens[28].

DGGE profile of 18S rRNA Genes

The total DNA from filtration samples were extracted and appeared as a single band on ethidium bromide stained agarose gel, indicating that the total DNA was acquired intact (data not shown). For analysis of eukaryotes community,

Table 1. The primers used in this study

Primer	Sequence	Saccharomyces cerevisiae positions at	Tm (^{0}C)
Euk1209f	TCC AGG CCC TAC GGG	1423 -1438	50
Uni1392r	GACGGGCGGTGTGTAC	1641 - 1627	51
GC-clamp	CGCCCGCCGCGCGCGG CGGGCGGGGCGGGGGC ACGGGGGG		

Table 2. Physicochemical properties of samples during composting process

Phase	Temp. (^{0}C)	pH	Moisture content (%)
1	28	7.8	88.32
2	50	7.9	75.5
3	60	8.9	65.05
4	50	7.6	55. 37
5	35	7.3	43.46

18S rRNA genes were amplified from the total DNA in each sample using Euk1209f-GC and Uni1392r primers. Total DNA from all phases of composting process were successfully amplified. All of amplicon produced single band in agarose gel electrophoresis with the size of approximately 200 bp (Fig. 2).

DGGE profiles of the eukaryotic communities showed some variation patterns among the bands from each phases of the process. During early mesophilic to early thermophilic phases more bands appeared in the gel compared to that at end thermophilic to maturation phases (Fig. 3). In the early mesophilic to thermophilic phases most of the bands appeared at the upper part of the gel, whereas at the end of the thermophilic to the maturation phases, the upper bands was disappeared. Meanwhile on the bottom of the gel, the bands showed decreased in intensity up to maturation phase. The numbers of appeared bands are relatively similar in each at composting phases. This indicates that the eukaryotic communities, especially at the thermophilic phase were persistent during the composting process. The thermophilic bacteria found in compost, thermophilic eukaryotes also play important role in the composting process[29].

Phylogenetic and Diversity of eukaryotic Community

To assess eukaryotic communities based on 18S rRNA gene sequences, from approximately 80 bands that appeared in all phases of cattle manure composting, 30 bands were successfully sequenced and analyzed.

The relative relationships of the sequence of the DGGE bands were described on Table 3. The majority of the sequences were closed to *Eimeriidae, Gregarina* (*Apicomplexa*), *Aspergillus, Penicillium,* and *Paecilomyces* (*Ascomycota*). *Gregarina* were found only in thermophilic phase. Meanwhile, some thin bands were characterized as Slime net (*Diplophrys*) and *Basidiomycota* (uncultured *Auriculariaceae*). At early mesophilic to end thermophilic phases, most of the commonly bands belonged to *Apicomplexa* and *Ascomycota*, whereas in the maturation phase only the phylum of *Ascomycota* was appeared. One sequence was not recognized yet at the GenBank database NCBI (EK 2.5).

Phylogenetic trees were constructed based on the distance matrices method. The sequences closed to *Eimeriidae* showed to cluster in distinct branch as well as *Ascomycota* (Fig. 4). Phylogenetic analysis of DGGE bands showed that there were 12 bands forming a new cluster, which is likely part of the class of *Apicomplexa*, meanwhile 10 bands are likely part of *Ascomycota* forming other new cluster. In addition there are two bands close to the *Gregarina* sp (99% -100%), and two bands close to *Penicillium* (99% -100%).

Table 3. Close relationship of DGGE sequence of cattle manure compost to the GenBank data

Composting Phases	DGGE Bands	Close relation			Similarity %
		Microorganisms	Phylogenetic Affiliations	Accession Number	
Early mesophilic (28 ^{0}C)	EK 1.4	*Eimeriidae* environmental sample	*Apicomplexa*	EF023442.1	93
	EK 1.5	*Aspergillus fumigates*	*Ascomycota*	JF708947.1	94
	EK 1.7	*Paecilomyces variotii*	*Ascomycota*	JF416647.1	93
	EK 1.8	*Penicillium glabrum*	*Ascomycota*	AF245270.1	99
Early thermophilic (50^{0}C)	EK 2.1	*Eimeriidae* environmental sample	*Apicomplexa*	EF023658.1	90
	EK 2.2	*Eimeriidae* environmental sample	*Apicomplexa*	EF023442.1	94
	EK 2.3	Uncultured eukaryote	*Eukaryote*	DQ104602.1	94
	EK 2.4	*Eimeriidae* environmental sample	*Apicomplexa*	EF023658.1	92
	EK 2.5	-	-	- -	
	EK 2.6	*Diplophrys sp.*	*Slime net*	AF304465.1	87
	EK. 2.7	*Aspergillus niger*	*Ascomycota*	JF925334.1	99
	EK 2.8	*Penicillium glabrum*	*Ascomycota*	AF245270.1	100
	EK 2.9	Fungal sp.	*Fungi*	JF719078.1	98
Thermophilic (60^{0}C)	EK 3.3	*Eimeriidae* environmental sample	*Apicomplexa*	EF023442.1	90
	EK 3.4	*Eimeriidae* environmental sample	*Apicomplexa*	EF023733.1	91
	EK 3.5	*Eimeriidae* environmental sample	*Apicomplexa*	EF023338.1	86
	EK 3.6	*Gregarina polymorpha*	*Apicomplexa*	FJ459748.1	99
	EK 3.7	Uncultured eukaryote	*Eukaryote*	DQ104585.1	94
	EK 3.9	Uncultured marine *dinoflagellate*	*dinoflagellata*	JF826395.1	94
	EK 3.10	Uncultured eukaryote	*Eukaryote*	GU825698.1	86
	EK 3.12	*Aspergillus nomius*	*Ascomycota*	JF416646.1	94
End of thermophilic (50^{0}C)	EK 4.1	Uncultured eukaryote	*Eukaryote*	DQ104601.1	95
	EK 4.2	*Eimeriidae* environmental sample	*Apicomplexa*	EF023733.1	93
	EK 4.3	*Gregarina polymorpha*	*Apicomplexa*	FJ459748.1	97
	EK 4.4	*Aspergillus oryzae*	*Ascomycota*	JF265070.1	93
	EK 4.7	Fungal sp.	*Fungi*	JF719120.1	97
Maturation (35 ^{0}C)	Ek 5.1	Uncultured *Ascomycota*	*Ascomycota*	HQ438170.1	93
	Ek 5.2	Uncultured *Ascomycota*	*Ascomycota*	HQ438145.1	94
	Ek 5.3	Uncultured *Auriculariaceae*	*Basidiomycota*	EF024332.1	89

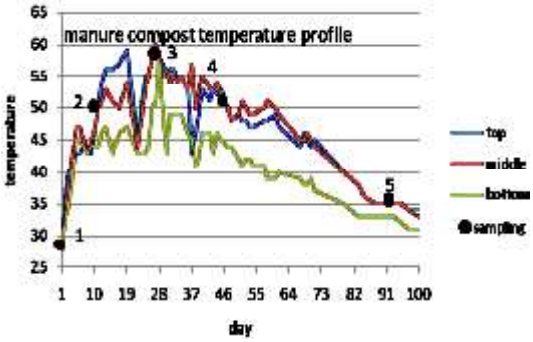

Fig.1. Temperature profile of manure composting process; temperature samples at (1). 28 0C, (2). 50 0C, (3). 60 0C, (4). 50 0C and (5). 35 0C.

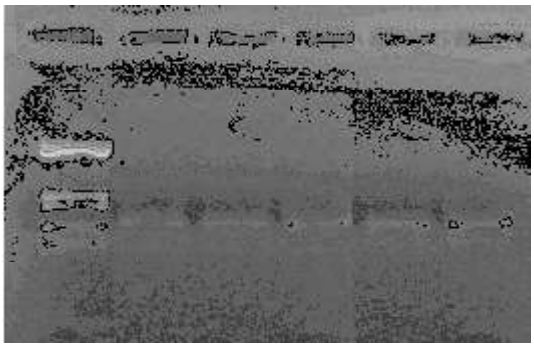

Fig.2. Agarose gel electrophoresis of 18S rRNA gene fragments from microorganism during composting process. 1. pUC19/*Hinf*I DNA marker; 2. early mesophilic (28 ^{0}C); 3. early thermophilic (50 ^{0}C); 43. thermophilic (60 ^{0}C); 5. end of thermophilic (50 ^{0}C); and 6. maturation phases (35 ^{0}C).

DISCUSSION

The activity of microorganism during composting process is highly dependent on environmental factors. The change of the physicochemical parameters during composting process is correlated with the succession of communities of microorganisms. Combining PCR with DGGE methods have been successfully probed the biodiversity of microbes in environment, such as compost[30;11;31]. However, information concerning succession of eukaryotic communities are still limited compared to that the bacteria. Phylogenetic studies of eukaryotes based on the difference of 18S rRNA gene sequence are inhibited by limitations of the availability sequence in GenBank.

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

Based on the DGGE profile and phylogenetic analysis of the selected bands, a shift of eukaryotic community was observed from the early mesophilic to thermophilic, and maturation phases. Following the increasing temperature at the thermophilic phase, dominant bands appeared at mesophilic phase were decreased in its intensity. Following thermophilic phase the diversity of eukaryotes spesies decreased significantly.

In this study, *Paecilomyces variotii* (DGGE band EK 1.7) and *Aspergillus fumigatus* (band EK 1.5) appeared in early of mesophilic but disappeared during thermophilic phase. *Paecilomyces* is a cosmopolitan filamentous fungus which inhabits in the soil, decaying plants, and food products. Some species of *Paecilomyces* are isolated from insects. *Paecilomyces* usually causes infections in humans, animals and emerging causative agents of opportunistic mycoses in immunocompromised hosts[32]. *Paecilomyces* is also able to degrade cellulose, xylan, starch, lignin, and chitin. *Aspergillus* was found in almost all of the composting phases. *Aspergillus* is known as organism to degrade cellulose, hemicellulose (xylan, pectin, arabinoxylan, and starch), lignin, and chitin[14;33]. Intensive cellulose decomposition occurs throughout the composting process are usually performed by fungi. The structure of fungal mycelium provides a competitive advantage in the

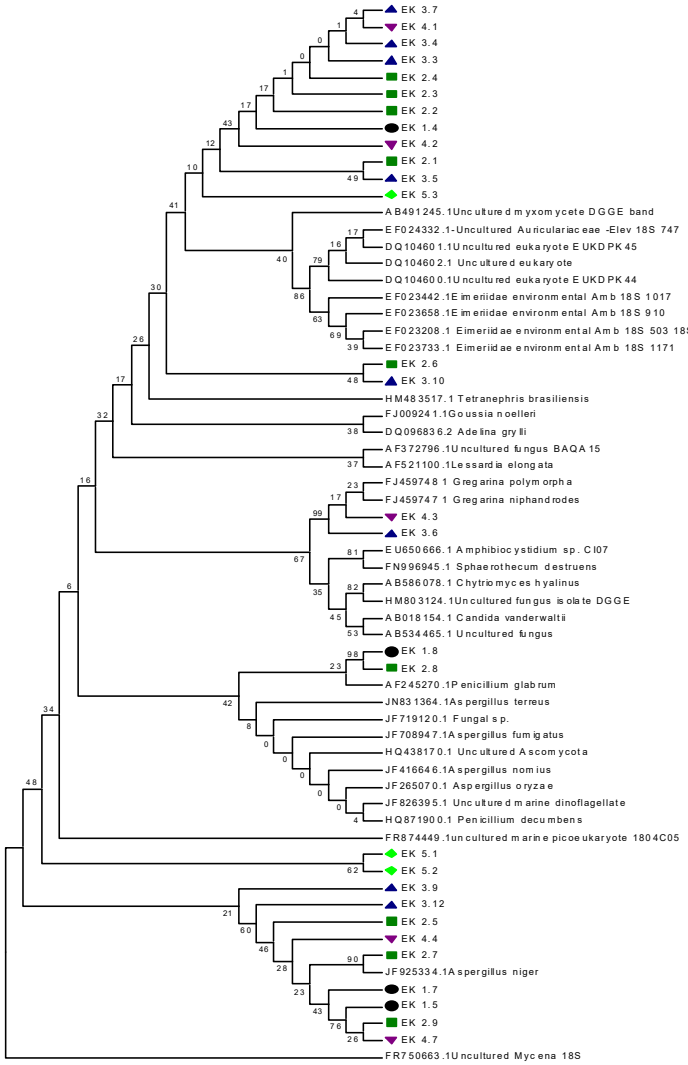

Fig. 4. Phylogenetic relationships of the 18S rRNA gene sequences during composting process. Phylogenetic tree was constructed by using neighbor-joining method of the MEGA 5.05 software with 1,000 bootstrap replicates.

degradation of cellulose as a nutrient since it could be transported into mycelium. Chitin is the building block of the exoskeleton of insects, some *Aspergillus* are able to use chitin both as N and C sources. Chitin is degraded through exoenzymes to N-acetylglucosamine, which is reabsorbed, transformed to fructose-6-P, and incorporated into the metabolism of carbohydrates[33]. *Aspergillus fumigatus* is a fungus of the genus *Aspergillus*, and cause disease in individuals with an immunodeficiency. *A. fumigatus* is usually found in soil and decaying organic matter such as compost. It plays an important role in recycling carbon and nitrogen[34]. *A. fumigatus* grow at temperatures ranging from 12 ° C to about 52-55 ° C. *A. fumigatus* is very common found at thermophilic phase during composting process since this fungus is strongly cellulolytic degrading microorganism[33].

Another class of Ascomycota have been reported in compost are *Geotrichum, Penicillium, Mucor, Cladosporium, yeasts, Absidiae, Rhizopus,*

Ketomium, Humicola, Coniochaeta, Tetracladium, and the various thermophilic fungi *Cladosporium, Aspergillus, Mucor, Rhizopus*, and *Absidiae*[29;12]. The majority of the sequences (EK 1.4, EK 2.1, EK 2.2, EK 3.3, EK 3.4, EK 3.5, EK 4.2) except for the bands found at the maturation phase were closed to *Eimeriidae*, but showing significant difference in the similarity of the sequence (86%-94%) (Table3). Although these sequences are phylogenetically grouped in the same phylum, limited sequence data restrict our representation of their deeper branch in species level.

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