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Succession of Bacterial Culture-independent During Manure Composting Process

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Composting process depends on the quality of organic materials, site of compost, composting methods, temperature, aeration systems, water content, C/N ratio and microorganisms. Community diversity of microorganisms can be revealed by grouping the microorganisms represented by DGGE bands. DGGE profiles of the bacterial from culture-independent during the composting process showed variation patterns from early mesophilic to maturation phases. On the top side of gel the DGGE bands showed less variation for all composting phases, except at maturation phase the number of the band were decrease significantly. Nevertheless on the middle side of gel, variation of the bands showed significantly differences. In this position, during peak of thermophilic phase, all bands were disappeared but reappeared at the end of the thermophilic and maturation phases. Detail analysis by comparing the sequence of 16S rRNA gene fragments to the GenBank showed that all of bands were closed to either *Gammaproteobacteria*, *Clostridia* or *Bacilli*. In the early of mesophilic and thermophilic phases most of bands appeared are closed to *Gammaproteobacteria*, however at the peak of thermophilic phase most bands are closed to *Bacilli*. Whereas *Clostridia* are found at mesophilic, end of the thermophilic and maturation phases.

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

Composting is a process of degradation of cattle manure by aerobic microbial activity. The purpose of composting process in general is to produce stable and high nutrient materials which are easily absorbed by plants (Kowalchuk, et al, 1999). Activity of microorganisms in the process depends on environmental factors. At optimum conditions needed by the microorganisms, the activity of microorganisms are the highest otherwise the decomposition process is slow down or even stops completely (Ryckeboer *et al*., 2003). Composting process depends on quality of organic materials, site of compost, methods, temperature, aeration systems, water content, C/N ratio and microorganisms (Neklyudov *et al*., 2008; Bernal *et al*, 2009).

The communities of microorganisms dominated during the composting phases depend on the environment (Gray et al, 1971; Bagstam, 1978; Crawford, 1983). Degradation of organic matter was performed by variety of different microorganisms in each phase. Microbial community involved in the process ussually are bacteria, fungi, yeast and protozoa (Ryckeboer *et al*., 2003; Bernal *et al*, 2009). The heterotrophic bacteria such as aerobic and anaerobic, mesophilic or thermophilic bacteria are the largest population

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in the compost while the hydrogen oxidizing bacteria, sulfur and ammonia are found in relatively low quantities (Blanc et al, 1997).

Culture-independent methods offer an alternative approach to study microbial diversity (Aminin et al, 2008a) and usually more sensitive to detect up to the species level compared to that culture dependent (Yohandini et al., 2008a). Biodiversity analysis of microorganisms by using PCR-DGGE during the composting process has been used widely and successfully revealed the existence of microorganisms in a variety of composting conditions (Hultman et al. 2010). In addition PCR-DGGE technique has also been successfully explained changing the composition of microorganisms in different phases. At each phase of composting process, bacterial species were found in variation while the dominant bacteria were related to composting conditions (Partanen et al, 2010).

The understanding of structure dynamics of microorganisms that play a role in the degradation of organic compounds in each phase of composting process is important to control the composting process effectively, especially roles of bacteria during degradation of organic materials in the process. In this report we would like to present diversity of bacteria during composting cattle manure.

MATERIALS AND METHODS

Composting set-up and Sampling

Composting process was conducted in Cigadung arround 3 km from Laboratorium 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. The bin 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, 47, and 91. Samples were immediately used for further analysis.

Isolation and Physicochemical analysis

Extract compost were prepared by shaking, 20 grams fresh sample in 180 mL of distilled water and then filtered. The supernatant 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 all microbial communities were stored at -20°C until used for DNA extraction. The pH was measured in water extract supernatan. The moisture content was obtained by drying the sample up to 70 ^{0}C until reached constant weight.

DNA Extraction

Total community DNA from each phase of compost samples were extracted using Zhou method (1996) with some modifications (Aminin et al, 2008b). 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 16S rRNA gene fragments

The 16S rRNA genes fragments were amplified by PCR method using a set of bacteria primers as described by Ferris et al, (1996). One primer lies on conserved region among members of the domain Bacteria (*Escherichia coli* positions 1055 to 1070). The other primer is based on a universally conserved region (*E. coli* positions 1392-1406) incorporated with a 40-bp GC-clamp in order to increase separation of DNA bands during DGGE analysis (Table 1). These primers amplify a fragment at approximately 390 bp long. PCRs were performed by using Taq polymerase according to

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 every cycle from 53 to 43°C. The final extension was performed for 10 min at 72°.

PCR product was determined by electrophoresis analysis, through 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 adding 40 µl of the PCR products loaded into a 8% (w/v) polyacrylamide-bisacrylamide (37.5:1) denaturing gels with gradients from 40 up to 70% (100% of denaturant corresponded to 7 M urea and 40% [v/v] deionized formamide) (Yohandini et al., 2008b). 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 (Bassam and Greshoff, 2007). 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 48°C, and 1 min at 72°C, and 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 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 5.10 Phylogenetic reconstruction was accomplished with the phylogeny MEGA 5.10 inference package (Saitou and Nei, 1987). Phylogenetic trees were constructed from distance matrices by the neighbor-joining method (Tamura et al, 2011), with substitution method Maximum Composite Likelihood (Tamura and Nei, 1993). The node reproducibility for tree topology was estimated by bootstrap analysis, which included 1000 replicate data sets.

RESULTS

Physico-chemical characteristic and 16S-rRNA gene fragment

Composting process was conducted in Kelurahan Cigadung, Bandung, Indonesia. Cattle manure and rice straw were mixed homogeneously at a ratio of 3:1 (cattle manure:rice straw). The process was traditionally carried out without using starter and self-acting aeration system. Physico-chemical properties during the process such as temperature, pH and moisture content were described in Safika et al (2013).

Total DNA from all phases of composting process were successfully extracted and used as template to amplify 16S rRNA gene fragment using primers as described in the methods. The results showed as a single band on agarose gel electrophoresis with size of about 400 bp as expected (Fig. 1).

Table 1. The primers used in this study

Primer	Sequence	*Saccharomyces cerevisiae* positions at	Tm (°C)
Euk1209f	ATGGCTGTCGTCAGCT	1055 -1070	50
Uni1392r	CACGGGCGGTGTGTAC	1392-1406	51
GC-clamp	CGCCCGCCGCGCCCCGCG CCCGGCCCGCCG CCCCCGCCC		

Table 2. Close relation of DGGE band sequences of cattle manure compost to the GenBank

Stages Composting	DGGE Bands	Microorganisms	Closest relatives Phylogenetic	composting	Similarity Bands
early mesophilic(28 ^{0}C)	BK1.1	*Peptostreptococcus anaerobius*	*Clostridia*	NR042847.1	92
	BK1.2	*Clostridium sporogenes*	*Clostridia*	AJ579907.1	79
	BK1.3	*Pseudomonas vranovensis*	*Gammaproteobacteria*	NR043313.1	89
	BK1.4	*Pseudomonas proteolytica*	*Gammaproteobacteria*	NR025588.1	99
	BK1.5	*Pseudomonas cichorii*	*Gammaproteobacteria*	EF101976.1	99
	BK1.7	*Tatumella sp. enrichment*	*Gammaproteobacteria*	HQ398263.1	84
	BK1.8	*Providencia vermicola*	*Gammaproteobacteria*	NR042415.1	100
early thermophilic(50^{0}C)	BK2.1	*Pseudomonas oryzihabitans*	*Gammaproteobacteria*	KC139422.1	98
	BK2.2	*Acinetobacter lwoffi*	*Gammaproteobacteria*	AF188302.1	76
	BK2.3	*Pseudomonas putida*	*Gammaproteobacteria*	JX120503.1	98
	BK2.4	*Uncultured compost bacterium*	*Bacteria*	JQ775316.1	99
	BK2.5	*Uncultured compost bacterium*	*Bacteria*	HM036038.1	99
	BK2.6	*Bacillus thermoamylovorans*	*Bacilli*	JQ670744.1	100
	BK2.7	*Bacillus licheniformis*	*Bacilli*	KC310461.1	100
thermophilic(60^{0}C)	BK3.1	*Ureibacillus thermosphaericus*	*Bacilli*	KC310463.1	83
	BK3.2	*Ureibacillus thermosphaericus*	*Bacilli*	AB671590.1	96
	BK3.3	*Uncultured Geobacillus sp.*	*Bacilli*	FN562413.1	80
	BK3.4	*Uncultured compost bacterium*	*bacteria*	AB034718.1	98
	BK3.5	*Uncultured compost bacterium*	*bacteria*	JQ775316.1	89
end of thermophilic(50^{0}C)	BK4.1	*Ureibacillus thermosphaericus*	*Bacilli*	KC310463.1	97
	BK4.2	*Uncultured Bacterium AW6 16S*	*Clostridia*	GU187298.1	88
	BK4.3	*Clostridium*	*Clostridia*	FR850659.1	86
	BK4.4	*Swine fecal bacterium*	*bacteria*	FJ753793.1	82
	BK4.5	*Clostridium sp*	*Clostridia*	JX575132.1	96
maturation(35 ^{0}C)	BK5.1	*Uncultured compost bacterium*	*Bacteria*	HM036043.1	97
	BK5.2	*Swine fecal bacterium*	*Bacteria*	FJ753822.1	97
	BK5.4	*Uncultured Eubacterium*	*Clostridia*	JN792356.1	94
	BK5.5	*Clostridium hungatei*	*Clostridia*	JX073559.1	86

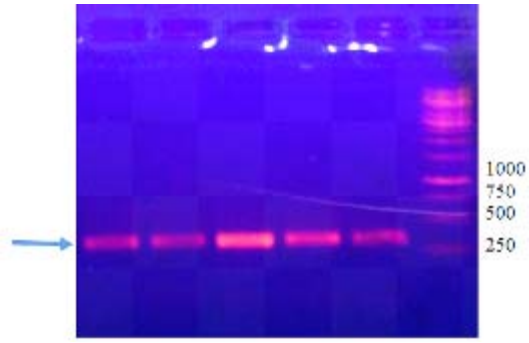

Fig. 1. Agarose gel electrophoresis of 16S rRNA bacterial gene fragments during composting process.1. At early mesophilic (28 ^{0}C); 2. At early thermophilic (50 ^{0}C); 3. At thermophilic (60 ^{0}C); 4. At the end of thermophilic (50 ^{0}C); and 5. At maturation phases (35 ^{0}C); 6. DNA marker 1 kb

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

DGGE profile of 16S rRNA Fragment

The DGGE profiles of bacteria communities in the composting of cattle manure was showed as in Fig 2. The profiles of bands during the process were variation throughout the gel. The bands at the middle of the gel are less variation compared to that at upper and bottom sides respectively. At the peak of the thermophilic phase the bands in the middle of gel were disappeared and reappeared at the end of the thermophilic and maturation phases. At the upper of gel, variation of the bands are relatively similar for all phases, except at the maturation phase are less variation. High intense bands were appeared at the bottom of the gel in all phases which then gradually decreased from thermophilic to maturation phase. The thickness of bands suggested the higher number of bacteria in the nature.

Phylogenetic and Diversity of Bacteria Community

All sequence 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 16S rRNA sequences of bacteria, 28 bands were successfully sequenced and analyzed. The relative relationships among the bands were described on Table 2. Nucleotide sequence accession number the sequences of 16S rRNA obtained in this study are available in the GenBank database NCBI under the accession numbers from KC811330 to KC811357.

Homology analysis showed that most bands close relationship to *Gammaproteobacteria*, *Clostridia*, and *Bacilli*, with sequence differences between 80-100%. BLASTN results of the bands at the mesophilic phase were closed to *Clostridia* (2 bands; 79-92%), and *Gammaproteobacteria* (6 bands; 84-100%). In the early of thermophilic phase, the nucleotide sequence were close to *Gammaproteobacteria* (3 bands; 76-98%), bacteria (2 bands; 99%) and *Bacilli* (2 bands; 100%). At the peak of thermophilic phase, the sequences were closed to *Bacilli* (3 bands; 80-96%) and bacteria (2 bands; 89-98%). At the end of the thermophilic phase the sequences were closed to *Bacilli* (1

band; 97%), *Clostridia* (3 bands; 86-96%) and bacteria (1 band; 82%). Meanwhile at the maturation phase, the bands were closed to bacteria (2 bands; 97%) and *Clostridia* (2 bands; 86-94%).

Phylogenetic analysis of the sequences showed that a few sequences formed a new cluster (Fig.3). 7 out of 12 bands of *Gammaproteobacteria* and 7 out of 9 bands of *Clostridia* formed a new cluster closed to uncultured bacterium (Fig.3). These microorganisms are typical bacteria acting during composting process.

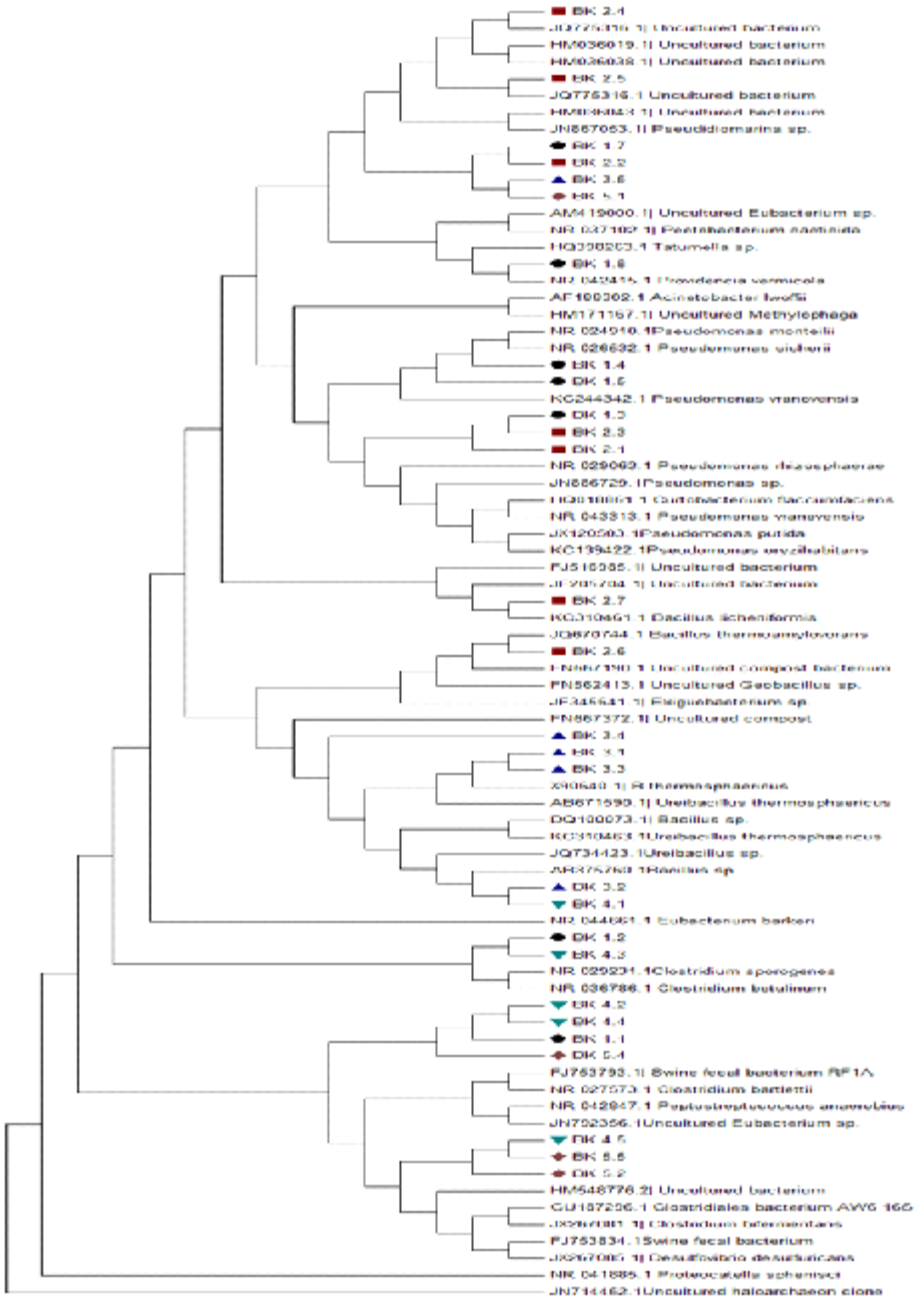

Fig. 3. Phylogenetic relationships of the 16S rRNA gene sequences during composting process. Phylogenetic tree was constructed by using neighbor-joining method of the MEGA 5.10 software with 1,000 bootstrap replicates

DISCUSSION

Microbial diversity in nature could be assessed by DGGE analysis of 16S rRNA gene fragment amplified from nature. The DGGE pattern showed variation of qualitative and quantitative microbes in nature (Muyzer, 1999). The result in this study showed that microbial communities during composting process were fluctuated due to changing in environmental properties.

At the mesophilic phase, some microbes such as *Pseudomonas, Providencia, Peptostreptococcus,* and *Clostridium* played an important role on degradation of manure. *Providencia* is known as xylanase producing bacteria, meanwhile *Pseudomonas* is known as nitrifying and cellulose producing bacteria (Insam et al. 2010). On the early of thermophilic phase, *Pseudomonas* was still detected however some *Bacillus* started to be detected. At peak of thermophilic phase where the temperature is at around 60 °C, *Bacillus thermosphaericus, Ureibacillus,* were observed. At the end of the thermophilic phase the structure of microbes was changed by revealing of *Clostridia*. While at the maturation phase *Clostridia* and *Gammaproteobacteria* were found, however the *Clostridia* and *Gammaproteobacteria* are different to that found during the mesophilic phase. *Bacillus* is known as bacterial indicated of the transition between mesophilic to thermophilic phases (Partanen et al, 2010), so that this result confirm the above statement.

Atkinson et al, (1996) estimates that 1% of all bacteria found in compost are anaerobic bacteria, and the genus *Clostridium* is one of anaerobic bacteria were detected in all types of composting. *Clostridia* are known to have role as a nitrogen-fixing bacteria (de Bertoldi et al, 1983). In the maturation phase, *Clostridia* degrade cellulose to produce cellulosomes large multi-enzyme complexes, which are bound to the outer surface of the microorganism (Wilson, 2008).

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