Indonesian Journal of Biotechnology, December, 2008 Vol. 13, No. 2, pp. 1085-1091

Succession of Actinomycetes During Composting Proccess of Dairy-Farm Waste Investigated by Culture-Dependent and Independent Approaches

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Abstract

Mesophilic, thermophilic, and maturation phases were recognized in composting proccess. Temperature changes influence the microbial communities in compost within composting proccess. Actinomycetes account for a larger part of compost microbial population. The aim of this research was to study succession of actinomycetes community during composting of dairy-farm waste investigated by culture-dependent and independent approaches.

In culture-independent method, the succession of actinomycetes community was analyzed by nested-polymerase chain reaction of ribosomal intergenic spacer (nested-PCR RISA) using spesific primer F243 and primer R23S followed by a second PCR using primers F968 and R23S. In culture-dependent method actinomycetes from compost were isolated on selective media, starch-nitrate medium and humic-acid + vitamins medium. DNA of actinomycetes was extracted and amplified by repetitive sequence-based PCR (rep-PCR) using primer BOXA1R. The banding patterns were used to generate dendrograms by UPGMA clustering with NTSYS program. Microcosm containing sterile rice-straw and water which is inoculated with each actinomycetes isolates was used for examining the ability of each isolate in rice-straw degradation.

The experiment results showed that succession of both bacteria and actinomycetes was occured within composting proccess of dairy-farm waste. Analysed by culture-independent method revealed that the highest community of compost's bacteria was on mesophilic, thermophilic, and maturation phases, respectively. Whereas PCR-nested RISA resulted the highest population of actinomycetes was on thermophilic, maturation, and mesophilic phases, respectively. By culture-dependent method was obtained 29 actinomycetes isolates from mesophilic phase, 23 isolates from thermophilic phase, and 19 isolates from maturation phase. Genetic diversity analysis of the obtained isolates showed the presence of phylogenetic grouping on each phase of composting proccess. This result illustrated the occurance of succession of actinomycetes community in compost. The ability of each isolates in rice-straw degradation was different, and SnT9 isolate was found to be a promising rice-straw degrader.

Keywords: succession, actinomycetes, composting, nested-PCR RISA, rep-PCR

Introduction

Many factors are involved in the complex process of composting, and they affect to a greater or lesser extent the direction of the process. The microorganisms have an essential role in the decomposition of organic matter. Despite the heterogeneous nature of organic source materials it can be divided into the following major elements: carbohydrates, proteins, fats, hemicellulose, cellulose, lignin and mineral matter (Subba Rao, 1997). As a result of microbial activity, heat is liberated and the temperature rises, if the heat is retained in the composting mass. Based on the development of temperature, the composting process can be divided into the mesophilic phase (temperatures below 45°C), the thermophilic phase (temperatures above 45°C), and finally the curing phase, which is characterized by a decrease in

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temperature.

Actinomycetes are a group of gram-positive bacteria (Class Actinobacteria) characterized by a high G-C content that perform a wide array of important functions in various habitats, including contributing significantly to organic matter processing. Actinomycetes are well known for their ability to decompose complex molecules, particularly lignocellulose components, which make them important agents in decomposition processes (Lacey, 1997). Additionally, the apparent widespread ability of actinomycetes to generate soluble lingo-carbohydrate from straw has been confirmed (Ball *et al.*, 1990).

Cultivation of microorganisms extracted from compost samples allows one to obtain pure cultures which can be used for further taxonomic or physiological characterizations (Belfa *et al.*, 1996). Rapid molecular PCR-based techniques, such as amplified ribosomal DNA restriction analysis (ARDRA), are useful for comparison of a large number of isolates at the phylogenetic level. This technique allowed the characterization of *Thermus* strains and Bacillus-related bacteria isolated from hot composting material (Belfa *et al.*, 1996; Blanc *et al.*, 1997). However, since any chosen cultivation approach will inevitably favor the growth of some community members while others are inhibited or not culturable at all, it is unlikely that any cultivation method will allow a full description of the microbial diversity.

Therefore, cultivation-independent methods have recently been used to characterize microbial-community successions during composting. These include assessment of the diversity of directly extracted nucleic acid-based techniques. The application of fingerprinting techniques such as temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), terminal restriction fragment length polymorphism (T-RFLP), or single-strand conformational polymorphism (SSCP) gives information about the types of microorganisms present in the environment. Peters *et al.* (2000) recently developed a protocol which allows the application of single-strand conformation polymorphism (SSCP) for the cultivation-independent assessment of microbial-community diversity. The aim of this research was to study succession of actinomycetes community during composting of dairy-farm waste investigated by culture-dependent and independent approaches.

Materials and Methods

Sampling

Composting windrows system in CV. Lembah Hijau Multifarm Surakarta was used for this investigation. Each windrow filled with a mixture of STARDEC (LHM Research Station), wood chips, and straw-bedded cow manure. This mixture was wetted before composting for the initiation of the self-heating phase. The windrows were turned every 7 days in order to enhance the composting process and avoid the formation of anaerobic compartments. Replicate samples were taken from a 50-cm depth. Samples designated for DNA extraction were stored immediately at -20°C.

E*xtraction of DNA from compost material and generation of nested-PCR-RISA and genetic profiles*

Compost samples were ground in mortar, and total DNA was extracted from samples of 0,5 g (dry weight) using the Gabor method (Gabor *et al.*, 2002; Arcuri *et al.*, 2003; Ishii *et al.*, 2000; Roose-Amsaleg *et al.*, 2001; Tien *et al.*, 1999; Zhou *et al.*, 1996). Two different primer systems were used to amplify 16S rRNA genes from total community DNA of compost. Each PCR was performed in a total volume of 12 ml in

micro-test tubes. Reaction mixtures contained 6 ml MegaMix Royal, 1 ml each primer, 1 ml of the diluted DNA extract and 3 ml bidestilate water. For nested-PCR with primers F243 (5'>GGATGAGCCCGCGGCCTA<3') (Invitrogen) and R23S (5'-GGGTTBCCCCATTCRG-3') (Invitrogen), cycle conditions for the reactions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 60 s, 50°C for 90 s, and 68°C for 8 min; and a final elongation for 10 min at 68°C (Heuer *et al.*, 1997). For PCR RISA using primer 968F (5'-AACGCGAAGAACCTTAC-3') (Invitrogen) and primer 23SR (5'-GGGTTBCCCCATTCRG-3') (Invitrogen), cycle conditions for the reactions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 1 min; and a final elongation for 5 min at 72°C (Ranjard et al., 2000). The DNA fragments were analyzed on 6% (wt/vol) polyacrylamide gels. To make the PCR products visible, gels were silver stained (Sambrook *et al.*, 1989).

Isolation and characterization of pure actinomycetes cultures from compost

In order to obtain actinomycetes cultures from composting material, samples taken at each phase of the composting process were suspended in sodium chloride solution (0.85%, wt/vol). Dilutions of this suspension were inoculated onto plate count agar with starch nitrate (SN) medium and humic acid+vitamin (HV) medium supplemented with 50 mg of cycloheximide $liter^{-1}$ for suppression of fungal growth. The inoculated plates were incubated at 30°C for 14 h before single colonies were transferred to fresh agar and subcultured (Hayakawa and Nonomura, 1987; Miyashita *et al.*, 1982). The Munsell® color charts for plant tissues was used for characterize pigmentation of colonies and soluble pigment in medium.

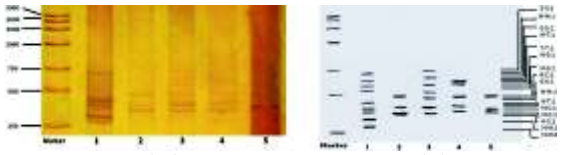
The repetitive-PCR technique was used for the characterization of isolates. Colonies of actinomicetes cells grown were suspended in 50 ml of lysis buffer (0.05 M NaOH–0.25% [wt/vol] sodium dodecyl sulfate) and incubated for 15 min at 95°C. The suspensions were diluted with 450 ml of water and centrifuged in a microcentrifuge at the highest setting for 5 min at room temperature. (Ausubel *et al.*, 1992; Song *et al.*, 2004). An aliquot (1 to 5 ml) of the centrifuged solution was used as a template for PCR. DNA was amplified with primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACGCTGACG-3') (Invitrogen) (Sadowsky *et al.*, 1996).. The DNA fragments were analyzed on 6% (wt/vol) polyacrylamide gels. Products were stained with silver nitrate (Sambrook *et al.*, 1989). The banding patterns were used to generate dendrograms by UPGMA clustering with NTSYS program.

Decomposition of rice straw under laboratory conditions

Rice straw was cut into 5 cm pieces and weighed at 2 gr after drying overnight at 105 °C. Actinomycete isolate was inoculated into microcosm containing rice-straw and sterile water, then incubated at 28 °C for 75 days. Observations were taken at 7-day intervals; any changes in colour or integrity of the straw strips, comparing with uninoculated (control) tubes, were recorded. At 10-week intervals, one straw piece from each of the microcosm was carefully removed, dried and reweighed, to calculate the percentage of weight loss (Xuan, 2007). Soluble medium in the microcosm was calculated for reduction sugar concentration (Somogyi, 1945; Somogyi, 1952).

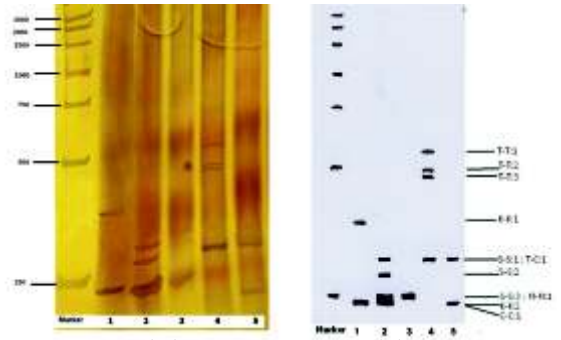
Results and Discussion

Culture-independent method



(A) (B)

Figure 1. Profile bacterial succession during composting proccess analyzed by PCR RISA with primers F968 and R23S. Note: (A)PAGE 6% visualization; (B)Schematic figure; (1)Stardec / M; (2)Cow manure / K; (3)Mesophilic / S; (4)Thermofilic / T; (5)Maturation / C. The band nomenclatur pattern as follow A-B:C, which A-B refers to each phases and C refers to serial number of band in the same phase.



(A) (B)

Figure 2. Profile actinomycetes succession during composting proccess analyzed by nested-PCR RISA with primers F243 and R23S continued with primers F968 and R23S,
Note: (A)PAGE 6% visualization; (B)Schematic figure; (1)Stardec / M; (2)Cow manure / K; (3)Mesophilic / S; (4)Thermofilic / T; (5)Maturation / C.

The experiment results showed that succession of both bacteria and actinomycetes was occured within composting proccess of dairy-farm waste. The succession of products in combination with increasing and decreasing band intensities during different composting stages, as detected with dendrogram profiles in this study. Analyzed by culture-independent method revealed that the highest community of compost's bacteria was on mesophilic, thermophilic, and maturation phases, respectively (Figure 1). The mesophilic stage is characterized by the growth and activity of mesophilic organisms, such as fungi, yeast, Gram-negative and lactic acid bacteria (Steger, 2002). Generally, the activity of the mesophilic community leads to an increase in temperature. In the next phase, thermophilic temperatures are reached and organisms adapted to these conditions (e.g. *Bacillus* spp., *Thermus thermophilus* and *Thermoactinomyces* sp.) take over the degradation process (Blanc *et al.*, 1998; Song *et al.*, 2001). The growth and activity of non-thermotolerant organisms, including pathogens and parasites, are inhibited during the thermophilic phase. The final curing stage / maturation is characterized by the development of a new mesophilic community. As the temperature declines, mesophiles and moderate thermophiles reappear, including fungi and actinomycetes.

The presence and development of actinomycetes populations and their potential use in characterizing the final compost product were investigated full-scale composting processes. The changes in the size of actinomycetes populations were determined by analysis of nested-PCR of 16S rRNA genes with actinomycetes specific primers. PCR-nested RISA resulted the highest population of actinomycetes was on thermophilic, maturation, and mesophilic phases, respectively (Fig. 2). Actinomycetes are commonly believed to play a significant role in the degradation of relatively complex, recalcitrant compounds (Goodfellow *et al.*, 1988). The ability of actinomycetes to degrade lignocelluloses implies that this group of bacteria may be suitable as potential indicator organisms for compost maturity.

Culture-dependent method.

By culture-dependent method was obtained 29 actinomycetes isolates from mesophilic phase, 23 isolates from

thermophilic phase, and 19 isolates from maturation phase (Figure 3).

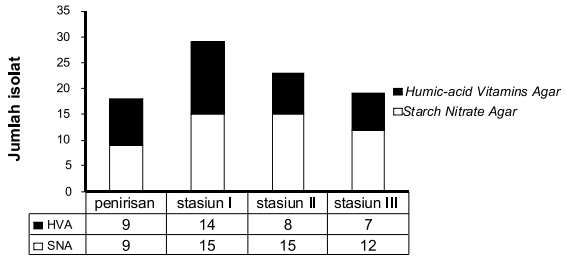


Figure 3.Number of actinomycetes isolated from each stage of composting process cultured on SNA medium and HVA medium.

The difference indicated succession of actinomycetes in composting proccess. Beside that, the number of actinomycetes isolate obtained from HV medium was lesser than which obtained from SN medium, indicated that HV medium was the better medium for actinomycetes isolation.

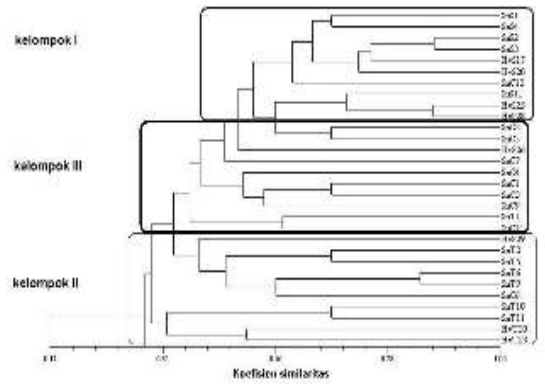


Figure 4. Profile of dendrogram actinomycetes isolates amplified with *rep*-PCR analyzed with UPGMA

Genetic diversity analysis of the obtained isolates showed the presence of phylogenetic grouping on each phase of composting proccess (Figure 4). This grouping consist of three groups, same as the phases of composting proccess. Group I was dominated with isolates which is taken from mesophilic stage in compost pile. Group II was dominated with isolates which is taken from thermophilic stage in compost pile, and group III was dominated with isolates which is taken from maturation stage in compost pile. This result illustrated the occurance of succession of actinomycetes community during composting proccess.

Rice straw degradation

(A) (B)

Figure 5.Photographs showing the degradation of rice-straw by SnT9 isolate compared to control after incubation for 10-weeks under static laboratory conditions, in microcosm containing rice straw as the sole source of carbon.

The ability of each isolates in rice-straw degradation was different, and SnT9 isolate was found to be a promising rice-straw degrader (Fig. 5). SnT9 isolate have strong hydrolytic activities to decompose rice straw, utilize it as sole carbon source and cause high weight loss (50%) and also produce reduction sugar (85 mg/l) under laboratory conditions (Fig. 6 and Fig. 7). Actinomycetes are able to degrade cellulose and solubilize lignin extensively as their primary metabolic activity, thus they are important agents of lignocellulose decomposition in soil (Abdulla, 2007).

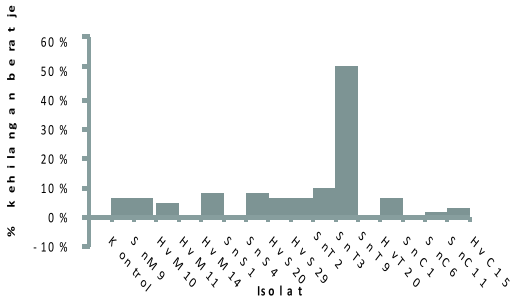


Figure 6. Percentage of rice-straw weight-lost vs. each isolate after incubation 30◦C for 75 days.

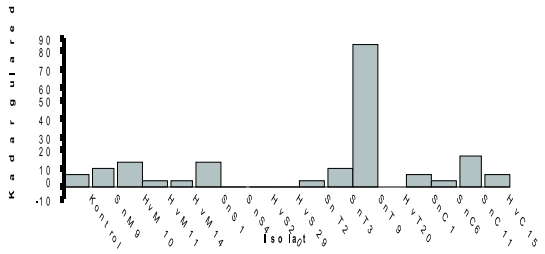


Figure 7. Concentration of reduction sugar vs. each isolate after incubation 30°C for 75 days.

Isolate SnT9 could represent important straw decomposers and further study is needed to verify its identification. Further detailed study is in progress to investigate the enzymatic system of the lignocellulytic actinomycete SnT9 and to optimise rice straw incorporation into soil using this isolate.

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