Bacterial Community Structure and Biochemical Changes Associated With Composting of Lignocellulosic Oil Palm Empty Fruit Bunch

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Bacterial community structure and biochemical changes during the composting of lignocellulosic oil palm empty bunch (EFB) and palm oil mill effluent (POME) anaerobic sludge were studied by examining the succession of the bacterial community and its association with changes in lignocellulosic components by denaturing gradient gel electrophoresis (DGGE) and the 16S rRNA gene clone library. During composting, a major reduction in cellulose after 10 days from 50% to 19% and the carbon content from 44% to 27% towards the end of the 40-day composting period were observed. The C/N ratio also decreased. A drastic change in the bacterial community structure and diversity throughout the composting process was clearly observed using PCR-DGGE banding patterns. The bacterial community drastically shifted between the thermophilic and maturing stages. 16s rRNA clones belonging to the genera *Bacillus*, *Exiguobacterium, Desemzia*, and *Planococcus* were the dominant groups throughout composting. The species closely related to *Solibacillus silvestris* were found to be major contributors to changes in the lignocellulosic component. Clones identified as *Thermobacillus xylanilyticus*, *Brachybacterium faecium*, *Cellulosimicrobium cellulans*, *Cellulomonas* sp., and *Thermobifida fusca*, which are known to be lignocellulosic-degrading bacteria, were also detected and are believed to support the lignocellulose degradation.

Keywords: Composting; Lignocellulose degradation; Denaturing gradient gel electrophoresis; 16S rRNA gene clone library

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INTRODUCTION

Malaysia is one of the world's largest oil palm producers, producing about 18 million tonnes per year, which is about 47% of the world's supply (Sumathi *et al*. 2007). In addition to palm-derived oil, the palm oil industry generates an abundant amount of

wastes, such as palm oil mill effluent (POME), empty fruit bunch (EFB), mesocarp fiber, and palm kernel shell. One way to create value-added products from these wastes is through the composting of POME and EFB, whereby the compost can be used as a soil amendment and as organic fertilizer (Baharuddin *et al*. 2010). The simultaneous treatment of different kinds of biomass in the palm oil industry through composting is a good practice for promoting recycling and zero waste strategies. Primary nutrients such as nitrogen, phosphorus, and potassium are important parameters for determining the quality of compost.

In previous studies on the composting of oil palm waste, the co-substrates used were chicken manure, decanter sludge, and sewage sludge. The primary nutrients found in the raw materials in those studies were less than 3% each, with the final C/N ratios of around 10 to 19; 60-90 days were required to complete the process (Baharuddin *et al*. 2009a; Kala *et al.* 2009; Kananam *et al.* 2011). However, a recent study on composting of EFB with POME anaerobic sludge in a closed anaerobic digester tank by Baharuddin *et al.* (2010) showed that composting could be accomplished within 40 days. The initial nitrogen, phosphorus, and potassium contents were 4.68%, 1.25%, and 5.16%, respectively, which is higher than in those studies mentioned above, and the final C/N ratio was 12.4. The continuous supply of microorganisms and also nutrients such as nitrogen and other nutritional elements from POME anaerobic sludge throughout composting process could be one of the factors that promote rapid composting through the effective degradation of lignocellulosic materials. As a result, the balance of plant nutritional elements (N, P, K) is improved, which is important for producing value-added fertilizer.

Previous studies on composting of agricultural wastes have focused on the relationship between the microbial diversity or succession and the change in the compost's physicochemical properties (Yu *et al.* 2007; Baharuddin *et al.* 2009b; Huang *et al.* 2010; Partanen *et al.* 2010). Earlier, the direct cultivation method of plating through bacterial colony counting was employed to determine the number of bacteria present during the composting of oil palm-derived EFB and mesocarp (Lim *et al.* 2009; Ahmad *et al.* 2011). However, the diversity of bacteria in compost is very large and complex, and it is difficult to distinguish the types of bacteria among the colonies. Furthermore, it is also known that only less than 10% of the bacteria existing in the environment can be cultured (Yu *et al.* 2007).

Recently, determination of the bacterial community during the composting process using culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), has become popular (Green *et al.* 2004; Danon *et al.* 2008; Nakasaki *et al.* 2009; Székely *et al.* 2009). The change in the microbial community can be visualized through the DGGE pattern. However, the DGGE results reveal DNA patterns with low resolution, and it is also difficult to identify the major DNA bands. In addition, the DNA sequence information is limited up to the genus level due to short sequences (< 300 bp). Nevertheless, DGGE still remains a useful method for monitoring shifts in community structure over time. In previous studies on microbial diversity and succession of oil palm EFB compost, DGGE and amplified rDNA restriction analysis (ARDRA) were used to study microbial diversity and succession (Baharuddin *et al.* 2009b; Liew *et al.* 2009). Explanations about the inter-relationship between bacterial community and succession and the change in biochemical properties, especially with respect to the lignocellulosic EFB composting through culture-independent methods, remain less known. The lignocellulosic component accounts for the majority of lignocellulose composting, and its degra-

dation is necessary during the composting process (Yu *et al.* 2007).

This study attempted to investigate the relationship between bacterial succession and biochemical changes throughout the composting process for a better understanding, especially of the changes of the lignocellulose composition, which could facilitate the composting process as well as compost maturity. In this study, PCR-DGGE analysis was used to observe the changes in community structure and also a direct identification using a 16S rRNA gene clone library to elucidate the bacterial succession and their associations during composting of lignocellulosic EFB.

EXPERIMENTAL

Composting and Sample Collection

Composting was carried out as described by Baharuddin *et al.* (2010). Pressed-shredded EFB with a size of around 15 to 20 cm was obtained after recovery of the remaining crude palm oil from the press-shredding machine. One tonne of pressed-shredded EFB (w/w) was mixed with 500 kg (w/w) of thickened POME anaerobic sludge. The moisture content of the compost was maintained at 55 to 60% with regular additions of 500 kg (w/w) of thickened POME anaerobic sludge at 3-day intervals, which were stopped a week before harvesting. The compost process took about 40 days with occasional turning using an excavator to provide sufficient aeration and mixing. One kilogram of sample collected at different depths was mixed. The samples were divided into two sections and were stored at -20 ^{o}C and -80 ^{o}C for further analysis. The carbon and nitrogen contents as well as the C/N ratio were determined using a CNHS analyzer 2000 (Leeco, USA) as previously described by Baharuddin *et al.* (2010). The structure of EFB was observed by environmental scanning electron microscopy (Philip XL30 ESEM). Prior to viewing, the samples were washed and rinsed with distilled water to remove the sludge and were then dried in an oven at 60 ^{o}C. The dried samples were then cut into sizes of 2 to 5 mm each and mounted onto a stub. Samples were coated with gold using a sputter coater (BALTEC, SOC 005). The coated samples were visualized under ESEM.

Lignocellulose EFB Compost Composition

The composition analysis of the cellulose, hemicellulose, and lignin components of EFB compost was performed using acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) as described by Goering and Van Soest (1970); the percentage of each component was calculated as follows:

$$\text{Percent of ADF (\%)} = (W_3\text{-}W_2) / W_1 \times 100 \quad (1)$$

$$\text{Percent of NDF (\%)} = (W_3\text{-}W_2) / W_1 \times 100 \quad (2)$$

$$\text{Percent of ADL (\%)} = (W_3\text{*-}\ W_2\text{*}) / W_1\text{*} \times 100 \quad (3)$$

where W_1 is the weight of the sample, W_1* is the weight of the sample from ADF determination, W_2 is the weight of the sintered filter funnel, W_2* is the weight of the sintered filter with the sample after reacting with H_2SO_4, W_3 is the weight of the empty

sintered filter funnel with the sample after drying, and W_3* is the weight of the empty sintered filter funnel after ashing.

Based on the results above, the percentages of cellulose and hemicelluloses were calculated as follows:

$$\text{Cellulose (\%)} = \text{ADF} - \text{ADL} \quad (4)$$

$$\text{Hemicellulose (\%)} = \text{NDF} - \text{ADF} \quad (5)$$

$$\text{Lignin (\%)} = \text{ADL} \quad (6)$$

DNA Isolation

Samples from day 0, day 10, day 20, day 30, and day 40 were used. Samples, which contained large fibers were mixed with Tris-EDTA (TE) buffer and cut into small sizes (1 to 2 mm) with sterilized scissors. One gram of each sample was then used for DNA extraction using a DNA extraction kit (MO-BIO, USA). The DNA was extracted based on the manufacturer's instructions, except as described below for hydrolysis. The samples in bead tubes were mixed with 500 μg mL^{-1} lysozyme chloride from egg whites (Nacalai tesque, Japan) and 220 μg mL^{-1} Mutanolysin from *Streptomyces globisporus* ATCC 21553 (Sigma-Aldrich, USA) and were then incubated at 37 ^{o}C for 30 min before cell disruption. The extracted DNA was then used for PCR-DGGE and 16s rRNA clone library.

PCR-DGGE Analysis

The 16s rRNA gene was amplified using primer 357F with a 40-bp GC clamp (5'-GGGGCCGCGGCCCCCGGG-3') and 518R (5'-ATTGCCCA-3'). The PCR reaction mixture and condition were performed according to Baharuddin *et al.* (2009b). DGGE analysis was performed with the DCode System for DGGE (Bio-Rad laboratories, USA). The PCR product was loaded onto a 1.5-mm-thick vertical denaturing gel containing 8% acrylamide with a gradient from 0% to 100%. One hundred percent of the denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed at 200 V at 60 ^{o}C for 5 h. After electrophoresis, the gels were stained with an ethidium bromide solution (1 mg/L) and viewed with the Gel Doc XR+ System (Biorad laboratories, USA).

PCR Analysis and Construction of 16S rRNA Gene Clone Library

The 16S rRNA gene was amplified with primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T-3'). PCR amplifications were performed in a 50-μL reaction mixture containing 1 μL of template DNA extracted from OPEFB, 10X buffer, 0.2 μL of ex Taq DNA Polymerase (TAKARA BIO INC, Japan), 4 μL of dNTP, 38.8 μL of sterilized ultra-pure water, and 0.5 μL of each primer with a concentration of 20 pmol μL^{-1}.

The PCR was carried out using a PCR thermal cycler (TAKARA, Japan) with conditions as follows: 0.5 min of denaturation at 96 ^{o}C, 0.5 min of primer annealing at 50 ^{o}C, and 1.5 min of primer extension at 72 ^{o}C. Serial dilutions of the original DNA were tested to obtain the optimal DNA concentration for PCR, as the compost samples contained several inhibitors. The optimal DNA dilution was used in the subsequent

amplification for the construction of a clone library. The PCR amplification cycles were also tested with 22, 24, 26, and 28 cycles.

The PCR products were analyzed using electrophoresis. The electrophoresis conditions were 1% (w/v) agarose gel and 1X TAE buffer. The electrophoresis was performed for 20 min at a constant potential of 100 V. The PCR bands were visualized by staining the agarose gel with gel red (Biotium, USA). The number of cycles was tested to reduce bias introduced during the PCR.

For construction of the 16S rRNA gene clone library, the DNA template was first amplified with the selected dilution and PCR cycles. The PCR products of approximately 1.5 kb were then ligated onto the cloning vector, pGEM-T (Easy) Vector Systems (Promega, USA), and transformed into *Escherichia coli* JM109 competent cells according to the manufacturer's instructions.

The cloned cells were spread onto an LB agar plate containing 100 μg/mL ampicilin-HCl (Nacalai tesque, Japan), 20 μg/mL X-gal (Nacalai tesque, Japan), and 10 mM IPTG (Nacalai tesque, Japan). A total of 606 positive colonies carrying a 1.5-kb insert were picked. The DNA insert was amplified through the colony PCR method using M13F (5'-TGT AAA ACG ACG GCC AGT-3') and M13RV (5'-CAG GAA ACA GCT ATG AC-3') as primers. The colony PCR was performed in a 50-μL reaction mixture containing a single clone of colony, 10X buffer, 0.2 μL of ex Taq DNA Polymerase (TAKARA BIO INC. Japan), 4 μL of dNTP, 39.8 μL of sterilized ultra pure water, and 0.5 μL of each primer with a concentration of 20 pmol μL^{-1}. The PCR was carried out using 25 cycles and conditioned as described above, except for the primer annealing temperature (55 ^{o}C). The PCR products were then purified using an Agencourt AMPure XP (Beckman Coulter, USA) kit according to the manufacturer's instructions prior to sequence analysis.

16S rRNA Gene Sequencing and Similarity Matrix Analysis

The partial 16S rRNA gene was sent to the 1st Base laboratory for sequencing analysis. The 750R (5'-TAC CAG GGT ATC TAA TCC-3') sequence primer was used for sequencing. Approximately 700 bases of sequences were analyzed for a similarity matrix between each sequence using the BioNumerics program (Applied Maths BVBA, Belgium). Sequences with ≥ 97% similarities were selected and were grouped in the Operationally Taxanomic Unit (OTU). Coverage of microbiota in the OPEFB compost was calculated using the method of Good (1953).

One clone from each group was randomly selected, and the sequence similarity to closest relative searches were conducted by matching its sequence to those contained in the databases at the National Center for Biotechnology Information (NCBI) using the nucleotide-nucleotide Basic Local Alignment Search online tool (BLASTn:http:// blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (RDPII) using the Sequence Match online tool (http://rdp.cme.msu.edu/seqmatch_intro.jsp).

The sequences were aligned using the ClustralW program contained in the MEGA 4.0.2 software (Tamura *et al.* 2007), and the phylogenetic tree was constructed based on the neighbor-joining algorithm with the same software. The representative sequence of each OTU has been deposited in the GenBank database under accession numbers JX286454- JX286470.

RESULTS AND DISCUSSION

Characterization of Biochemical Changes during the Composting Process

The composting of EFB with POME anaerobic sludge was conducted under aerobic conditions. After 10 days of composting, the temperature increased to 62 ^{o}C, which indicated that the thermophilic stage had been reached (Fig. 1a). The pH of the composting process was constant, ranging from 7 to 8 throughout the composting process (Fig. 1a). The initial C/N ratio of the compost was 45, and it decreased to 12 by the end of composting process (Fig. 1b).

Figure 1c shows the results of the cellulose, hemicellulose, and lignin compositions throughout the 40 days of the composting process. At the beginning of composting, the compositions of cellulose, hemicelluloses, and lignin were about 52%, 26%, and 19%, respectively, and the temperature was around 34 ^{o}C. The initial amount of cellulose at day 0 was higher than the amount of cellulose for the rest of the days during the composting process.

In the initial (day 0) composting process, fresh press-shredded EFB and POME anaerobic sludge were added and mixed together. Baharuddin *et al.* (2010) suggested that higher volatile suspended solids in POME anaerobic sludge was due to the presence of beneficial microbes, which provide a good source of bacteria. The highest temperature of composting of this study was 62 ^{o}C, indicating that it was mainly caused by microbiological activity, which released fermentation heat (Bertoldi *et al.* 1983). The pH slightly increased due to the degradation of organic acids in POME sludge, which was constantly added during the composting process.

Compared to previous studies (Baharuddin *et al.* 2009a), the composting process of this study could be completed within 40 days, and the C/N ratio could be achieved at almost the same value (~12). Yahya *et al.* (2010) reported that composting of EFB with decanter cake slurry with the addition of POME was completed after 51 days at a C/N ratio of 18.65, which take a long period of time and has a higher C/N ratio than present study. The improved composting of EFB-POME anaerobic sludge in the present study indicates that the composting process has been facilitated by the increased nutrients and bacterial diversity due to the continuous supply of sludge during the composting process.

The quality of compost was measured based on the amount of nutrient such as nitrogen, phosphorus, and potassium, as shown in Table 1. It was found that the amount of those nutrients was comparable with previous studies done by Thambirajah *et al*. (1995), Baharuddin *et al*. (2009), and Yahya *et al*. (2010).

Table 1. Nutrient of POME Anaerobic Sludge and EFB Compost Final Product

Nutrients	POME anaerobic sludge	EFB compost
1. Nitrogen (%)	37.5	2.2
2. Phosphorus (%)	1.2	1.4
3. Potassium (%)	5.16	2.8
4. C/N ratio	4.70	12.5

Data represent duplicate samples.

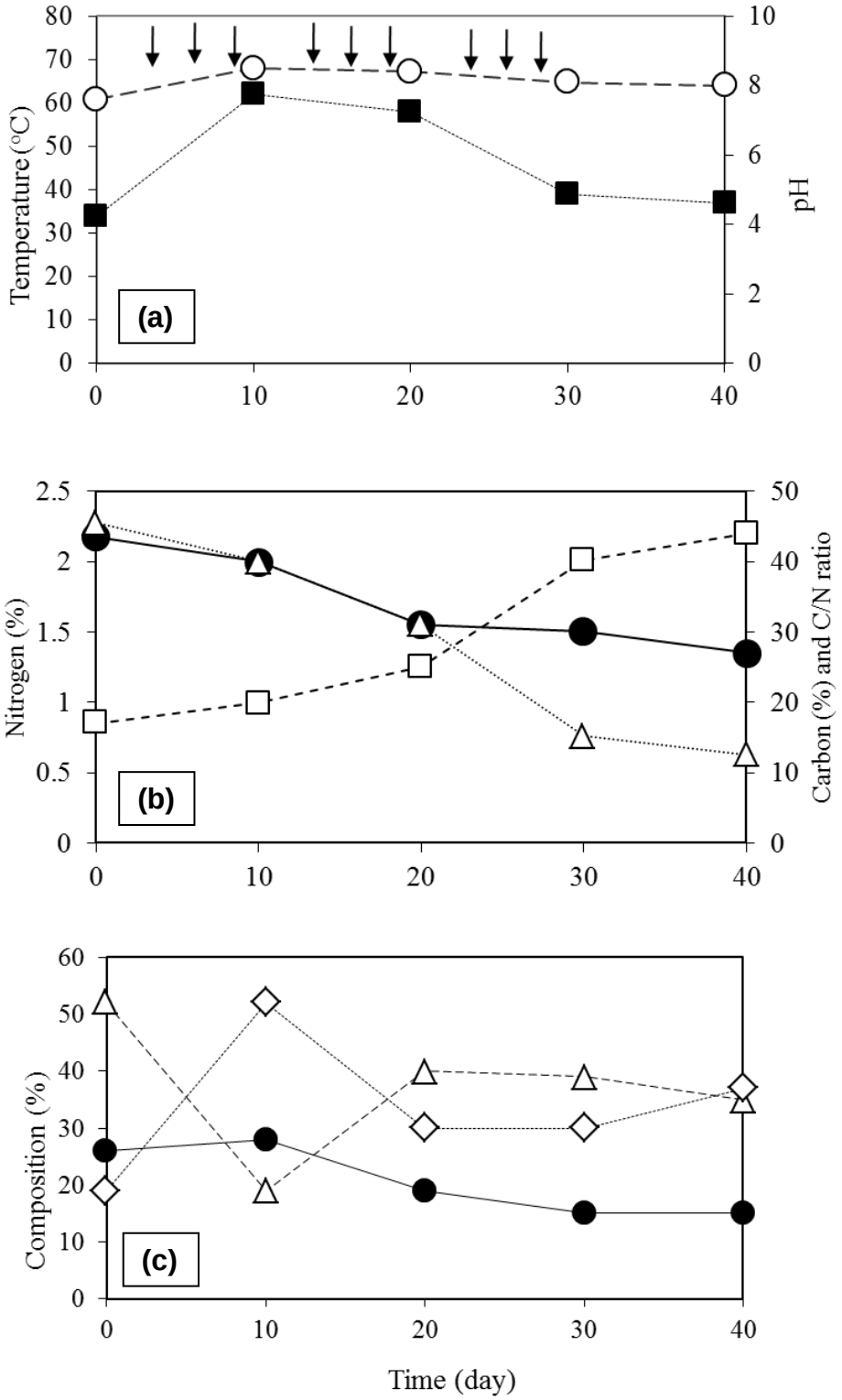

Fig. 1. Changes in physico-chemical properties of the compost during the co-composting process. (a) Temperature and pH, (*circles*) pH and (*filled boxes*) temperature; (b) profile of carbon, nitrogen, and C/N ratio, (*filled circles*) carbon, (*boxes*) nitrogen, and (*triangle*) C/N ratio; and (c) lignocellulose composition of EFB compost (*triangles*), cellulose, (*filled circles*), hemicellulose, and (*diamonds*) lignin. An arrow indicates the addition of POME during the process. The data represents the average of duplicated samples.

Scanning Electron Microscopy (SEM)

With SEM analysis, it was observed that the structure of intact EFB at day 0 displayed a smooth surface, as the composting process had not yet started (Fig. 2a). After 10 days of composting, it was found that the cellulose content decreased from 52% to 19% and also that the material was slightly disrupted, with a mildly rough surface (Fig. 2b). However, some microfibril structures could be observed from the fiber materials after 20 days of composting, and the number of silica bodies was reduced (Fig. 2c), as reported earlier by Baharuddin *et al*. (2009b).

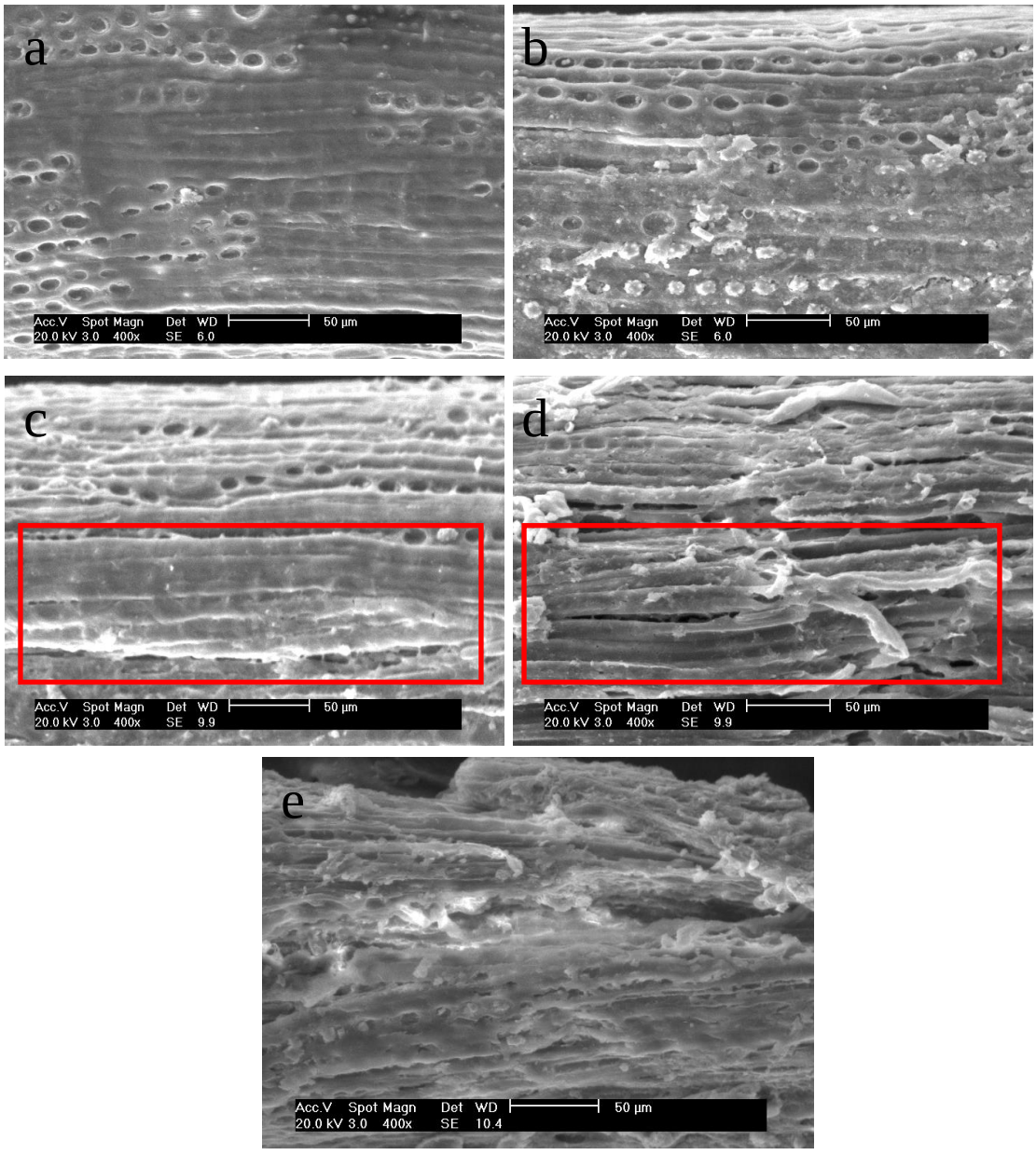

Fig. 2. SEM images of the structure of EFB compost at (a) 0 days, (b) 10 days, (c) 20 days, (d) 30 days, and (e) 40 days. The *box* indicates the microfibril structure of lignocellulose.

In addition, the microfibrillar structure of EFB fibre could be observed, as indicated in the figure. The thermophilic stage continued for 20 days, during which the temperature slightly decreased to 58 ^{o}C. At this stage, the hemicellulose and lignin compositions were around 19% and 30%, respectively. The degradation of lignocellulosic material is affected by the existence of aerobic mesophilic and thermophilic bacteria, which are capable of producing lignocellulose-degrading enzymes (Kala *et al.* 2009; Nakasaki *et al.* 2009). The reduction of the cellulose and hemicellulose components within 20 days of composting was due to the presence of thermophilic bacterial groups capable of degrading lignocellulosic material. The EFB structure in Fig. 2d shows that the microfibril structures were broken after 30 days; it was also observed that after 40 days the structures were strongly disrupted (Fig. 2e). During this stage, the temperature of the compost decreased to around 37 to 39 ^{o}C toward the end of the composting process, which indicates the cooling and curing stage of the compost. Based on the analysis of the compositions of cellulose, hemicelluloses, and lignin in the compost, it was found that no drastic alteration in the composition occurred after 20 days of composting.

PCR-DGGE of EFB Compost

From the results of DGGE (Fig. 3), it can be observed that the difference in banding patterns by DGGE community fingerprinting revealed a drastic change between each stage of composting. A few prominent bands (A, B, and C) were found at the initial stage of composting.

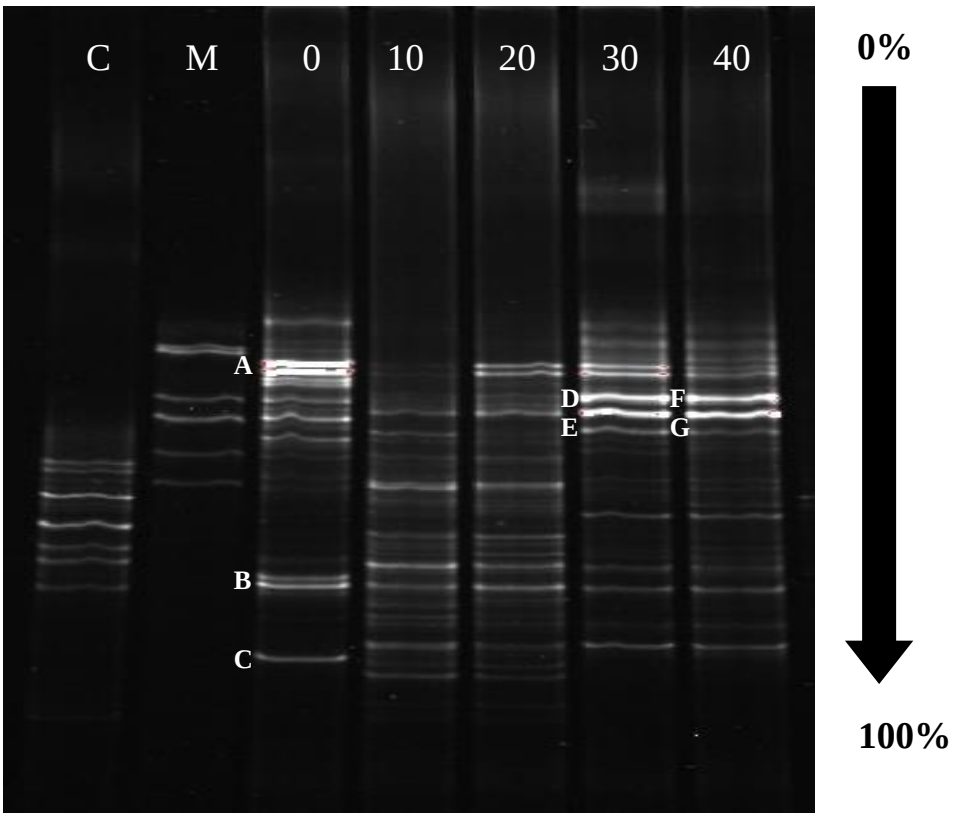

Fig. 3. PCR-DGGE analysis of EFB compost. The arrow indicates the directions of electrophoresis and percentages of DNA denaturant. The numbers correspond to the sampling days. C (Control- DGGE compost) and M (DGGE ladder)

At the thermophilic stage (10 and 20 days), the number of bands increased, while the number decreased as composting reached the cooling and maturing stage (30 and 40 days). Toward the end of the composting process, the most prominent bands (D, E, F, and G) were detected. In this study, DGGE was performed to demonstrate the bacterial community change at different stages of the composting process. The drastic change in the DGGE profile suggests the presence of high and low G+C content in the DNA sequence of bacteria at different stages of composting. The difference in band patterns from the initial stage until the end stage of composting suggests that the bacterial community structure may be important for the changes in temperature and carbon content.

According to Zheng and Wu (2010), the G+C content level of DNA is correlated with the temperature range condition when the analysis is applied to thermophilic and mesophilic organisms. This result correlates with the abundance of bacteria from phylum *Actinobacteria* (high G+C content DNA bacterium) and *Firmicutes* (low G+C content DNA bacterium) at the thermophilic stage (10 days and 20 days) and cooling and curing stage (30 days and 40 days) obtained from the clone library. The change in microbial community may play an important role in the biochemical changes during the composting process.

EFB Compost Clone Library and Bacterial Population

Table 2 shows the number of clones and the analysis coverage of OPEFB compost. The number of clones in the OPEFB compost clone libraries ranged from 96 to 171 clones. The clone sequences were checked for their similarity matrix for grouping purposes. Each group of clones (OTU) was formed based on the similarity matrix of at least two clones. After grouping, it was found that the percent of analysis coverage for 0, 10, 20, 30, and 40 days were 61%, 85%, 71%, 82%, and 73%, respectively.

Although the analyzed clone number of the day 0 sample was the highest when compared to the other samples, the analysis coverage of the day 0 sample was lower than the other samples. The low percentage of analysis coverage suggested that at the initial stage of composting, the samples contained various types of bacteria. When all of the sequences were combined together, the analysis coverage showed a high total percentage of 81.3%.

The total number of single clones decreased from day 0 until day 40 of composting (Table 2). The number of single clone groups at initial composting (day 0) was 66, and the number decreased to 20 to 33 for the rest of the samples. The number of groups after 30 days was similar to that after 40 days, being 31 clones. The day 10 sample showed the lowest number of single clone groups amongst the samples, with only 20 groups.

The 16s rRNA clone library analysis was done to clarify specific features of the bacterial community during composting. The microbial community structure shown in this study was consistent with previous reports that used DGGE (Green *et al.* 2004; Baharuddin *et al*. 2009b; Liew *et al.* 2009). The increase in coverage value after the initial stage of the composting process could be the reason that a diverse bacterial community was present in the early stage of composting and decreased toward the end. The results were reflected in the DGGE patterns, in which many bands could be seen in the first 20 days.

Table 2. Analysis of Microbial Community at 10, 20, 30, and 40 Days

Day of Composting	0	10	20	30	40	Total
1. No. of clones	171	138	115	96	119	606
2. No. of clone groups (≥ 2 clones)	91	43	50	43	43	186
3. No. of single clone groups (one clone)	66	20	33	31	31	113
4. Coverage (%)	61.4	85.5	71.3	82.3	73.9	81.3

Analysis coverage was calculated as follows:

$$(\%) = \left[\frac{\text{1-Number of single clone groups}}{\text{Number of clones}}\right] \text{X } 100$$

Figure 4 shows the change in bacterial population during the composting process.

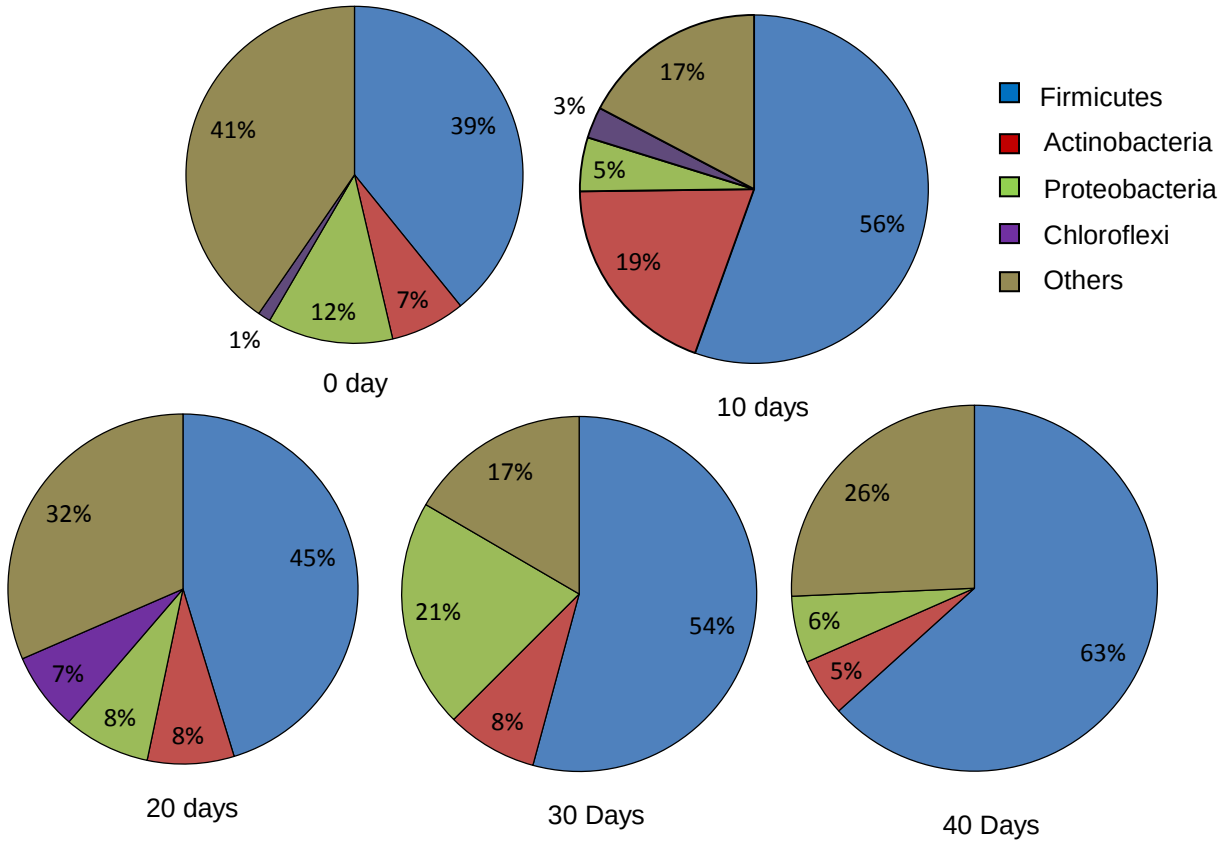

Fig. 4. Population of dominant bacteria at phylum level obtained during the EFB composting process after their similarity matrices were calculated by BioNumerics and classified by the sequence match program of the Ribosomal Database Project II.

From the figure, the phylum *Firmicutes* was found to be the most dominant group throughout the composting process. This bacterium was detected in all of the samples, with a population of about 39%, 56%, 45%, 54%, and 63%, respectively. The second dominant group detected belonged to the phylum Proteobacteria (52%), followed by the phylum *Actinobacteria* (47. Phylum Chloroflexi was found in the day 0 (1%), day 10 (3%), and day 20 (7%) samples. The population of Chloroflexi increased until day 20 and disappeared after 30 days of the composting process. The other groups constituted less than 2% of the population. It was found that the population of the other groups decreased after the composting process started. *Actinobacteria* was found to be the major lignocellulose degrader, whereas Firmicutes mainly decomposed easily degradable organic compounds (Peters *et al.* 2000).

Bacterial Succession during EFB Composting

The analysis of all sequences in the library showed that clones related to the *Bacillus* species were found to be one of the most dominant groups during the composting process. Figure 5 shows the changes in the population of the dominant groups for clones related to the *Bacillus* species. The similarity matrix of the entire clone sequence related to the *Bacillus* species was analyzed, and it was found that three different groups amongst the *Bacillus* species were present throughout the process. During the composting, the *Bacillus* species was found to be the most dominant form of bacteria when the temperature of composting was around 60 ^{o}C. The *Bacillus* species has been widely identified and isolated during the thermophilic stage (50 ^{o}C to 70 ^{o}C) of composting (Dees and Ghiorse 2000; Ohnishi *et al.* 2011; Wang *et al.* 2011; Xiao *et al.* 2011; Charbonneau *et al.* 2012). The dominant group detected in the day 20 sample, representing 26% of the population, was a species related to *Lysinibacillus massiliensis*. *Lysinibacillus massiliensis* is a gram-negative, aerobic, rod shaped, spore-forming bacterium originally isolated from cerebrospinal fluid (Glazunova *et al.* 2006). At this stage, the increase in population of this bacterium indicated the availability of organic material generated from lignocellulose degradation for their growth. According to Hanajima *et al.* (2011), a clone related to this bacterium was found to be accumulated during the active stage of organic carbon decomposition in cow manure slurry. The increase in the population of *L. massiliensis* suggests that the optimum condition for this bacterium to grow is thermophilic, as it was only detected at the end of the thermophilic stage of the composting process.

The second dominant group, representing 21% of the population, was closely related (99% similarity) to *Solibacillus silvestris*. This group was detected at day 0, day 10, day 20, and day 40. From Fig. 5, it can be seen that the population of this bacteria increased after 10 days and decreased toward the end of the composting process. In this study, it was seen that the closely related *S. silvestris* StLB046 clones made an important contribution to the lignocellulose degradation. This bacterium was found to be dominant during the thermophilic stage, at which cellulose decomposition occurred. Recent genomic analysis of *S. silvestris* StLB046 through the carbohydrate active enzyme database (CAZY) showed that it contains genes for cellulose and hemicellulose degradation (Morohoshi *et al.* 2012). *S. silvestris* is a gram-positive, aerobic, rod-shaped, peritrichous flagellation, round endospore-forming bacterium originally isolated from a forest soil (Rheims *et al.* 1999). Another dominant group, which was detected at day 0, day 10, and day 20, belonged to *Bacillus* sp. R-7413 (97% similarity). The population for

this bacterium was slightly reduced after 20 days of composting. It can be seen that the decrease in cellulose composition and increase in temperature correlated with the increase and decrease in the population of this species, especially during the thermophilic stage. Two groups of this species were detected at day 10, and three groups were detected at day 20. It was found that the changes in the lignocellulosic components, especially cellulose at 10 days, mainly occurred when *Bacillus* species were dominant.

Another group of bacteria, which was found to be dominant, were clones related to the *Exiguobacterium* species. Three groups of clones related to this bacterium were observed throughout the composting process (Fig. 5). The dominant group of these clones was related to *Exiguobacterium* sp. ERGBD-1 (99% similarity) and it was detected at day 0, day 10, and day 40 with populations of about 6%, 18%, and 13%, respectively. The populations of this group changed throughout the composting process; the population increased at the early stage of composting, especially up to day 10, and slightly decreased toward the end of the composting process. The *Exiguobacterium* species, which was identified as the second most dominant group, has been reported previously by Guo *et al.* (2007). Based on cellulose composition and temperature profile, an increase in temperature along with a decrease in cellulose indicates that these groups of bacteria might play important roles in the alteration of composting components and conditions. The second dominant group was found to be *Exiguobacterium* sp. MN3 (98% similarity). This group was only detected at day 30. The group that belonged to *Exiguobacterium acetylicum* TSWCSN13 (97% similarity) was detected at day 10 sample with a population of 1%. Two groups of this species were detected at day 10. Similar to the *Bacillus* species, the results show that the cellulose and temperature pattern correlated with the population of this species. The *Exiguobacterium* species was observed to have the ability to produce cellulase and xylanase. In our screening and isolation of bacteria from the compost in this study, we managed to isolate one bacterium that was related to *E. acetylicum* (98% similarity), which has cellulase and xylanase activity on selective agar. Complete genome sequencing of the thermophilic bacterium *Exiguobacterium* sp. AT1b showed that this bacterium was found to contain genes encoding for cellulose- and hemicellulose-degrading enzymes (Vishnivetskaya *et al.* 2011).

Other dominant groups detected were clones related to *Desemzia incerta* and *Planoccoccus* sp. Both of these bacteria were found to be dominant during the cooling and curing stages (30 days and 40 days) of the composting process, with populations of about 26% and 32%, respectively. It was found that the population of these bacteria increased when the temperature decreased to 34 ^{o}C and when there were also slight reductions in carbon and lignocellulose. Towards the end of the composting process (30 days to 40 days), only a slight change in lignocellulose composition and reduction of carbon occurred due to the decreased microbial activity of EFB degradation. Previous studies have reported that cellulose-degrading bacteria decreased during the curing process (Dees and Ghiorse 2000).

A reduction of lignin component was observed during the composting process. It could be suggested that the decrease in lignin content was due the presence of uncultured bacteria which belonged to the detected phylum *Actinobacteria*. Previous studies showed that *Actinobacteria* is able to degrade lignin in a compost environment (Tuomela *et al.* 2000). SEM micrograph observation showed that the EFB structure was almost broken at cooling and curing stage (Fig 2d and 2e). Similar types of structures were also observed in the study done by Baharuddin *et al.* (2009b). The broken structure of EFB at the final

stage of composting suggests that cellulose and hemicellulose components were degraded. At this stage, the bacterial population was dominated by bacteria from the class *Bacilli* under phylum *Firmicutes*, which could utilize carbohydrates such as sugars from the decomposition of lignocellulose during the thermophilic stage. This result correlated with the presence of clones related to *D. incerta* and *Planoccocus* sp. at the cooling and curing stages. *D. incerta* has been isolated from spent mushroom compost and is phylogenetically related to the order *Lactobacillales* (Ntougias *et al.* 2004; Hammes and Hartel 2006). The presence of this bacterium as a dominant group is related to readily used carbohydrates, such as sugars from the decomposition of lignocellulose, which become substrates for this bacterium. Despite the availability of readily degradable organic materials during the cooling and curing stage, a small amount of lignocellulose degradation still occurred at this stage. This is in agreement with the presence of *Planococcus* sp., which was previously isolated and found to have endoglucanase and cellobiase activity, which are important for cellulose and oligosaccharide degradation (Barman *et al.* 2011).

Figure 5 also shows that other groups of bacteria that are known to have the capability to degrade lignocellulose material were detected during the thermophilic stage. Five groups represented these bacteria. Four groups of clones, which were detected at day 10, belonged to *Thermobacillus xylanilyticus* (3%), *Brachybacterium faecium* (7%), *Cellulosimicrobium cellulans* (2.1%), and *Thermobifida fusca* (1.4%). The group that was represented by *Cellulomonas* sp. (2%) was only detected at day 20. The group that belonged to *B. faecium* DSM 4810 was also detected at day 30, constituting only 2% of the population. The presence of this group of bacteria reflected the decreased in cellulose and hemicellulose, particularly after 10 days and 20 days of the composting process. Nakasaki *et al.* (2009) reported that, during composting of sludge containing 14.8% fibers, lignocellulose degradation occurs at the later stages of thermophilic composting, after most of the easily degradable organic materials have been consumed.

The results reported by Nakasaki *et al.* (2009) were opposite to those from this study, whereby the initial EFB compost consisted of more than 90% of the lignocellulosic component (Fig. 1c). To obtain carbon sources for their growth, these bacteria can first degrade the complex structure of lignocellulose. The hole-like structures on EFB, as shown in the SEM photograph, indicate that the silica bodies have been removed. Baharuddin *et al*. (2009b) reported that some silica body removal was observed on the outer surface of pressed-shredded EFB. The removal of silica bodies, which provide the space for the bacteria or enzymes to penetrate the EFB structure, may explain the potential of these bacteria to degrade lignocellulose. At this stage, five groups of clones related to species that are well known for their ability to degrade lignocellulose were obtained from the library.

One of the species detected related to *T. fusca* is a moderately thermophilic soil bacterium that belongs to the phylum *Actinobacteria*. This bacterium was previously isolated from heated organic materials such as composted horse manure, sewage sludge compost, or mushroom growth medium (Bachmann and McCarthy 1991; Béki *et al.* 2003). Genome analysis of this strain revealed that it has a glycosidizing module identified as cellulase and xylanase (Lydkis *et al.* 2007). The phylogenetic position showed that clones from the group of uncultured bacteria (2-017, 3-037 and 4-107) had the same lineage as uncultured bacteria that belong to phylum *Actinobacteria*. The other four groups of clones, which belong to *T. xylanticus*, *C. cellulans*, *Cellulomonas* sp., and

B. faecium, were also detected. *T. xylanticus*, an aerobic moderately thermophilic bacterium, has been isolated from farm soil situated underneath a manure heap (Touzel *et al.* 2000). *C. cellulans* has been isolated from food waste, garden waste, and continuous thermophilic composting (Ryckeboer *et al.* 2003; Xiao *et al.* 2011).

Cellulomonas terrae, a novel species of cellulolytic and xylanolytic bacterium, has been isolated from soil (An *et al.* 2005). The CAZY database shows that the *B. faecium* DSM 4810 genome includes the gene encodings for cellulose- and hemicellulose-degrading enzymes (http://www.cazy.org/). The availability of these bacteria during the composting process is believed to support lignocellulose decomposition, especially at the thermophilic stage.

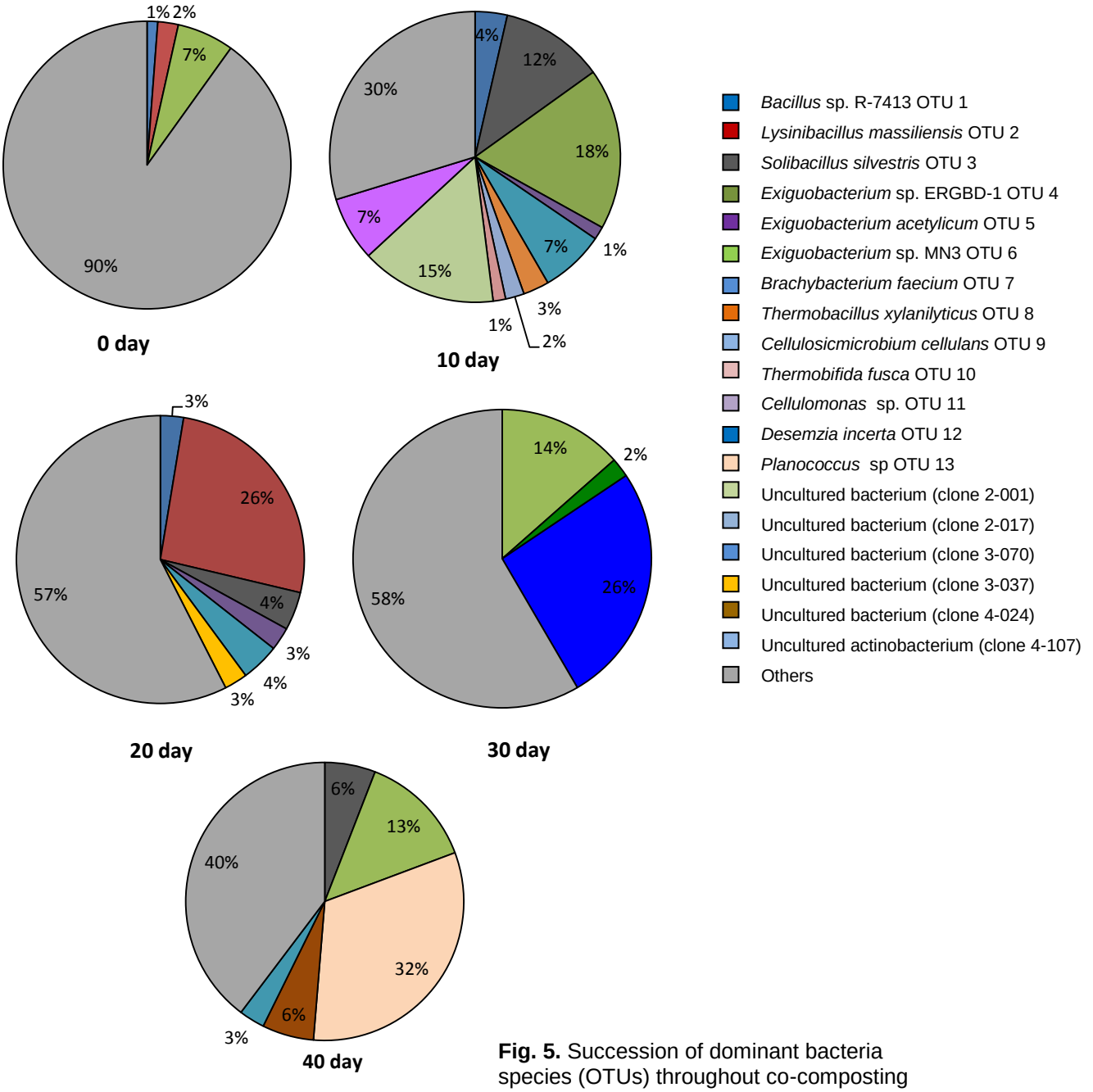

Fig. 5. Succession of dominant bacteria species (OTUs) throughout co-composting process

Two groups of uncultured bacteria were detected at day 10, day 20, and day 30 of composting. The phylogenetic tree of the clones that belong to the uncultured bacteria show that the clone represented by 2-001 shares a lineage with *S. silvestris* (Fig. 6). However, the clone represented by 3-070 and 4-024 formed a lineage distinct from other *Bacillus* species and uncultured bacterial clones. The uncultured bacterial clones represented by 2-017, 3-037, and 4-107 shared a lineage with uncultured *Actinobacterium*, an uncultured *Cellulomonas* sp. clone, and uncultured *Actinobacterium*, respectively. The sequence match by RDPII indicates that the percent similarity of these bacteria was less than 89%; they are thus believed to be novel bacteria and could have potentially been lignocellulose degraders during the composting process.

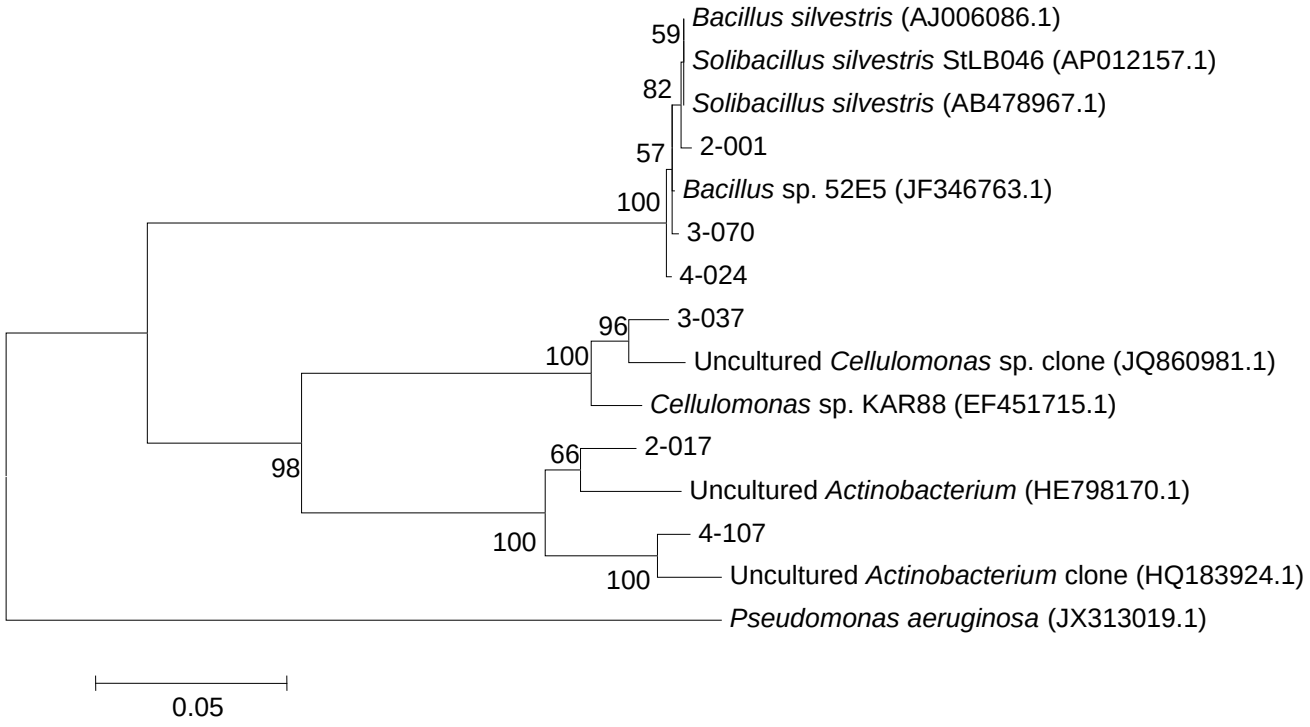

Fig. 6. Phylogenetic tree of the dominant uncultured bacteria in EFB compost presented in Fig. 4. The neighbor-joining method was used, and the sequence of *Pseudomonas aeruginosa* was used as the outgroup taxon. The scale bar represents 0.05 changes per nucleotide position.

CONCLUSIONS

1. The presence of *Solibacillus silvestris* and *Exiguobacterium* sp. as dominant groups of bacteria throughout the composting process showed major impacts in the change of composting conditions, especially in the lignocellulosic component.
2. *Thermobacillus xylanilyticus*, *Brachybacterium faecium*, *Cellulosimicrobium cellulans Cellulomonas* sp., and *Thermobifida fusca*, which have previously known to involve in the lignocellulosic degradation, were predominantly detected during the thermo-philic stage. The changes in physiochemical properties, particularly in lignocellulose composition and the structure of EFB compost, throughout the

composting process were mainly due to the actions of these bacteria, which are supported by the continuous supply of nutrients from the anaerobic POME sludge.

3. The results of this study suggest that the continuous addition of POME sludge and the change in bacterial community are important factors contributing to the production of higher quality compost.

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