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MICROBIAL SUCCESSION IN CO-COMPOSTING OF CHIPPED-GROUND OIL PALM FROND AND PALM OIL MILL EFFLUENT

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ABSTRACT

Succession and phylogenetic profile of microbial communities during co-composting of chipped-ground oil palm frond (CG-OPF) and palm oil mill effluent (POME) were studied by applying polymerase chain reaction-denaturant gel gradient electrophoresis (PCR-DGGE) analysis. The results indicated that the dominant microbial community detected was γ-Probacteria *such as* Pseudomonas *sp. at almost throughout the composting process. Whilst* Bacillales *such as* Bacillus psychrodurans *were found toward the end of the composting process.* Bacteroidetes *such as* Pedobacter solani *were detected at the final stage of composting. This study contributed to a better understanding of microbial shifting and functioning throughout CG-OPF composting. Therefore, PCR-DGGE is recommended to be used as a tool to identify potential microbes that can contribute to a better performance of composting process.*

Keywords: chipped-ground oil palm frond (CG-OPF), palm oil mill effluent (POME), composting, PCR-DGGE, *Pseudomonas* species.

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INTRODUCTION

In recent decades, wastes generated from oil palm industries have increased tremendously (Lim *et al*., 2009). The wastes generated could contribute and led to environmental pollution. The solid wastes generated in the mill are typically used as the burning fuel for electricity generation. However, liquid wastes are partially treated in the ponding system before being discharged to the river (Klammer *et al*., 2008). The total production of oil palm frond (OPF) as a by-product during oil palm pruning and harvesting is recorded about 45.49 t per year (MPOB, 2011). Based on a report by Astimar and Basri (2006), Najib *et al*. (2014) and MPOB (2011), the OPF dry matters during oil palm replanting and pruning were estimated at about 14.47 t ha^{-1} and 10.40 t ha^{-1}, respectively. In the current practice, OPF is not utilised in composting, but instead being left to rot in the plantations to provide some nutrients and organic matter.

Composting is an aerobic and self-heating process by microbial organic degradation and stabilisation. Szekely *et al*. (2009) interpreted composting as the microbial succession which continuously adapting to different nutrient changes and environmental condition. Composting process typically being dominated initially by mesophilic microorganisms which utilise degraded materials easily. However, intense metabolic activity has contributed to the heat generation that led to rapid temperature increment. This phenomenon has led to thermophilic microorganisms taking over the declining mesophilic community. Baffi *et al*. (2006)

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reported that lignin, being a complex and stable materials, can be degraded in the thermophilic phase. On the other hand, new mesophilic community is formed to substitute thermophilic community in the maturation phase.

One of the composting benefits is to change organic wastes into beneficial products for better plant growth (Baharuddin *et al*., 2009a). The main target of composting is to produce a worth compost that consists of high nutrients, as fertiliser as well as efficient soil conditioner (Baharuddin *et al*., 2009a). Composting of empty fruit bunches (EFB), mesocarp fibres and organic wastes, have been well documented by Baharuddin *et al*. (2009a) and Lim *et al*. (2009). Unfortunately, little information on physico-chemical characteristics during full complete co-composting process of chipped-ground oil palm frond (CG-OPF) and palm oil mill effluent (POME) was published, especially in pilot scale operation. Since ample OPF are being produced during replanting and harvesting, it is important to find the best way for the disposal of OPF, instead of leaving them to rot in the plantations. In this study, composting methods have been suggested to convert OPF to worthy product by addition of POME for better performance of compost. Hence, the aim of this study is to evaluate and identify the microbial succession during co-composting of CG-OPF and POME.

MATERIALS AND METHODS

Pilot Scale Composting Site

The co-composting of CG-OPF and POME was conducted in windrow semi-open system, inside a brick blocks to ensure better mixing, aeration and maintain the generated heat during composting process. The CG-OPF was produced through chipping and grinding processes using chipper machine and hammer mill machine, respectively, which were located at the Biomass Pilot Plant, Agro Product Unit, Malaysian Palm Oil Board (MPOB)-Universiti Kebangsaan Malaysia (UKM). The sieve size of 0.5 cm was used to produce small particle size of the CG-OPF. The composting material was located inside a brick blocks (2.1 m length, 1.5 m width and 1.5 m height) (Baharuddin *et al*., 2009a; Lim *et al*., 2009; Najib *et al*., 2011; 2014). The experimental work was carried out under shade and inside the brick blocks on cement base at composting site of the Department of Bioprocess Technology, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Composting Treatment and Sampling

A total of 1 t CG-OPF was loaded using backhoe into the brick block. POME, 50 litres, containing naturally occurring beneficial microorganisms for co-composting was sprayed using a centrifugal pump to the composting pile at every three-day interval. This is to maintain the optimum moisture content for good aeration for bioactivity of the microorganisms within the range of 55% to 65% (Baharuddin *et al*., 2009a; Lim *et al*., 2009; Najib *et al*., 2011; 2014). After adding POME, the composting material was turned manually for mixing in order to aerate the system, to maintain the optimum moisture content as well as to control the generated heat produced during composting. During the curing stage, which was about a week before harvesting, addition of POME was stopped and the material was turned frequently in order to obtain a final compost product with a good texture and size. Throughout the composting period, the composting material was covered at the middle of the heap using 1 m × 1 m hard plastic to avoid drastic fluctuation in humidity and temperature during the process. The composting cycle was completed within 40-60 days, depending on the maturity of the composting material based on C/N ratio.

A total of 1 kg sample was collected every three days at different depths and points in the piles (surface and core). The samples were then divided into two parts. One part was stored at 4°C while the other part was stored at -20°C until further analysis. All analysis was done in triplicates.

DNA Extraction

The extraction of microbial DNA from compost samples stored at 4°C, was done with the Soil Master DNA Extraction Kit (Epicentre Technologies, USA) adopted from a cell disruption protocol by Yeates *et al*. (1998). About 10 ml extraction buffer which contains 100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA pH 8.0 and 1.5 M NaCl was mixed with 2 g wet basis compost sample as proposed by Najib *et al*. (2011). A cell lysis was conducted through vigorous vortex mixing for 2 min by using 0.5 g of 2 mm glass bead (APS Finechem, Australia). Finally, by using DNA purification kit, Qiaquick Gel Extraction Kit (Qiagen Inc, Valencia), the purification of the extracted DNA samples was carried out.

Polymerase Chain Reaction

The polymerase chain reactions (PCR) were conducted according to Muyzer *et al*. (1993) and Najib *et al*. (2011). DNA samples were diluted with sterilise ultra-pure water to minimise the inhibition effects of co-extracted contaminants before PCR reactions. In order to perform PCR amplifications, 50 µl volume consisting of 1 µl template DNA, 25 µl Ex Taq DNA polymerase (Takara Shuzo, Japan), 20 µl ultra-pure water (Millipore, USA) and 2 µl of each primer (First Base Laboratory, Malaysia) was

prepared. With the primer set of forward primer (341f) with a 40 bp GC-rich clamp, 5'-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG* -3' and reverse primer (518r), 5'-*ATT ACC GCG GCT GCT GG*-3' the amplification of 16S rDNA was performed (Najib *et al*., 2011). A total volume of 25 μl of PCR mixture containing 10 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate (Vivantis, Finland), 2.5 μl of 10X PCR buffer (Vivantis, Finland) containing 100 mM Tris-HCl, 15X mM MgCl, 500 mM KCl; pH 8.3, 1.25 U of Taq DNA polymerase (Vivantis, Finland) and 5 μl of DNA template and made up to 25 ml with sterile water was used during PCR amplifications. The PCR Thermal Cycler (MasterEP Gradient, Eppendorf, Germany) was used for PCR cycling process which were operated with cycle conditions of 94°C for 3 min. Later, it was run in 30 cycles of 52°C for 1 min, 72°C for 1 min, 94°C for 1 min. Thereafter, a setting 52°C for 1 min with final additional steps at 72°C for 10 min was run (Najib *et al*., 2011).

Denaturant Gradient Gel Electrophoresis

Denaturant gradient gel electrophoresis (DGGE) was performed with the DCodeTM system (BioRad Laboratories, USA) according to Muyzer *et al*. (1993) protocol. About 1.0 mM of 6% (w/v) polyacrilamide (37.5:1; acrilamide: bisacrilamide) (BioRad Laboratories, USA) with a denaturing gradient of 30% to 70% was prepared and used to detach 16S rDNA PCR product where 100% denaturant conform to 7 M urea and 40% (v/v) deionised formamide. Each specific lane was loaded with 15 μl PCR products and DGGE was set and ran at 60°C and 200V with 1X TAE buffer (BioRad Laboratories, USA) for 5 hr. The SYBR^R Green nucleic Acid Gel Stain (Invitrogen, USA) was used to discolour the gel for about 30 min, followed by water cleaning and snapped on a UV transillumination table (Labnet, USA) (Najib *et al*., 2011). The gel bands were then excised by using Pasteur pipettes and eluted in 50 μl TE buffer for overnight. The DNA fragments were washed and re-amplified by using the same primer. The single replicate PCR product was then purified by using Qiaquick PCR Purification kit, Qiaquick Gel Extraction Kit (Qiagen Inc, Valencia) before sequencing.

Sequencing and Band Characterisation

Sequencing of the PCR products was conducted to search for sequence similarity by using a BLAST (Basic Local Alignment Search Tool) network service of the Gen-Bank Database (http://www.ncbi.nlm.nih.gov/) (Najib *et al., 2011).* The sequences were aligned by using BioEdit and MEGA4 to refer sequences downloaded from Gen-Bank.

RESULTS AND DISCUSSION

DGGE Analysis using 16S rDNA Universal Primers

In this baseline study, co-composting of CG-OPF and POME at 1.0 t scale was analysed in terms of biochemical aspects and bacterial populations evaluated by DGGE analysis. The results of DGGE analysis in *Figure 1* showed that the position of most bands did not change significantly (based on observation on DGGE banding patterns) as the age of composting proceeded. Whereas, the intensities of several bands changed significantly, especially during 40 to 60 day of composting (DOC). Overall, it is suggested that the bacterial communities did not change significantly throughout 60 DOC. Details of analysis of each sample showing the different bacterial species in each community (*Table 1*). Different environmental condition and substrate characteristics had contributed to the colonisation of certain dominan microbes throughout composting process.

The results obtained in this study indicated that the major members were mainly uncultured and unidentified bacteria. The same finding was also reported by Baharuddin *et al*. (2009b). In this study, the major member was *Pseudomonas* sp. with phylogeny of γ-*Proteobacteria*. The recovered sequences were mainly from seven phylogenetic groupings: γ-*Proteobacteria* (28 sequences),

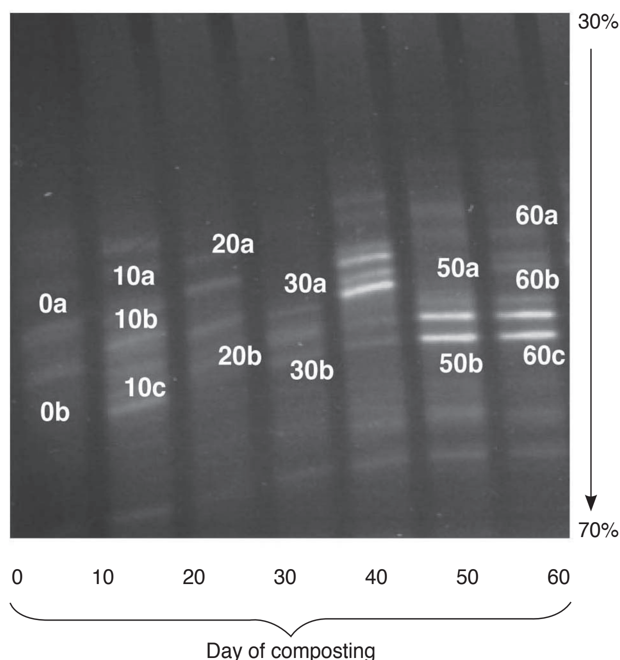

Figure 1. Comparison of the denaturant gradient gel electrophoresis (DGGE) banding patterns of microbial communities of the compost samples from composting of chipped-ground oil palm frond (CG-OPF) at various composting time. The arrow on the right indicates the gradient of DNA denaturants.

TABLE 1. PHYLOGENETIC AFFILIATION OF EXCISED DENATURANT GRADIENT GEL ELECTROPHORESIS (DGGE) BANDS

Band	Nearest relative (accession)	Similarity (%)	Source	Phylogeny
0a 1	*Pseudomonas libanensis* strain BF1 16S ribosomal RNA gene, partial sequence	98	Compost	γ-*Proteobacteria*
0a 2	Soil bacterium Trim-S2N- M1LLLSSL-2 16S ribosomal RNA gene, partial sequence	98	Compost	Bacteria
0a 3	Bacterium 9-gw1-10 16S ribosomal RNA gene, partial sequence	98	Compost	Bacteria
0b 1	*Pseudomonas fluorescens* strain LMG 14675 16S ribosomal RNA gene	96	Compost	γ-*Proteobacteria*
0b 2	*Gamma proteobacterium* PB3 16S ribosomal RNA gene, partial sequence	96	Compost	γ-*Proteobacteria*
0b 3	Soil bacterium Mafe-S1N-M1LLLSSL-1 16S ribosomal RNA gene, partial sequence	96	Compost	Bacteria
10a 1	*Paenibacillus* sp. JAM-FM32 gene for 16S rRNA, partial sequence	98	Compost	*Bacillales*
10a 2	*Paenibacillus glucanolyticus* strain FR1_105 16S ribosomal RNA gene, partial sequence	98	Compost	*Bacillales*
10a 3	Uncultured *firmicute* clone R61 -03-00r43 small subunit ribosomal RNA gene, partial sequence	98	Compost	Bacteria
10b 1	*Alishewanella* sp. Tabriz4 16S ribosomal RNA gene, partial sequence	92	Compost	γ-*Proteobacteria*
10b 2	Bacterium ZY-2006c 16S ribosomal RNA gene, partial sequence	92	Compost	Bacteria
10b 3	Endosymbiont of *Acanthamoeba* sp. AC309 16S ribosomal RNA gene, partial sequence	92	Compost	Bacteria
10c 1	*Rheinheimera* sp. R942 16S small subunit ribosomal RNA gene, partial sequence	95	Compost	γ-*Proteobacteria*
10c 2	*Rheinheimera aquimaris* strain JS-47 16S ribosomal RNA gene, partial sequence	94	Compost	γ-*Proteobacteria*
10c 3	*Chromatiaceae* bacterium D10-48 partial 16S rRNA gene, isolate D10	94	Compost	γ-*Proteobacteria*
10c 1 (rep)	Uncultured bacterium partial 16S rRNA gene, clone X25	96	Compost	Bacteria
10c 2 (rep)	*Rheinheimera* sp. R923 16S small subunit ribosomal RNA gene, partial sequence	95	Compost	γ-*Proteobacteria*
10c 3 (rep)	*Alishewanella* sp. 620 16S ribosomal RNA gene, partial sequence	94	Compost	γ-*Proteobacteria*
20a 1	*Pseudomonas* sp. G32 gene for 16S rRNA, partial sequence	99	Compost	γ-*Proteobacteria*
20a 2	*Pseudomonas marginalis* strain EII-2 16S ribosomal RNA gene, partial sequence	99	Compost	γ-*Proteobacteria*
20a 3	*Pseudomonas aurantiaca* strain PB26 16S ribosomal RNA gene	99	Compost	γ-*Proteobacteria*
20b 1	*Gamma proteobacterium* ML-173 16S ribosomal RNA gene, partial sequence	96	Compost	γ-*Proteobacteria*
20b 2	Bacterium enrichment culture clone HS_5 16S ribosomal RNA gene, partial sequence	96	Compost	Bacteria
20b 3	*Bradyrhizobium* sp. Gc148 16S ribosomal RNA gene, partial sequence	96	Compost	α-*Proteobacteria*
30a 1	*Pseudomonas* sp. MIXRI75 16S ribosomal RNA gene, partial sequence	99	Compost	γ-*Proteobacteria*
30a 2	*Pseudomonas* sp. B2-67 partial 16S rRNA gene, strain B2-67	99	Compost	γ-*Proteobacteria*
30a 3	*Pseudomonas anguilliseptica* partial 16S rRNA gene, strain KB37	98	Compost	γ-*Proteobacteria*
30b 1	*Cryobacterium* sp. g20 gene for 16S rRNA, partial sequence, strain: g20	76	Compost	*Actinobacteridae*
30b 2	*Cryobacterium* sp. 5003 16S ribosomal RNA gene, partial sequence	76	Compost	*Actinobacteridae*
30b 3	*Hymenobacter* sp. 1009 16S ribosomal RNA gene, partial sequence	76	Compost	Bacteroidetes
50a 1	*Rheinheimera* sp. KIN89 16S ribosomal RNA gene, partial sequence	98	Compost	γ-*Proteobacteria*

TABLE 1. PHYLOGENETIC AFFILIATION OF EXCISED DENATURANT GRADIENT GEL ELECTROPHORESIS (DGGE) BANDS (continued)

Band	Nearest relative (accession)	Similarity (%)	Source	Phylogeny
50a 2	*Gamma proteobacterium* QLW -Oulin5D partial 16S rRNA gene, isolate QLW-Oulin5D	98	Compost	γ-*Proteobacteria*
50a 3	Uncultured *delta proteobacterium* partial 16S rRNA gene, clone GW_1	96	Compost	δ-*Proteobacteria*
50b 1	*Rheinheimera* sp. CF12-10 16S ribosomal RNA gene, partial sequence	98	Compost	γ-*Proteobacteria*
50b 2	*Rheinheimera* sp. E407-8 16S ribosomal RNA gene, partial sequence	98	Compost	γ-*Proteobacteria*
50b 3	Uncultured bacterium G3Clone38 16S ribosomal RNA gene	98	Compost	Bacteria
60a 1	Uncultured bacterium clone 16S ribosomal RNA gene, partial sequence	100	Compost	Bacteria
60a 2	Uncultured *alpha* proteobacterium clone TFS-11 16S ribosomal RNA gene, partial sequence	100	Compost	α-*Proteobacteria*
60a 3	Uncultured *Pseudomonas* sp. clone L6B-494 16S ribosomal RNA gene	100	Compost	γ-*Proteobacteria*
60b 1	*Gamma proteobacterium* QLW-Oulin 5E partial 16S rRNA gene, isolate QLW-Oulin5E	99	Compost	γ-*Proteobacteria*
60b 2	Uncultured *Chromatiaceae* bacterium clone GC12m-2-16 16S ribosomal RNA gene, partial sequence	98	Compost	γ-*Proteobacteria*
60b 3	Uncultured *Chromatiaceae* bacterium clone GC12m-3-12 16S ribosomal RNA gene, partial sequence	98	Compost	γ-*Proteobacteria*
60c 1	*Rheinheimera* sp. MOLA 75 partial 16S rRNA gene, culture collection MOLA:75	88	Compost	γ-*Proteobacteria*
60c 2	Uncultured bacterium clone Bio1ac08 16S ribosomal RNA gene, partial sequence	88	Compost	Bacteria
60c 3	Uncultured *Thiocapsa* sp. clone DR548SW228 16S ribosomal RNA gene, partial sequence	87	Compost	γ-*Proteobacteria*

Bacteria (11 sequences), *Bacillales* (2 sequences), α-*Proteobacteria* (2 sequences), *Actinobacteridae* (2 sequences), *Bacteroidetes* (1 sequence) and δ-*Proteobacteria* (1 sequence) (*Table 1*). Most of the band hit high similarity which is greater than 90%, although two sequences, *Actinobacteridae* from sample of Day 30, one sequence, *Bacteroidetes* from sample of Day 30, two sequences, γ-*Proteobacteria* and one sequence, Bacteria, each from sample of Day 60, was lesser than 90% similarity.

At the initial stage of composting, after the POME was introduced onto CG-OPF heap, two major groups consisting of γ-*Proteobacteria* and Bacteria were dominant with sequence similarity greater than 95%. In the previous work reported by Baharuddin *et al*. (2009b), three major groups known as *Cyanobacteria*, δ-*Proteobacterium* and *Firmicutes* were traced during initial stage of co-composting of empty fruit bunch (EFB) and POME. This study which used OPF as a compost substrate led to γ-*Proteobacteria* namely *Pseudomonas* sp. present dominantly. According to Lim *et al*. (2009), *Pseudomonas libanensis* from major band number 0a 1 is a Gram-negative, rod-shaped, fluorescent, motile bacterium isolated from natural springs in Lebanon. Based on 16S rDNA analysis, *P. libanensis* has been placed in the *P. fluorescens* group, which is a plant growth promoting bacteria. Besides, major band number 0a 2, 0a 3 and 0b 3 consisted of Bacteria namely soil bacterium was found during initial stage of composting. They act as decomposers that break down organic materials to produce detritus and other breakdown products. When the temperature increased to 50°C at 10-20 DOC other bacterial groups were present especially γ-*Proteobacteria*, Bacteria and *Bacillales*. The *Bacillales* are an order of Gram-positive bacteria, placed within the *Firmicutes*. Although major band number 10c was repeated, the closest and dominant known species for γ-*Proteobacteria* group during

thermophilic phase was *Rheinheimera* sp. (*Figure 2*). At Day 30 to 60 of composting, the major group for known species is γ-*Proteobacteria*. This major species was detected at mesophilic and curing phase during composting process. According to Baharuddin *et al.* (2009b), *proteobacteria* was detected as prominent microbial community in soil. Furthermore, at 30 DOC, the phyla *Actinobacteridae* was detected at sequence similarity as low as 76%. The closest species traced was *Cryobacterium* sp. In addition, the uncultured bacterium with similarity over than 95% was detected at 50 to 60 DOC or during curing phase.

In this study, we can see the stability of microflora which can stand for about 60 DOC. The phylum γ-*Proteobacteria* existed at most of major bands from Day 0 until Day 60 of composting.

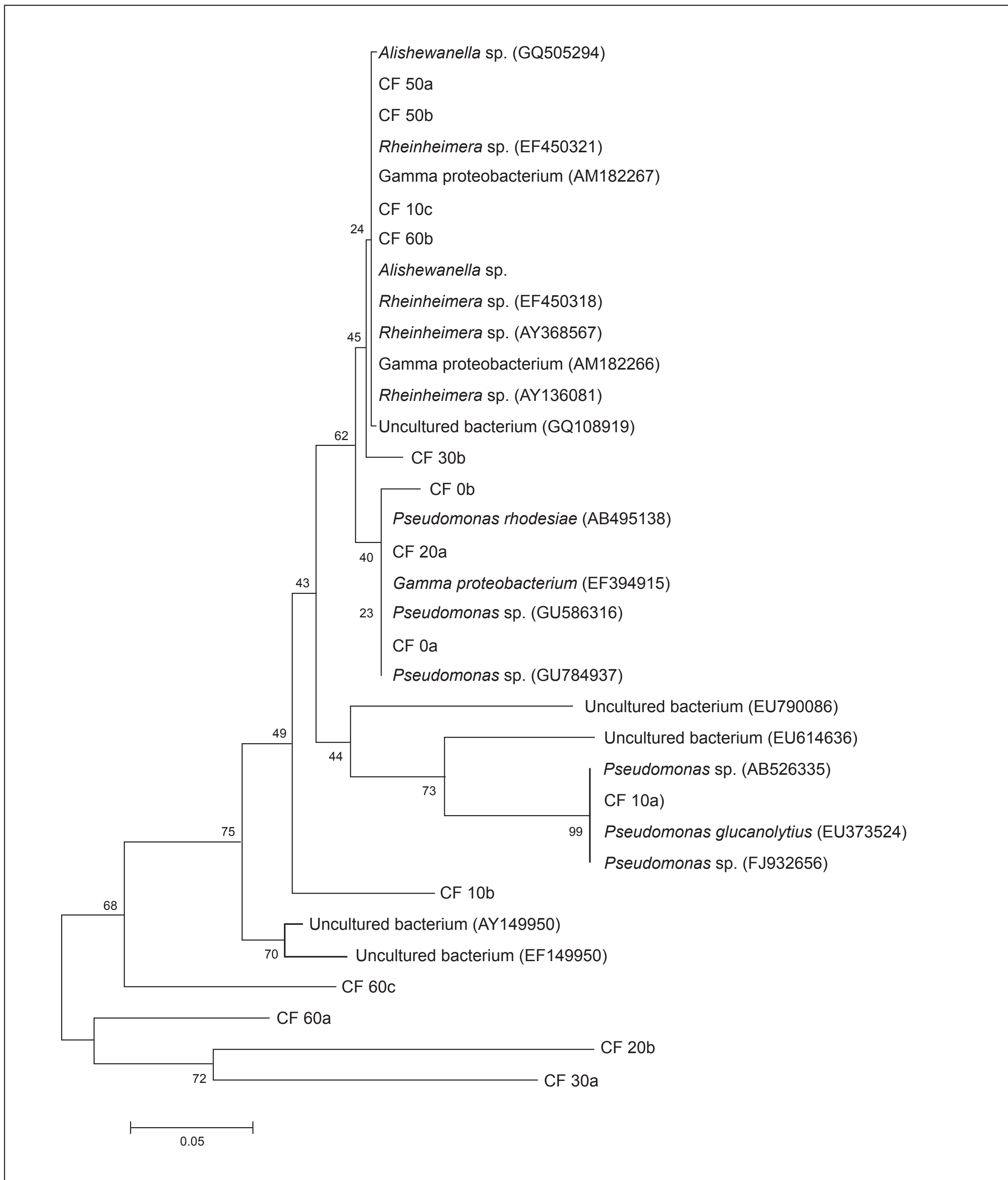

Figure 2. Neighbour-Joining tree representing the phylogenetic relationship of the most abundant 16S rDNA sequences from chipped-ground oil palm frond (CG-OPF) compost samples to various closely related sequences obtained from Basic Local Alignment Search Tool (BLAST) searches.

CONCLUSION

In conclusion, co-composting of CG-OPF and POME had demonstrated that the condition such as temperature, moisture content, pH, C/N ratio were important factor in the development of microbial population in compost. The structural changes of the substrate might also relate to the shift of microbial succession. The PCR-DGGE result indicated that the stability of microbes which could stand for about 60 days of composting. The PCR-DGGE analysis also revealed that phylum γ-*Proteobacteria* existed at most of major band from Day 0 until Day 60 of composting. The results indicated that the dominant microbial community detected was γ-*Probacteria* such as *Pseudomonas* sp. at almost throughout the composting process. Hence, PCR-DGGE is a useful tool to identify potential microbes that can contribute to a better performance of composting process.

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