DYNAMIC CHANGES OF BACTERIAL COMMUNITY DURING THE COMPOSTING OF OIL PALM EMPTY FRUIT BUNCHES (OPEFB) AS ANALYZED BY DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) ANALYSIS

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ABSTRACT

In this study cultivation-independent approach was performed to characterize and understand the bacterial community dynamics during the oil palm empty fruit bunches (OPEFB) composting process. OPEFB was composted in a compost heap of 1m × 1m × 1m. During the process, bacterial community structure in specific composting time were characterized by denaturing gradient gel electrophoresis (DGGE) analysis based on the hypervariable V3 region of 16S rRNA gene. The DGGE banding patterns revealed significant change in the bacterial community structure throughout the composting process. A total of 38 bands were selected for sequencing analysis. Majority of the DGGE bands’ sequences were related to known counterparts with low percentage of identity indicating novel composting bacterial populations. These DNA sequences were related to culturable relatives of Firmicutes, Proteobacteria, Acidobacteria, Bacteroidetes, Fibrobacteres and Chloroflexi. In general, distinctive predomination by Firmicutes followed by succession by Proteobacteria were revealed during the OPEFB composting process. Information attained in this study may be useful for improving the efficiency of OPEFB composting by indigenous bacterial population. The diverse sequences from known bacteria indicated novelty in the composting bacterial populations and potentially their functions.

Key words: Oil palm empty fruit bunches (OPEFB), compost, bacterial communities, denaturing gradient gel electrophoresis (DGGE), 16S rDNA

INTRODUCTION

Oil palm (scientific name, Elaes guineensis Jacq.) is an important commodity crop in tropical countries, including Malaysia and Indonesia. In Malaysia, 97.56 million tons of fresh fruit bunches were processed in year 2015 to extract crude palm oil (Anonymous, 2016). During the process, oil palm empty fruit bunches (OPEFB) that accounted for 23-25% of total fresh fruit bunches (H-Kittikun et al., 2000), is rapidly being generated by the palm oil processing mills at increasing volume by year due to huge increasing demand for crude palm oil.

OPEFB consists of 77.7% of holocellulose, 44.2% of alpha-cellulose, 33.5% of hemicellulose and 20.4% of lignin (Khalid et al., 2008) which is highly resistant to microbial degradation. Successive groups of microbes in each composting stages are believed to play different physico-chemical role(s) in degradation of the OPEFB. The understanding of microbial community at each successive OPEFB composting stage would therefore provide in-depth information useful towards enhancement of composting efficiency and compost quality by revealing the novel microbial structures. Although studies have been carried out extensively to optimize the composting process, there are limited reports on the microbial dynamics during composting of OPEFB (Thambirajah et al., 1995; Baharuddin et al., 2009; Yahya et al., 2010).

While most studies reported on culturable microbial communities, Zainudin et al (2014) reported on
the bacterial diversity of OPEFB compost based on DGGE. However, the research group analysed 7 DGGE bands which did not represent the richness of the community.

The recent advancement of molecular techniques in culture-independent methods including PCR-DGGE enabled more thorough investigation on microbial ecology. In this regard, these advance techniques help to overcome the limitation of culture-dependent approaches which were known to reveal less than 1% of the bacterial communities from the environmental samples (Amann et al., 1995; Davis et al., 2005). PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is one of the cultivation-independent techniques that can provide a higher resolution in determination of predominant microbial community from environments (Muyzer, 1999) and provide a promising method to monitor the microbial community shift during a process. Microbial diversity studies from various sources using DGGE have been reported. These include samples from animal manure compost (Song et al., 2014), crude oil (Liew & Jong, 2008), microbial fuel cell (Jong et al., 2006), rhizosphere soil (Yang et al., 2016), ryegrass silage (Li & Nishino, 2011), agricultural vegetable compost (Adams & Frostick, 2009; Wang et al., 2015), biogas digester (Liu et al., 2009), kitchen waste compost (Wang et al., 2015) and many more.

It is general known that bacterial communities in each composting process greatly differ from one to the other depending on the initial composting material & the composting method (Ishii & Takii, 2003; Haruta et al., 2005; Wang et al., 2015). Although Liew and co-workers (2009) reported the bacterial community in mature OPEFB compost, to the authors’ knowledge, there is no study focusing in an oven at 105°C until constant weight value is obtained.

### Experimental design

The traditional composting process was carried out outdoor in a pyramid-like compost heap of 1m × 1m × 1m (length × width × height). The main composting materials included mixture of raw OPEFB and chicken manure. A total of 1.5 tonnes of raw unshredded raw OPEFB was obtained from the local palm oil processing mills. Topsoil and urea were added as additives. The compost heap consisted of repeated layers of feedstock (OPEFB, chicken litter, topsoil and urea). During the composting, water was added when needed to maintain the moisture content of compost heap. The compost heap was turned using a track loader during the process to achieve homogeneity and provide enough aeration. Sampling was performed at day 0, 2, 4, 7, 10, 14, 18, 23, 30, 37, 44, 51, 59, 66, 81 and 137. Approximately 50 g of samples from the core (0.5 m depth) of the compost heap were collected in three replicates. All samples were processed immediately after sampling.

### Physico-chemical analyses

The temperature of the sampling point was measured using a thermometer with type R thermocouple probe (OAKTON Instruments, USA). The pH value and moisture content were performed according to Takaku and co-workers (2006) with some modifications. To measure the pH value, 5 g of sample was suspended into 50 ml of pure water and mixed by shaking at 200 rpm for 3 hours under room temperature. The pH value of the suspension was determined using a pH meter (Eutech instruments, Singapore). Moisture content was determined by drying 10 g of fresh compost sample in an oven at 105°C until constant weight value is obtained.

### Denaturing gradient gel electrophoresis (DGGE) analysis

Total genomic DNA of all compost samples was extracted using PowerMax™ soil DNA isolation kit (MO BIO laboratories, USA). Amplifications of 16S rDNA fragments were carried out as described previously (Liew et al., 2009) using the universal bacterial primer pair, 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). The PCR products were then visualised on a 1% SeaKem® LE agarose gel (Lonza, USA) using a Syngene gel documentation system (Syngene, UK) after staining with 1 μg/mL ethidium bromide solution. Subsequently, nested PCR was performed to amplify the hypervariable region V3 using the primer 341fGC (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534r (5′-ATTACCGCGCTGCTGCTGG-3′) to generate PCR product (~200bp) containing 40 bp of GC-clamp as described previously (Liew & Jong, 2008).

The GC-clamp PCR products were separated using a DCode universal mutation detection system (Bio-Rad laboratories, USA). PCR products with GC-clamp were examined on a 10% (wt/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) gel with a denaturant ranging from 30% to 60%, where a 100% denaturant is defined as 7 M urea. The GC-clamp PCR products were sequenced for the hypervariable region V3 using the primer 341fGC (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534r (5′-ATTACCGCGCTGCTGCTGG-3′) to generate PCR product (~200bp) containing 40 bp of GC-clamp as described previously (Liew & Jong, 2008).

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and 40% (v/v) formamide. Electrophoresis was carried out in 1 × TAE (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA) for 5 hours at 130 volts, 60°C. After electrophoresis, the gels were stained with ethidium bromide and documented using a Syngene gel documentation system (Syngene, UK).

**Sequencing analysis**

Selected DGGE bands were excised from the gel and re-amplified using primer pair 341f/534r (without GC-clamp). The reamplified PCR products were cloned and sequencing reactions were carried out using DYEnamic ET terminator cycle sequencing kit as suggested by manufacturer (GE Healthcare, UK). Sequencing of the sample was carried out commercially. Partial 16S rDNA sequences were aligned to the non-redundant sequence database at the National Center for Biotechnology Information (NCBI) using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) program. GenBank accession numbers of these bands’ sequences are indicated in Table 1. The sequence data for representative DGGE bands (34) were deposited into GenBank database under the accession numbers of EU725866, EU725869- EU725874, EU725876- EU725878, EU725880- EU725881, EU921820, EU921823, GU586692, GU586694, GU586699- GU586701, GU586709- GU586710, GU586715- GU586717, GU586721- GU586723, GU586725, GU586727- GU586728 and GU586731- GU586734.

**RESULTS**

**Physico-chemical properties’ changes during composting**

The changes of the measured mean temperature, pH and moisture contents for the compost are shown in Figure 1. The compost temperature drastically increased from 39.3°C at Day 0 to 65.8°C at Day 2, which is the highest temperature recorded. The temperature dropped gradually and maintained at approximately 60°C during the thermophilic phase (Day 2-10). During the cooling phase (Day 14-59), the temperature declined slowly and maintained around 45°C. The compost core slowly attained ambient temperature during maturation phase (Day 59-137). The pH increased within the first 2 days from an initial value of 7.7 at Day 0 to approximately 9 at Day 2. The alkaline pH maintained between pH 8.4 to pH 9.0 onwards. On the other hand, the compost’s moisture content fluctuated between 38.6 – 61.7% throughout the composting process. The initial moisture content was about 61% after setting up the compost heap. At the end of composting, the moisture content was around 45%.

**DGGE analysis**

In this study, DGGE bands adopting the same migration rate during gel electrophoresis were decided to represent the same bacterial species. Bacterial community structure dynamic over composting time is observed based on changes in DGGE banding patterns. The decreasing number of bands indicates reduction in the bacterial diversity over the period of composting (Figure 2). In total, 38 DNA bands were excised aseptically from the DGGE gels. DNA sequencing analysis (Table 1) revealed that these bands were distributed among 6 bacterial phyla, specifically *Firmicutes* (18 bands), *Proteobacteria* (sub-division alpha- (3 bands), beta- (6 bands), delta- (1 band), gamma- (3 bands)), *Acidobacteria* (2 bands) *Bacteroidetes* (3 bands), *Fibrobacteres* (1 band) and *Chloroflexi* (1 band). Seven bands were matched to cultivated bacterial sequences at species level with an identity index of >97% and eight bands matched to cultivated bacterial sequences at genus level with index of >95% but < 97%. The remaining 19 bands exhibited percentage of identity less than 95% to their cultivated counterparts. However, all 16 bands show an identity index of ≥97% with their uncultivable relatives in GenBank database, 8 with an identity index of ≥95% but < 97% and 10 bands with percentage of identity less than 95%.

The bacterial diversity at Day 0 and 2 were the most diverse among other sampling days. At Day 0, the initial phase, bands related to *Acidobacteria, Bacteroidetes, Fibrobacteres, Firmicutes, alpha-Proteobacteria* and *beta-Proteobacteria* were detected. During the thermophyllic phase, maximum banding patterns was observed on Day 2 when the temperature of the compost heap peaked (65.8°C) but the number of bands reduced thereafter. DNA sequences of these bands were distributed among five bacterial lineages, including *Bacteroidetes, Chloroflexi, Fibrobacteres, Firmicutes* and *Proteobacteria*. The most intense band appeared on Day 2 was distantly related to *Bacterium Ellin 5299* (accession number EU029132, originating on Day 2 was distantely related to *Bacterium Ellin 5299* (accession number EU029132, originating from soil, 95% sequence similarity).

During Day 10-23 of the cooling phase, most of the dominant/intense bands detected were related to *Firmicutes*. The *Firmicutes* group consisted of Bacillales bacterium (P4), *Bacillus* (P14, P29), *Clostridium* (P5, P33), *Eubacterium* (P28), *Natronarobiaceae* bacterium (P16), *Oceanobacillus* (P7, P19) and *Paenecoccus* (P20). Successive predominance by *Proteobacteria* was observed from Day 37 onwards until Day 66 of maturation phase, including *Burkhillia psedomallei*, (P27), and *Hydrogenophaga* sp. (P15, P17, P17a and P17b) of the *beta-Proteobacteria*. The bands recovered during the maturing phase were
Table 1. BLAST results of DGGE band sequences from OPEFB compost

<table>
<thead>
<tr>
<th>Band</th>
<th>Accession no</th>
<th>Closest relative (accession no)</th>
<th>Culturable Id (%)</th>
<th>Source</th>
<th>Affiliation</th>
<th>Closest relative (accession no)</th>
<th>Unculturable Id (%)</th>
<th>Source</th>
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<td>Dried rice straw</td>
<td>Bacteroidetes</td>
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*Id (%): % Identity.
Fig. 1. Temperature, moisture content and pH value profiles of the compost heap during the composting process. Type of parameters: (●) Temperature; (□) Moisture content; (△) pH.

Fig. 2. DGGE profiles of bacterial communities during the composting of OPEFB. The black arrow (right) indicates the linear denaturant gradient. The numbers indicate the position of the excised DGGE bands.
related to *Firmicutes* (*Eubacterium* sp.), similar migration distance with P28, *delta-Proteobacteria* (*Desulfitobacteriumacetoxidans*, P34); *gamma-Proteobacteria* (*Chromatium* sp., P9; *Steroidobacter* sp., P24) and *Bacteroidetes* (*Flavobacterium* sp., P22, *Chitinophagaceae* bacterium, P1; *Parapedobacter korensis*, P2).

**DISCUSSION**

Based on the DGGE bands analysis, the bands with sequences related to *Firmicutes* and *Proteobacteria* are two most abundant phyla throughout the composting process. The abundant DGGE bands observed on Day 0 of OPEFB composing suggesting a highly diverse bacterial community structure was expected (Figure 2). Most of these bands showed similarity to sequences originating from soil, rhizosphere and other environmental sources (Table 1). This is in agreement with the outdoor setup for the composting heap and the composting inputs including OPEFB and chicken manure. A DGGE band P8 was affiliated to an uncultured bacterium clone CS2_153 with 95% identity. The uncultured counterpart was previously documented in similar OPEFB compost at the same composting heap. Similar finding was also reported by Schloss et al. (2009). Besides, DNA sequence of P8 was also distantly related to a previously uncultured soil bacterial isolates, *Bacterium Ellin6067* (AY234719) from *beta-Proteobacteria* with 93% identity. The bacteria was originating from an activated sludge system.

However, most bands gradually disappeared or reduced in bands’ intensities and similar findings were also reported by Song et al (2014) during the composting of chicken and pig manure. Based on the DGGE bands *Firmicutes* emerged as the predominance bacterial populations from Day 2 until Day 10. The disappearance or reduced predominance as described by the less complicating banding patterns and lower DGGE bands’ intensities of most bacterial members suggested that the high temperature may have killed or reduced most of the initial bacterial species and subsequently contributed to the decline in microbial biomass. Similar finding was also reported by Schloss et al (2003).

Predomination by *Firmicutes* persisted from Day 10 onwards until Day 23 while composting started cooling phase. Similar findings were also reported by others (Zainudin et al., 2014; Antunes et al., 2016). The predominant *Firmicutes* populations are represented by bands P4, P5, P7, P14, P16, P19, P25, P29, P31, P31a, P31b, P32 and P33. The composting temperature recorded was ~50°C. The persistence of *Firmicutes* at the high temperature could be due to their specialized abilities to form spore which are able to tolerate higher temperatures. Most of the bands from *Firmicutes* including P18, P25, P29, P31, P31a, P31b and P32 were closely related to sequences that previously reported from environment sources agreeable to the natural presence of *Firmicutes* members (Blanc et al., 1999; Ohno et al., 2000; Pedro et al., 2001; Takaku et al., 2006; Watanabe et al., 2009). Bands distantly related to other *Firmicutes* members specifically *Alkalibacterium* (P32, 91% identity) and *Clostridium* (P33, 91% identity) were also detected in the late thermophilic phase. These bands however showed closer affiliation (≥ 97% sequence similarity) to their uncultured counterparts, uncultured bacterium clone CK8 (GU320655) and uncultured bacterium clone M55_D21_H_B_A07 (EF586054) isolated from organic waste compost and solid waste digester, respectively. Both *Alkalibacterium* (alkaliphile bacterium) and *Clostridium* were anaerobic organisms. This may indicate the anaerobic niche inside the compost heap. Similar findings were reported in other studies (Blanc et al., 1999; Ryckeboer et al., 2003).

*Staphylococcus thermophilus* of phylum *Chloroflexi*, a Gram-positive, non-spore-forming thermophile was the closest cultivable relative to band P23 which appeared only on Day 7 (Figure 2). This bacterium is previously reported in the thermophilic sludge (Hensel et al., 1989). However, its presence in the OPEFB composting was not known.

From the later stage of cooling phase (Day 37 - 59) until compost maturation, the decline in compost heap temperature and the alkaline condition are believed to promote the proliferation of bacterial groups belonging to *beta-Proteobacteria*, *gamma-Proteobacteria* and *delta-Proteobacteria*. The predominant DGGE bands of *Proteobacteria* include P15, P17, P17a, P17b and P27 which were affiliated to *beta-Proteobacteria*, P9 and P24 which were affiliated to *gamma-Proteobacteria* and P34 which was affiliated to *delta-Proteobacteria*. The presence of *gamma-Proteobacteria* (P9, P24) in cooling phase was also reported by several studies (Peters et al., 2000; Ishii & Takii, 2003; Takaku et al., 2006). Beffa et al. (1996) in their study described that water-soluble depolymerised lignocellulose compounds was found in the cooling and maturation phases which promote the growth of a large variety of mesophilic bacterial species. DGGE band P34 which was affiliated to *delta-Proteobacteria* started to increase in band intensity from Day 44 towards compost maturation (Day 137).

Overall, bacterial members related to phyla *Firmicutes* predominated Day 10-23 of the cooling phase, which were then succeeded by predomination by bacterial members of *Proteobacteria* until the
end of the maturation phase. *Firmicutes* re-emerged at Day 51 (bands P20, P28) to co-dominate the compost community together with *Proteobacteria* until end of composting. *Hydrogenophaga* of *Beta-proteobacteria* (bands P17, 17a P17b) was reported as a chemoorganotrophic bacterium which utilizes hydrogen and carbon dioxide as their energy and carbon sources, respectively (Willems et al., 1989). It was one of the hydrogen-oxidizers previously found in maturation phase of a green waste composting (Beffa et al., 1996). Hence, it was suggested that the presence of *Hydrogenophaga* in the compost most possibly play important role in the sulfur- and hydrogen- oxidation activities during the maturation phase of the OPEFB compost. *Bacteroidetes* was present on Day 137 but with low band intensity. This indicated that it is a less abundant group in the maturation phase bacterial community. In the present study, *Flavobacterium* (P22) was found in the compost environment despite the fact that it is a common freshwater inhabitant (McBride et al., 2003). Besides, the bacterium was also reported in the cooling phase of composting by Ryckeboer and co-workers (2003). According to McBride et al (2003), although *Flavobacterium* cannot degrade compost, it can decompose certain types of polysaccharides. This may explained the detection of the bacterium at the final composting stage of maturation.

The indigenous bacterial communities in a composting environment are responsible for the biodegradation of organic materials and they play an important biological factor affecting the composting process. It is generally known that the composting environment changes continuously in terms of physicochemical and microbial diversity due to the interaction between the biotic and abiotic factors within the compost as reported (Haruta et al., 2005; Franke-Whittle et al., 2014). Several reports (Beffa et al., 1996; Ryckeboer et al., 2003; Haruta et al., 2005; Takaku et al., 2006; Kopec et al., 2015) also suggested that biotic factors are presumably the main determinant to the succession of microbial community reside in the compost. In the present study, succession/predomination by distinct microbial groups at different composting phases maybe direct or indirectly influenced by the distinctive physicochemical conditions which favored their proliferation.

**CONCLUSION**

In conclusion, our study provided an insight of the bacterial community shift/dynamic assisted by documentation of the physical parameters including temperature and pH during OPEFB composting. We believed that the knowledge is important for ultimate improvement of the composting efficiency and quality. Future works by pyrosequencing may provide a better understanding of the microbial diversities in the oil palm empty fruit bunches composting process.

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