Periodic addition of anaerobic sludge enhanced the lignocellulosic degradation rate during co-composting of oil palm biomass

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Abstract. The main objective of this work was to investigate the effects of the controlled periodic addition of anaerobic sludge during composting to increase amount of microbial DNA, which appears to be correlated to soluble sugar content which may relate to rate of lignocellulosic degradation. In this study, the composting of pressed-shredded oil palm empty fruit bunch with the periodic addition of palm oil mill effluent anaerobic sludge for moisture control in a newly designed in-vessel type composter was carried out. A control experiment was also conducted over the same period but with the periodic addition of water for moisture control instead of the anaerobic sludge. The lignocellulosic composition and the reducing sugar content were determined via fibre analysis and the spectrophotometric method respectively. The bacterial profile throughout the composting process was quantified by using qPCR. The growth of bacteria reached its peak at 48°C and the degradation of lignocellulose was highest during the thermophilic stage. The highest content of reducing sugar coincided with the highest degradation rate of lignocellulose and the highest DNA copy number during the thermophilic stage. Under the controlled experimental condition of increasing the microbial community, the composting was accelerated to 2.07% OM degradation per day compared to the water addition control at 0.60% OM per day.

Keywords: composting, oil palm empty fruit bunch, quantitative PCR, lignocellulosic fraction, anaerobic sludge

INTRODUCTION

The palm oil industry generates huge quantities of oil palm biomass. Omar *et al.* (2011) reported that the wastes are 14% fibre, 7% shell and 23% oil

palm empty fruit bunch (OPEFB). The palm oil mill effluent (POME) generated from OPFFB is estimated to be about 65% by Chin *et al.* (2013).

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Degradation of the OPEFB which piled up in the mills led to pollution before they are distributed in the plantations for nutrient recycling (Bukhari *et al.*, 2014). It is estimated that 63 million tonnes of POME and 22 million tonnes of OPEFB were generated (MPOB, 2015). Managing this agricultural waste through composting by mixing it with organic anaerobic sludge is a suitable way to allow the waste to be recycled (Guardia *et al.*, 2010; Juan *et al.*, 2013). The high content of lignocellulosic material is currently the main obstacle to achieving a high rate of the degradation process of agricultural waste (Gabhane *et al.*, 2012; Zhong, Bian and Zhang,

2018). Composition of lignocellulosic in OPEFB are mainly cellulose, and lignin (Omar *et al.*, 2011). As shown in Table 1, there had been many studies on modification of the initial waste content such as mixing different waste material in composting, closed and open system of composting, adding additives and determining the types of microbes involved within OPEFB and POME. Quantifying the essential composition of lignocellulosic degradation and microbial succession in relation to the composting stage process parameter could correlate microbial degradation activity and plant biomass composition (Wei *et al.*, 2012; Juan *et al.*, 2013).

Table 1. Microbial quantification and identification techniques in OPEFB composting.

Materials	Highest temperature (°C)	Microbe quantification and identification	C/N final	Period composting	C/N at day 30	Effective volume (m ³)	References
OPEFB + cow dung	>70		18:1	60	-	-	(Thambirajah, Zulkali, and Hashim, 1995)
OPEFB + goat dung	>70	Plating + culturing	14:1	60	-	-	
OPEFB + chicken manure	>70		12:1	60	-	-	
OPEFB + partially treated POME	60.2	Plating + culturing + PCR + DGGE + sequencing	12.8:1	60	24.6:1	90	(Baharuddin <i>et al.</i> , 2009)
OPEFB + POME anaerobic sludge	67	Plating + culturing	12.4:1	40	15.3:1	2.4	(Baharuddin <i>et al.</i> , 2010)
OPEFB + POME anaerobic sludge	70	Plating + culturing	13.9:1	40	14.3:1	10	(Wan Razali <i>et al.</i> , 2012)
OPEFB + POME anaerobic sludge	62	Plating + culturing + PCR + DGGE + sequencing	12.4:1	40	14.6:1	2.4	(Mohd Zainudin <i>et al.</i> , 2014; 2013)
OPEFB + POME anaerobic sludge	48	QPCR + PCR	13.5:1	30	13.5:1	1.2	This study

C/N: carbon to nitrogen ratio; DGGE: denaturing gradient gel electrophoresis.

Juan *et al.* (2013) provided an in-depth analysis on plant biomass composting via the dynamics of organic matter composition during the initial, thermophilic, mesophilic and maturing, and matured composting stages in relation to

microbial biomass carbon. Microbial biomass carbon determination was done by the fumigation-extraction method based on Vance, Brookes, and Jenkinson (1987). Microbial biomass carbon which represents the amount of

microbes was discussed by comparing it with soluble organic carbon and reducing sugars in the compost. They also evaluated the changes of soluble substrate sugars, protein, and non-soluble lignocellulosic compositions in relation to the physical, chemical, and biological parameters studied to observe the degradation mechanism. Evaluation of the soluble organic carbon, microbial biomass carbon, pH and inorganic nitrogen can be of great use in identifying the composting stage. Another study by Wei *et al.* (2012) quantified the microbial shift via quantitative PCR and measured rDNA. The results were correlated among rDNA, transcription of representative functional degradation enzyme genes, enzymatic assay, and imaging data of biomass degradation. However, no degradation of the lignocellulosic profile was reported, with only initial and final lignocellulosic contents provided. Therefore, there is potential for improvement of measuring rDNA and lignocellulosic mass composition interaction by using qPCR and acid digestion technique as a composting measuring parameter.

The addition of waste or sludge during composting been practiced in food waste composting. Food waste will be added continuously into composter with pre-filled bulking agent such as saw dust. The same concept applied to OPEFB composting where sludge will be added continuously with regards to regulate moisture content level. The sludge addition in OPEFB composting have been identified as the main factors that influence organic matter reduction of composting.

As shown in Table 1, several studies on the composting of OPEFB with the addition of POME had been done. Composting with the addition of treated POME to maintain moisture content during the process was reported by Baharuddin *et al.* (2009) to have increased the rate of degradation. OPEFB and POME composting using windrow was carried out to identify the microbial community succession during the composting period by Mohd Zainudin *et al.* (2014). The degradation was due to the presence of hemicellulolytic and cellulolytic strains of bacteria. The clones related to *Bacillus* species most dominantly detected when changes of lignocellulosic occurred. It is proven that degradation in terms of carbon to nitrogen ratio

reduce at a higher rate compared to batch type. However, because of lack of data such as amount of added sludge was not fixed and rely on moisture content as an indicator, it is difficult to conclude that the addition of sludge could function as moisture content regulator or nutrient supply for microbes towards degradation of lignocellulose (Narihiro and Hiraishi, 2005). Furthermore, few studies have been carried out to investigate degradation of lignocellulose during periodicals addition of sludge during composting of OPEFB and previous work have not comprehensively considered tracking degradation composition in organic matter and microbial quantification in composting. Therefore, a substantial study on the composition degradation and microbial growth in composting is essential to provide comprehensive relative importance of degradation mechanism in periodic addition composting. The objectives of this study were to correlate an in-vessel type composting of OPEFB with periodic addition of anaerobic sludge POME in order to develop optimum environmental control of dominant microbial population for lignocellulosic degradation.

MATERIALS AND METHODS

Raw materials. Raw materials of pressed-shredded OPEFB with shredded size of 15 cm to 20 cm were obtained from the Jugra Palm Oil Mill Sdn Bhd. Selangor, Malaysia. The thickened POME anaerobic sludge was from the FELDA Palm Oil Mill, Besouk, Perak, Malaysia.

Composting apparatus. An in-vessel type of composter (Figure 1) made of high density polyethylene was constructed for this study. The composter had a total volume of 2700 L, 151 cm height and 156 cm diameter. The apparatus consisted of an agitator with high torque and low speed electrical motor, flow meter, a compressor, pump for sludge intake, and leachate recycling. The vertical agitator and compressor were operated intermittently for agitation and aeration of the compost material. The aeration flow rate was maintained at a set point of 0.27 m³/hr. This was provided for by using a compressor with a humidifier which contained water in the same

pipeline. Agitation was done for 15 minutes per agitation, four times per day. Aeration was kept at a minimum of 10% oxygen (Vargas-García *et al.*, 2010). The temperature and oxygen level were recorded using a compost temperature and oxygen sensor STAN BURRAGE (UK) Oxygen and Temperature Sensor Model CP11.

Procedure and analysis. Initially pressed shredded OPEFB were fed into the compost with the agitator on. POME anaerobic sludge was pumped from the sludge tank into the composter gradually and evenly distributed on the top of the composter tank. The amounts of OPEFB and POME used were 130 kg each being equivalent to (1:1) ratio wet basis for all the experiments of the study and 1200 L of working volume. The moisture content (MC) was maintained by adding sludge periodically at 3 days as established by (Mohd Zainudin *et al.*, 2013) until a week before the 30th day of the composting experiment coded

as RUN 1 and was done in triplicate. For the control experiment, water was added periodically to the compost with mass ratio as in RUN 1 and coded as RUN 2. RUN 3 had the same initial compost mass ratio but without the periodical addition of either water or sludge for the entire experiment. MC was determined by drying the samples at 105°C for 24 hours. The total carbon content (TOC) was calculated based on the equation from organic matter (Haug, 1993). Organic matter was determined by loss on ignition at 550°C for 4 hours using a muffle furnace (KSL-1700X, MTI Corporation, USA). Nitrogen was determined according to the Organic Elemental Analyzer instrument manufacturer's manual. Reducing sugar was analysed in an extract of 10 g compost in 40 ml 0.5 M K₂SO₄ shaken at 200 rpm for 30 minutes and filtered through a filter paper stated by Juan *et al.* (2013) and determined using dinitrosalicylic acid (DNS) as stated by Miller (1959).

Figure 1. Schematic diagram of composter.

Lignocellulose and other compost composition. Acid Detergent Fibre (ADF), Acid Detergent Lignin (ADL) and neutral detergent fibre (NDF) were used to determine hemicellulose, cellulose, and lignin contents respectively. The method was performed as described by Goering and Van Soest (1970), and the percentage of each component was calculated according to Omar *et al.* (2011). Degradation ratio was calculated according to Wang *et al.* (2011).

DNA extraction from compost samples. Samples of large fibres were mixed with sterile distilled water and cut into small pieces (1 to 2 mm) with sterilized scissors. Ten grams 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.

Polymerase chain reaction, cloning, and sequencing. The purified DNA was amplified using primer pair 27F (5'-AGAGTTTGATC CTGGCTCAG-3') and 1492R (5'-GGTTACC TTGTTACGACTT-3') and the PCR was carried out according to Mohd Zainudin *et al.* (2013). The PCR mixture contained 4.5 µl, nuclease free water, 12.5 µl 2X PCR buffer, 5 µl of 2 mM dNTPs, 0.75 µl each forward and reverse primer of 10 µM, 1 µl DNA template and 0.5 µl thermostable polymerase (1.0 U/µl). PCR amplification was run using the following cycling conditions: 2 minutes at 94°C for 1 cycle; 25 cycles for each cycle of 10 seconds at 98°C, 30 seconds at 52°C; followed by extension for 1 minute 40 seconds at 68°C. Each PCR product was visualized on 1% TAE agarose gel ran at 100 V, for 60 minutes. The PCR products were sent to First Base Laboratories, Selangor, Malaysia for cloning, sequencing and further analysis.

RESULTS AND DISCUSSION

Physico-chemical evolution. Table 2 shows the carbon, nitrogen, C/N, degradation rate and OM loss. In view of the results obtained, RUN 1 achieved the highest degradation rate of 2.07% OM degradation per day. The periodic addition of sludge increased degradation rate and OM loss.

Water addition in RUN 2 slightly increased degradation rate compared to RUN 3 which had no addition during the composting. The results suggested that the highest value for degradation rate, OM loss and lowest final C/N were obtained by using periodic addition of sludge compared to the addition of only water and no addition at all regardless of the initial C/N ratio of the different experiments. The results obtained agreed with those of the previous work carried out by Mohd Zainudin *et al.* (2014) and Wan Razali *et al.* (2012). Clearly, as expected, periodic addition of sludge allowed the organic matter loss increase to justify addition not only for moisture content regulator but supporting nutrient needs for microbial to grow. Hence, further detailed analysis of RUN 1 was carried out to assess its process characteristics.

Table 3 shows the temperature profile during the composting. It increased to 48°C within 5 days. This temperature was slightly lower compared to the results obtained by Baharuddin *et al.* (2009), Mohd Zainudin *et al.* (2014), and Wan Razali *et al.* (2012), which were 58.2°C, 62°C, and 70°C, respectively, which operate on higher scale. This phenomenon occurs due to size of composting potential heat loss becoming less when size increases (Mason and Milke, 2005a). However, the highest temperature obtained from this experiment was still within the thermophilic range which was defined by Mason and Milke (2005) as greater than 40°C. The thermophilic range lasted for about 5 days, starting from day 3 to day 8. However, this experiment differed substantially from the previous studies due to the use of enclosed type of composting, medium scale, controlled aeration and moisture, plus the ability to obtain a C/N ratio of 13.45 and in the mesophilic and maturing stage where more stable composting was achieved. A higher maximum temperature might not be able to be achieved due to the agitation being done four times per day which could have caused some heat loss during the composting.

The ratio of C/N is supposed to be reduced to acceptable level as mentioned by Baharuddin *et al.* (2010) if the level of nitrogen controlled for this experiment fell to 1% of nitrogen. This obstacle as already mentioned by Baharuddin *et al.* (2010) and Yoshizaki *et al.* (2012), is influenced by mill operation and aging condition of the sludge

POME. The ratio of C/N value decreased from 47.81:1 to 16.49:1 within less than 10 days. The temperature of the process which occurred within the time frame from 0 to 8 days was above 42°C, indicating that high reduction of C/N occurred during the thermophilic stage in agreement with the results of Wan Razali *et al.* (2012). The amount of nitrogen gradually increased while carbon decreased throughout the process. As explained by Said-Pullicino, Erriquens, and Gigliotti (2007),

general increasing of nitrogen was inherent to concentration effect as a consequence of the degradation of organic carbon compounds which also reduced the dry mass. The shift of nitrogen to higher content can also be explained due to addition of sludge consisting higher nitrogen (1.11%). This suggested that improved degradation for this composting was achieved through addition of sludge and measurement of organic matter loss as an indicator of degradation.

Table 2. Value of carbon, nitrogen, degradation rate, and OM loss

Experiment	Carbon (%)		Nitrogen (%)		C/N		Degradation rate (OM%/day)	OM loss (%)
	Initial	Final	Initial	Final	Initial	Final		
RUN 1	52.98	49.30	1.11	3.66	47.81	13.45	2.07	62.00
RUN 2	52.54	51.61	1.07	1.68	49.00	30.80	0.83	25.00
RUN 3	51.33	50.48	1.73	2.28	30.00	22.00	0.60	18.00

Table 3. Physico-chemical parameters measured during composting for RUN 1.

Day	Temperature (°C)	pH	Moisture content (%)	C (%)	N (%)	C/N
0	37±6.0	6.71	63.07±5.6	52.98±0.8	1.11±0.5	47.81
3	44±3.5	7.06	70.36±3.7	51.81±1.7	1.83±1.1	28.29
5	48±4.0	7.41	68.68±2.6	51.57±1.5	2.42±1.5	21.34
8	42±2.7	8.38	70.77±3.5	51.03±2.2	3.09±1.7	16.49
12	40±0.0	7.78	66.97±4.3	50.56±2.7	3.06±1.5	16.50
22	39±2.7	8.72	72.28±0.5	49.49±4.0	3.55±1.6	13.95
26	37±6.2	7.80	73.42±3.2	49.39±2.9	3.61±1.5	13.70
30	37±3.7	8.61	74.67±1.8	49.30±1.0	3.66±1.5	13.45

Growth dynamics of microbes. The isolation and identification of the bacteria responsible for the rapid composting of OPEFB compost had been done by Mohd Zainudin *et al.* (2013). The major genera present based on 16S rRNA clones belonged to *Bacillus*, *Exiguobacterium*, *Desemzia*, and *Planococcus*. *Bacillus* species were present during the entire period of the composting process and were widely identified and isolated in many composting studies (Jurado *et al.*, 2014; Mohd Zainudin *et al.*, 2014). Hence, for this study, the quantification of bacterial DNA from the genus *Bacillus* was selected due to the strains being closely related and under the phylum *Firmicutes* (Mohd Zainudin *et al.*, 2014). As can be seen in Table 4, growth of bacteria reached maximum DNA copy number within days 11-20 and contributed to the highest temperature recorded in the vessel. This result was almost the same as that of Wan Razali *et al.* (2012), on the rapid growth of bacteria for the same time frame of their study. The DNA copy number decreased when the temperature dropped to 42°C and continued to decrease on days 21-30

when the temperature dropped to 42°C and below. In previous researches by Baharuddin *et al.* (2010) and Wan Razali *et al.* (2012), when the growth profile was assessed through microbial unit counting or colony unit counting, it was shown that growth increased during the early stage of composting, just as was found in this study though the use of qPCR. López-González *et al.* (2015) used DNA quantification to divide composting into stages of: bio-oxidative or highly active, cooling, and maturation. This study of co-composting OPEFB with POME sludge might not have entered maturation yet but could be divided into two stages of thermophilic indicated as thermophilic and decrease thermophilic, with less stage numbers compared to López-González *et al.* (2015) due to a shorter experimental time frame. The thermophilic stage lasted for 10 days and the decreased thermophilic stage lasted for 9 days, while the mesophilic and maturing stage lasted for 9 days based on DNA copy number and temperature values.

The percentage of carbon also started to decrease between day 3 and day 5, which indicated the consumption of carbon elements in the compost paralleled the trend with reducing sugar. The reducing sugar measure was done using potassium sulfate according to Hofman and Dušek (2003) and Juan *et al.* (2013), and was able to be used as a measurement of the fraction of the total organic matter. Significant decreased amounts of reducing sugar during the peak of the temperature occurred simultaneously opposite to the highest bacterial growth copy number reading (Table 4). Eventually, reducing sugar increased quickly within the thermophilic range of

temperature, reaching a peak of 1.1% dry weight. Its reading continued to reduce following stage of decrease thermophilic to mesophilic and maturing stage. This trend of reducing sugar was more related to microbial activity and temperature, as mentioned by Juan *et al.* (2013). There are relatively few references with information on the soluble fraction, such as reducing sugar in composting that could be used to compare to the results of this study and that the degradation depended on continued mineralization of the soluble organic fraction (Said-Pullicino, Erriquens and Gigliotti, 2007).

Table 4. Growth dynamics of microbes for RUN 1.

Day	Temperature (°C)	DNA copy number (range)	Soluble sugar (% dry weight, d.w.)	Stage
0-10	32-48	0-301,714	0.52-1.07	Thermophilic
11-20	40-42	57,045-91,663	0.41-0.42	Decrease thermophilic
21-30	30-42	42,248-83,103	0.25-0.57	Mesophilic and maturing

Compost substrate degradation. The OPEFB lignocellulosic composition of the initial compost consists of 47% cellulose, 24% hemicellulose, and 19% lignin. The high degradation ratio of cellulose started from day 10 until day 21 (Table 5). The degradation behaviour of cellulose showed that it started to degrade when soluble degradable compounds such as reducing sugar were decreased in days 0-10 as shown in Table 4. This behaviour of degradation was already reported by Juan *et al.* (2013) and Huang *et al.* (2010). The increased cellulose degradation ratio might be due to less availability sugars or other readily biodegradable compounds within the compost substrate. However, the reducing sugar value kept increasing from day 0 to day 10 corresponding to the total sugar degradation and liberation from the solid to the liquid form fraction. The degradation ratio of cellulose demonstrated that species of the genus *Bacillus* have cellulolytic activities. *Bacillus* have more influence on the breakdown of cellulose according to Jurado, López, and Moreno (2014). Compared to cellulose, hemicellulose did not degrade significantly. The degradation ratio of hemicellulose began to increase during the peak temperature of the thermophilic stage and kept

slowly increasing within the 40°C range and started to decrease after day 21 when the temperature range dropped to 30°C. This showed that the thermophilic stage was the main contributor of hemicellulose degradation and that *Bacillus* sp. might produce higher endoglucanase at all temperatures and less xylanase activity, as mentioned by Seo *et al.* (2013).

Lignin showed no indication of degradation throughout the composting period. The structure of lignin provides recalcitrance when chemically bonded or wrapped with hemicellulose and the cellulose complex structure. The result was almost similar to that of Juan *et al.* (2013) where no lignin degradation occurred until the end of the composting.

Figure 2 shows the thermogravimetric analysis (TG) and derivative thermogravimetric (DTG) analysis for the thermophilic stage, decrease thermophilic, and mesophilic and maturing stage of lignocellulosic degradation. Figure 2 also shows the TG and DTG curves of the composting matters for the different stages of the composting period. It was apparent that there were three major weight losses due to thermal decomposition, as shown on the DTG graph (I, II, and III). The initial stage was from 50°C to

150°C, which contributed up to 10% of weight loss. This process occurred due to the vaporization of moisture and other volatile substances (Pasangulapati *et al.*, 2012; Talib *et al.*, 2014).

The subsequent stage was the significant decomposition which occurred in the range of 250°C to 400°C. This decomposition achieved major weight loss of around 61% for the thermophilic stage, decreasing to around 58% of composting materials at the decrease thermophilic stage, and decreasing to 52% for the mesophilic and maturing stage. These major losses were consequences of the degradation of the hemicellulose and cellulose contents within the biomass (Carrier *et al.*, 2011; Pasangulapati *et al.*, 2012; Talib *et al.*, 2014). This result supported the data obtained from the analysis of the lignocellulosic content, indicating that lignocellulosic materials on the final day was lower

than in the initial composting, directly due to degradation of the OM material.

The final stage of the thermal degradation happened from 450°C to 600°C. This constituted about 10% losses in material weight attributed to degradation composting of lignin as explained by Omar *et al.* (2011) and hemicellulose structure (Lyons *et al.*, 2006). The TG degradation curves for the initial, middle composting had a different slope than at the final composting day. This difference could be explained in part due to the degradation of structural hemicellulose, and the result did not show any significant lignin degradation since the lignocellulosic analysis indicated that the lignin content did not change throughout the composting period and furthermore, lignin was slowly degraded within a broad temperature range (Barneto *et al.*, 2010).

Table 5. Lignocellulosic mass composition degradation ratio in OPEFB composting for RUN 1.

Day	Temperature (°C)	Degradation ratio (%)			Stage
		Hemicellulose	Cellulose	Lignin	
0-10	32-48	0-4	0-7	0	Thermophilic
11-20	40-42	4-5	7-11	0	Decrease thermophilic
21-30	30-42	2-5	11-16	0	Mesophilic and maturing

Figure 2. TG and DTG curves of materials in the composting process for RUN 1.

CONCLUSION

The presence of a bacterial community of specific species during composting was demonstrated using DNA quantification via qPCR. This study showed that other types of quantification of microbes could also be done instead of using DNA hybridization. The dynamics of the growth of bacteria described the relationship among the lignocellulosic fraction, composition as the major carbon source and temperature trends. The characteristics of the lignocellulosic degradation pattern depended on compost process parameters such as the periodic additional of nitrogen source into the system for rapid composting. Even though the composting was done for 30 days, there were several stages occurring per time line, where the degradation ratio of the lignocellulose started on days 0-10 and started to decrease after days 21-30, which almost concurrently paralleled the genus *Bacillus* growth trends which reduced after day 20. The controlled condition of the composter in RUN 1 was able to accelerate composting due to the microbial growth condition, which reduced the composting time to 2.07% OM degrade per day, compared to the water only addition (RUN 2) of 0.60% OM degrade per day and no addition (RUN 3) with lower C/N value.

Several microorganisms such as species related to *Exiguobacterium* and *Solibacillus* genera which were detected and also considered as dominant groups in previous studies may serve as guides for future work to measure growth in terms of specific species, similar to what was done in this study. Known dominant groups alone may not be adequate to access degradation in composting. Hence, it is recommended that dominant microbial growth information should be paired with information on functional genes encoding degradation enzymes. Functional gene profiling related to the lignocellulosic degrading process should be conducted to understand the dynamics of lignocellulose composting.

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