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Pyrosequencing Analysis of Bacterial Communities in Composts Dominated by Beneficial Microorganisms

Shin Young[1], Ha, In-Soo Kim[1]

[1]*Department of Environment Engineering, Korea Maritime and Ocean University, Busan, Korea*

Abstract- In this study, wastewater and sewage sludge treated with beneficial microorganisms (BM) were converted into compost by using food waste as a supplementary nitrogen source. Comparative microbial community analyses based on the pyrosequencing technique were performed on BM treated sludge and control sludge, and on the resulting compost samples. Analysis of the diversity of microorganisms showed 559 operational taxonomic units (OTUs) out of 1580 reads for the control sludge and 594 OTUs out of 2174 reads for the BM treated sludge; thus, higher diversity was detected in the control sludge. Pyrosequencing of the BM treated sludge revealed that Proteobacteria (41.30%) was dominant, followed by Gemmatimonadetes (14.12%). Additional microbial diversity analyses showed 249 OTUs out of 3406 reads in the control compost and 183 OTUs out of 2632 reads in the BM treated compost. Although the diversity of microorganisms was relatively high in the sludge samples, the diversity decreased as composting progressed and similar levels of diversity were detected in the compost samples. High proportions of Bacteroidetes and Proteobacteria were present in the compost samples derived from both sludges.

Keywords – Beneficial microorganisms; Microbial diversity analysis; Sewage sludge treatment; Composting; Food waste

I. INTRODUCTION

Organic waste is a resource that can be converted into useful materials by microorganisms, and it can be recycled in a variety of ways. In our work, wastewater and sewage sludge treated with beneficial microorganisms (BM) is being converted into compost by using food waste as a supplementary nitrogen source. For investigative research, samples consisting of control sludge and sludge innoculated with BM are first treated in a laboratory-scale reactor similar to an Annamox system. Then, the composting characteristics of BM treated sludge and control sludge can be compared. The feasibility of using coffee grounds and sawdust as bulking agents has also been examined. These earlier tests revealed that composting of BM treated sludge proceeded at a faster rate than the composting of control sludge, and higher temperatures were reached in the BM treated samples. When coffee grounds were used as a bulking agent, most of the odors were effectively absorbed; similar results were also seen with the sawdust.

In this study, extensive results on the compositions of microbial communities in control sludge and BM treated sludge samples as well as compost samples are presented. In addition, the BM treated compost was compared with composts available in the market to gain further information on the differences in BM communities that can be attributed to various composting methods. Compost consisting of pig manure treated with BM was produced in this study by using photosynthetic organisms, similar to commercial pig manure compost production techniques. In order to examine the differences in the composting reactions of various microbial communities, a relatively new technique called pyrosequencing was employed. Pyrosequencing has been shown to be a valuable tool for the analysis of complex microbial communities present in the environment.

Pyrosequencing analysis of microbial communities does not require laborious cloning such as when Escherichia coli is used to construct clone libraries, and the cost of pyrosequencing is $1/100^{th}$ of the cost of conventional Sanger sequencing. Pyrosequencing also allows for the mass sequencing of large amounts of material, which is tracked by the use of barcodes. This new sequencing method is now being widely used for investigations of complex microbial communities because of its ability to analyze large sequences of genes faster and cheaper than conventional methods (e.g., Bae, 2011; Lim et al., 2010).

There are several of methods available for quantifying the microbial species richness, which indicates the number of species in a sample. This study used operational taxonomic units (OTUs), where each unit represents individual living organisms or communities of species with 97% sequence similarity. Rarefaction curves representing the OTUs analyzed from each sample were plotted and the differences in species richness were analyzed. Alpha diversity, which represents the diversity of species within a certain region or ecosystem, was also analyzed. In addition, beta diversity was studied to examine the differences in species diversity between the BM treated and control sludge samples and the corresponding compost samples (Heck et al., 1975).

II. METHODS

2.1 Sampling

Control sludge samples were acquired from an A-level sewage treatment facility in Busan city, South Korea. The BM treated sludge was prepared by treating the sludge with BM and stabilizing the material in a laboratory-scale bioreactor. The control and BM treated sludge were composted under aerobic conditions, and samples were extracted on the 28th day of composting and stored at -20°C until further analysis. Hereafter, SCL stands for the compost derived from the control sludge, while BCL denotes the compost derived from the BM treated sludge (the term L refers to the use of low temperature experiments). Furthermore, both control pig manure compost and BM treated pig manure compost were utilized in order to make comparisons with composts on the market, and here, these materials are abbreviated as PMP and BMP, respectively.

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2.2. DNA extraction

The DNA was extracted by using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) (Ufnar et al., 2006; Viau and Peccia, 2009). Briefly, the sludge and compost samples were filtered through 0.2 μm cellulose nitrate membrane Whatman filters and the material on the filters was retained for analyses. Large soil particles, which may inhibit the polymerase chain reaction (PCR), were removed from the samples. Then, a 0.25 g portion of the membrane filter was mixed with beads, which facilitate the breakdown of cell membranes, and DNA-protecting buffers within a Power Bead Tube. Reagents were added to the samples followed by 60 μL of solution C1 and the tubes were vortexed in a cell disruptor set at the maximum speed for 10 min. The samples were then centrifuged at a speed of 10,000 g for 30 s and the supernatant was transferred to new collection tubes. Approximately 250 μL of solution C2 was then added to the samples to remove soil particles, pieces of cells, proteins, and organic and inorganic inhibitors (e.g., humic acids); this step was necessary to purify the DNA. The samples were subsequently vortexed for 5 s and incubated at 4°C for 5 min. The samples were then centrifuged again at 10,000 g for 1 min at room temperature (20–25°C) and 600 μL of the supernatant liquid was transferred to new collection tubes. For the second stage of inhibitor removal, 200 μL of solution C3 was mixed into the samples and the samples were incubated at 4°C for 5 min. Then, the tubes were centrifuged at 10,000 g for 1 min at room temperature and 750 μL of the supernatant was transferred to new collection tubes. To attach the DNA to the silica filter membrane, 1200 μL of solution C4, a high salinity liquid, was added to the supernatant and samples were vortexed for 5 s. The mixed liquid was centrifuged in a spin filter at 10 000 g for 1 min at room temperature. To remove alien substances absorbed to the filter, 500 μL of solution C5 was added to the filter and samples were centrifuged at 10,000 g for 30 s at room temperature. Filtered supernatant was then discarded and the spin filters were moved to new collection tubes for DNA elution with 100 μL of solution C6. Lastly, samples were centrifuged at 10,000 g for 30 s and the extracted DNA was stored at -20°C.

2.3. Pyrosequencing method

Pyrosequencing of the extracted DNA was performed by Chunlab in Korea. The process of pyrosequencing can be divided into six stages. In the first stage, barcodes were arrayed and included in the PCR primer in each sample and 2-bp linkers were removed. The second step involved removal of sequences with unambiguous bases that were shorter in length. In the third step, cut off primers were used for amplification and detections were conducted by using a pair-wise alignment algorithm. In the fourth step, reads of other types other than the target gene for analysis, namely, the 16S rRNA gene, were excluded. This step was essential to exclude recognition of new sequence types and avoid the resultant analytical errors. The fifth step was to remove chimeras generated during the amplification of sequences from multi-templates. The last step involved taxonomical identification of sequences through a similarity search. Prokaryotic microorganism identification was performed by using the analysis methods described in the EzTaxon server and EzTaxon-e-databases (Chun et al., 2007; Kim, 2012; Kim et al., 2012).

The nucleic acid sequences obtained through pyrosequencing were analyzed by using CLcommunityTM, which is a community analysis program provided by Chunlab (Chun et al., 2010). The CD-HIT program was used for OTU definitions (Li and Godzik, 2006). The OTUs were determined and species richness was estimated by analyzing the rarefaction curves. Alpha and beta diversity were computed. Fast UniFrac analysis was performed by using the CLcommunityTM program to study the relationships between microbial "ecosystems" (Hill et al., 2003). Chunlab provided the pyrosequencing results of the bacterial community analysis on its website (Chunlab, 2014).

III. RESULTS AND DISCUSSION

3.1. Metagenome analysis with the rarefaction curve

3.1.1 Sludge

The number of reads of nucleic acid sequences and the OTUs estimated by the program were as follows: 1580 reads

and 559 OTUs in the control sludge, and 2174 reads and 594 OTUs in the BM treated sludge. In each sequence, the length of reads was about 450–500 bp, which was suitable for bacterial community analysis. As evident from the rarefaction curves shown in Fig. 1, the diversity of the control sludge was higher than that of the BM treated sludge.

3.2.2 Compost

The diversity of bacterial communities in the control compost and BM treated compost was analyzed by using the pyrosequencing technique. The number of reads of the nucleic acid sequences obtained and the OTUs estimated by the program were as follows: 3406 reads and 249 OTUs in the control compost, and 2632 reads and 183 OTUs in the BM treated compost.

Fig. 2 shows the rarefaction curves for the OTU values obtained from each compost sample. From these data, it was evident that diversity was higher in the control compost than in the BM treated compost. It is likely that the relatively low levels of diversity in the BM treated compost were due to dominant bacterial activity during composting. In other words, the control compost probably displayed higher diversity because it contained lower numbers of dominant species than the BM treated sludge.

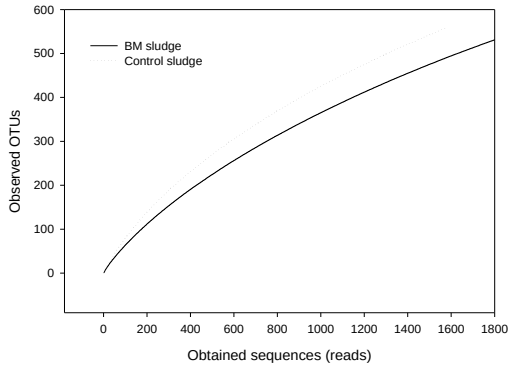

Fig. 1. Comparison of rarefaction curves for BM sludge and control sludge.

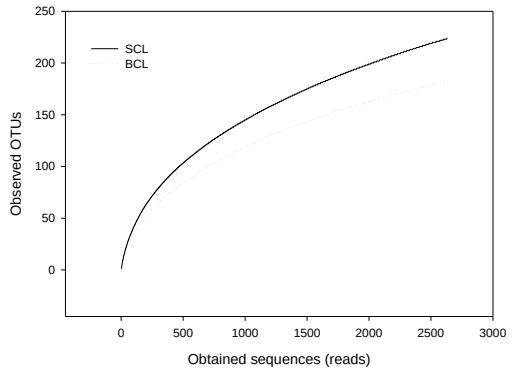

Fig. 2. Comparison of microorganism diversity in BM compost and control compost as indicated

3.2- Diversity of bacterial communities analyzed by use of the pyrosequencing technique

3.2.1 Sludge

The phylum diversity of bacterial communities found in the control sludge and the BM treated sludge was analyzed by using the pyrosequencing technique, and the results are illustrated in Fig. 3. The bacterial phylums present in the control sludge in descending order were as follows: 31.2% Proteobacteria, 23.1% Bacteroidetes, 17.4% Chloroflexi, 6.01% Actinobacteria, 5.1% Firmicutes, and 15.25% others (each with levels less than 5%). The bacterial phylums present in the BM treated sludge in descending order were as follows: 41.30% Proteobacteria, 14.12% Gemmatimonadetes, 11.45% Firmicutes, 7.58% Bacteroidetes, 7.17% Actinobacteria, and 16.65% others (each with levels less than 5%).

The most abundant phylum reported in both sludge samples was Proteobacteria, which are known to prosper well in highly organic environments. A study on the RABC (rotating activated Bacillus contactor) process that is often used to treat urban sewage reported that Firmicutes (43.9%) was the most dominant phylum, followed by Bacteroidetes (17.6%) and β-Proteobacteria (14.3%) (Jeris and Regan, 1973). Thus, Proteobacteria appear to be common constituents of sludge.

Photosynthetic bacteria absorb and utilize carbon dioxide and hydrogen sulfide and can prevent contamination and odor problems during sewage treatment (Lee, 2011). In this study, Gemmatimonadetes were relatively abundant in the BM treated sludge samples (14.12%) but were relatively rare in the control sludge samples (0.3%). These bacteria may help with biological phosphorus removal during the activated sludge treatment process (Zhang et al., 2003). Bacteroidetes were detected in the control sludge at higher abundances (23.1%) than in the BM treated sludge (7.58%). Dominant Bacteroidetes levels have also been observed in the repetitious aerobic and anaerobic environments used in Y treatment facilities (Jung, 2014). Higher levels of Actinobacteria were found in the BM treated sludge (7.17%) than in the control sludge (6.01%). These bacteria can produce antimicrobials that fight pathogenic bacteria, and they can also consume chitinous substances, which may help to control harmful fungi and bacteria and render the environment favorable for other BM. Actinobacteria are common in healthy soil (Chaudhary et al., 2013). It is possible that the increase of Actinobacteria in the BM treated sludge will help prevent the proliferation of harmful bacteria during composting.

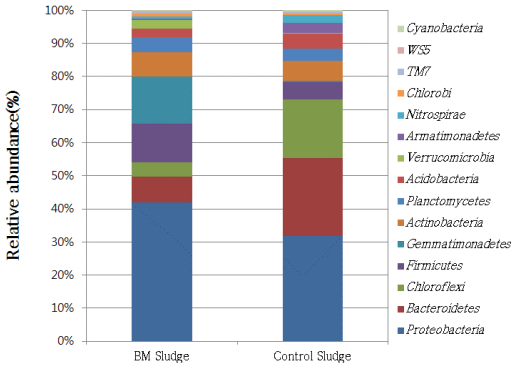

Fig. 3. Comparative analysis of dominant phyla in BM sludge and control sludge.

The results of the analysis of the bacterial communities at the species level are illustrated in Fig. 4. Analysis of the control sludge revealed that Saprospiraceae_uc_s were the highest in proportion at 3.60% followed by Caldilineaceae_uc_s (3.35%), FM213038_s (3.22%), HQ014633_s (2.97%), Nitrospira defluvii (2.02%), Fimbriimonas_uc (1.96%), and FJ710748_s (1.83%). Nearly 69% of the community was dominated by species with levels less than 1%, and these species included Cloacibacterium rupense (1.77%), Cloacibacterium normanense (1.64%), GU455152_s (1.58%), GU454905_s (1.51%), GU455152_g_uc (1.329%), Macellibacteroides fermentans group (1.26%), AB298726_s (1.26%), and FJ936783_f_uc_s (1.26%). In the species level analysis results for the BM treated sludge, FQ658948_s was the highest at 12.55% followed by Bacillus funiculus (9.75%), Pseudomonas monteilii group (7.03%), Nakamurella multipartita (2.62%), AY548945_g_uc (2.25%), Pseudomonas hunanensis (2.16%), and Flavimonas oryzihabitans (1.37%). Further, 55% of the community was dominated by species with levels less than 1%, and these species included EU135435_s (1.37%), Dokdonella_uc (1.28%), HQ440080_s (1.19%), FJ936783_f_uc_s (1.10%), DQ660884_s (1.05%), and Saprospiraceae_uc_s (1.01%).

In the BM treated sludge, Bacillus (9.75%) participated in denitrification, iron reduction, and manganese oxidation reactions, while Pseudomonas (7.03%) removed phosphorus (Usharani and Lakshmanaperumalsamy, 2010; Vasudevan et al., 2002). AY548945_g_uc, which are microorganisms mainly found in ANAMMOX sludge, were dominant in the BM treated sludge at a level of 2.25%, and they probably contributed to nitrogen removal during the water treatment process (Pöhle, 1993).

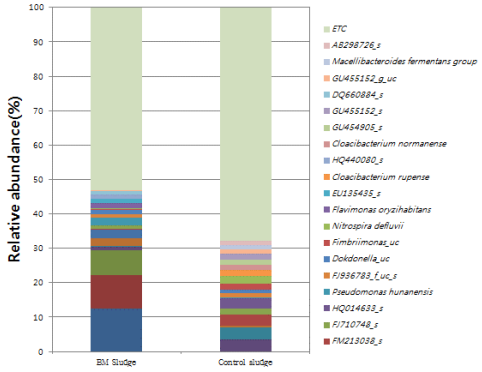

Fig. 4. Comparative analysis of dominant species in BM sludge and control sludge.

3.2.2 Compost

Results for the phylum level analysis of the bacterial communities in the control compost and BM treated compost are illustrated in Fig. 5. The bacterial phylums present in the control compost in descending order were as follows: 58.86% Bacteroidetes, 30.53% Proteobacteria, 6.01% Acidobacteria, 2.75% Actinobacteria, 1.7% Firmicutes, and 0.11% others (with levels less than 1%). The bacterial phylums present in the BM treated compost in descending order were as follows: 62.61% Bacteroidetes, 29.33% Proteobacteria, 4.71% Actinobacteria, 3.26% Firmicutes, and 0.07% others.

Takaku et al. (2006) reported that Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were present in the bacteria communities of control composting samples. The bacterial communities observed in this study were similar to those in the above report. Here, Bacteroidetes levels were the highest among all phylums in both compost types. In general, Bacteroidetes levels gradually increase as composting progresses. Thus, in this study, the high levels of Bacteroidetes can be attributed to the fact that aged compost samples were examined. Firmicutes were detected at a level of 11.4% in the initial BM treated sludge, but their levels declined to only 3.26% in the BM treated compost. This corresponds with a report that the detection range for this phylum was wider during the initial phase of composting but narrowed during the process of decomposition (Takaku et al., 2006). The second most dominant bacteria detected in this study was Proteobacteria, whose levels were similar in the control compost and BM treated compost. Actinobacteria levels were higher in the BM treated compost than in the control compost. Actinobacteria supply fixed nitrogen to the microbial "ecosystem," and the activity of these bacteria tends to increase with increasing temperatures as composting progresses. In this study, the BM treated sludge's decomposition temperature was probably higher than that of the control sludge, as such differences were observed in preliminary tests (see Section 1). Other thermophiles such as Chloroflexi were not detected in the control compost but were detected in the BM treated compost. This finding was also probably related to the temperature differences during the composting period. Acidobacteria, which are usually found in mineral rich soils or well controlled soils, were observed in the control compost. Thus, this compost could potentially have a positive effect on similar soil types.

The results for the species level analysis of bacterial communities in the control compost and BM treated compost are illustrated in Fig. 6. Analysis of the control compost revealed that Sphingobacteriales_uc_s were the highest in proportion at 29.53% followed by Chitinophaga terrae (14.26%), Sphingobacteria_uc_s (5.56%), Novosphingobium lindaniclasticum (4.22%), Luteibacter jiangsuensis (3.22%), Achromobacter pulmonis (2.67%), and Achromobacter ruhlandii group (2.23%). Others accounted for 19% and included Chitinophaga_uc (1.67%), Ochrobactrum anthropi group (1.64%), Burkholderia tropica (1.43), Pandoraea pnomenusa group (1.29%), Burkholderia glumae (1.26%), 4P005153_s (1.17%), Granulicella mallensis (1.17%), Bryocella_uc (1.11%), Hephaestia caeni (1.05%), Granulicella_uc (1.02%), and others (<1%).

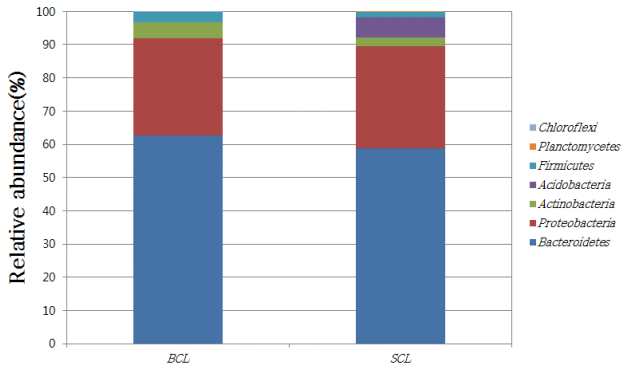

Fig. 5. Comparative analysis of the dominant phyla in BM compost and control compost.

In the species level analysis results for the BM treated compost, C. terrae was the highest at 36.96% followed by Burkholderia multivorans (12.27%), Olivibacter jilunii (5.01%), Luteimicrobium xylanilyticum (3.38%), AB274846_s (3.26%), Sphingobacteriales_uc_s (2.58%), and Flavobacterium anatoliense (2.50%). Bacillus coagulans (1.89%), Bordetella hinzii (1.74%), Ochrobactrum pseudintermedium (1.67%), FJ936783_f_uc_s (1.10%), DQ660884_s (1.05%), Saprospiraceae_uc_s (1.01%), and others (<1%), which accounted for 14% of the distribution. The characteristics of microorganisms in the control compost and the BM treated compost were quite

different from each other. The most dominant species in the BM treated compost, namely, C. terrae, helps to reduce nitrates, and Chitinophagaceae_uc_s (11.6%) aids in the decomposition of anthracene derivatives (Kim and Jung, 2007; Zhao et al., 2014). Phaga_uc (11.66%) and B. hinzii (1.67%) decompose chitin, and thus the cell walls of fungi, and they can also produce antimicrobial agents that are capable of controlling the growth of harmful microorganisms during the composting period (Funke et al., 1996; Li et al., 2011). B. multivorans (12.27%) produces lipase, which is a fat digesting enzyme, and this helps to decompose the fatty acids in coffee, which is often used as a bulking agent (Gupta et al., 2006).

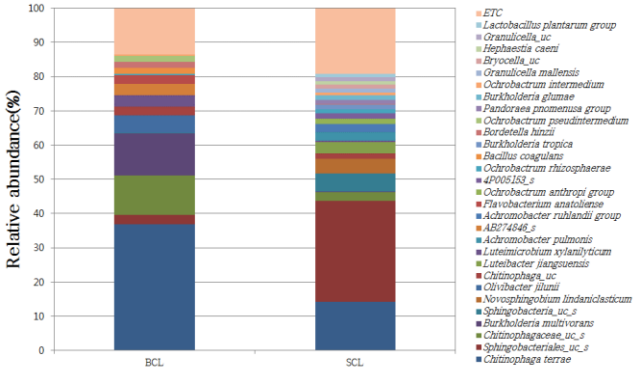

Fig. 6. Comparative analysis of dominant species in BM compost and control compost.

3.2.3. Community analysis of BM treated sludge and compost

The number of reads of nucleic acid sequences and the OTUs estimated by the program were as follows: 2174 reads and 594 OTUs in the BM treated sludge, and 2632 reads and 183 OTUs in the BM treated compost.

Fig. 7 illustrates the results of the community analysis on the BM treated sludge and the corresponding compost that was produced. First, BM treated sludge had more diverse micro-organic properties than the compost. The species level analysis indicated that Proteobacteria were the most dominant species in both materials; the detection levels were 41% in the sludge and 29.3% in the compost. Species of the Bacteroidetes phylum were the most dominant after composting. The family properties were markedly different before and after composting. Before composting, Pseudomonadaceae were the most dominant at 11.08%, but a value of only 0.68% was detected after composting. Conversely, Burkholderiaceae were only at 0.27% before composting, but levels increased to 15.15% after composting. Likewise, Chitinophagaceae were 3.08% before composting, but levels increased to 51.93% after composting. This means that the type of dominant BM changes depending on the composting conditions. In addition, micro-organic properties simplified as the composting proceeded, and this finding agrees with the results of other studies on micro-organic properties during each period of the composting process (Takaku et al., 2006). The properties of microorganisms present in the final compost samples were similar. Therefore, it appears that the initial sludge microbial communities or combination of microorganisms innoculated into the samples do not determine the ultimate micro-organic properties of the compost, although they do help with composting.

Recall that PMP represents pig manure compost treated with photosynthetic bacteria (a product purchased at the market) and BMP represents pig manure compost treated with BM (a product also purchased at the market); furthermore, SCL stands for the compost derived from the control sludge, while BCL denotes the compost derived from the BM treated sludge (the term L refers to the use of low temperature experiments). The microbial analysis results for these materials are shown in Fig. 8.

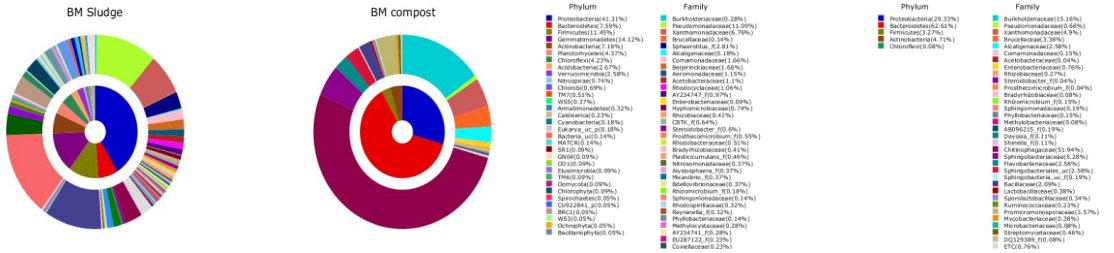

Fig. 7. Double pie charts showing the microbial communities of different samples from BM sludge and BM compost. The inner pie represents the phylum compositions, and the outer pie represents the family compositions.

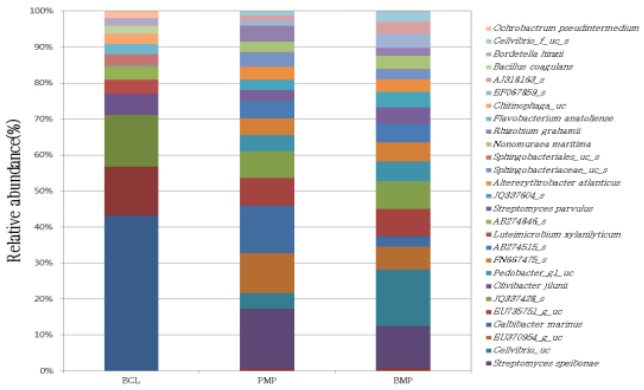

Fig. 8. Comparative analysis of the dominant species in the PMP, BMP, and BCL composts.

IV.CONCLUSION

The main conclusions of this research are as follows:

1. A phylum level analysis using pyrosequencing of the bacterial communities present in BM treated sludge indicated that Proteobacteria (41.30%) and Gemmatimonadetes (14.12%) were dominant. Proteobacteria are usually found in highly organic environments such as those present in sewage and wastewater treatment processes. Photosynthetic bacteria can absorb and utilize carbon dioxide and hydrogen sulfide and are effective in contamination and odor prevention. Gemmatimonadetes are aerobes found in activated rainwater treatment facilities. They were at 0.3% in the control sludge but at 14.12 % in the BM treated sludge; thus, these findings indicate that activated BM sludge processing can lead to higher levels of such bacterial activity.
2. The results of the analysis on the microbial diversity of the BM treated compost and control compost corresponded with the results of other studies. Specifically, microbial communities were simplified during the composting process and dominance of Bacteroidetes, Proteobacteria, and Firmicutes increased. Actinobacteria, which supply fixed nitrogen to the microbial "ecosystem," had a dominance level of 4.17% among the microorganisms in BM compost. The activity of these bacteria increased with increases in temperature as the composting progressed.
3. The PMP and BMP composts, which are on sale in the marketplace, were compared with the BM compost developed in this study. The totals for the "other" category of bacteria, which were ones detected at levels of less than 1%, accounted for 49% and 48% in the PMP and BMP composts, respectively, but these bacteria accounted for only 15% in the BM treated compost. Thus, BM treated compost had a lower diversity than other composts currently on sale; however, the compost was dominated by microorganisms that enabled symbiosis.
4. In conclusion, the results of this study suggest that the application of a complex BM agent during the composting process is a valuable organic matter treatment method since appropriate microorganisms dominated the composting process during the testing. The presence of such microorganisms is known to help to accelerate the rate of composting. Moreover, high maturing temperatures in the compost can inhibit the growth of pathogens, which increases the feasibility of using the compost in diverse applications.

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