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Characterization of the Microbial Community in A Continuous Garbage Treatment Process

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Abstract

Previously, we developed a "static type" garbage treatment system whose performance exceeds that of conventional garbage treatment systems. However, we could not explain the system's excellent performance especially from a microbial ecosystem's stand point (Matsuda *et al*, 2017). Microbial activity causes the decomposition of organic materials in a garbage treatment process. However, we do not know which microbes are effective in garbage decomposition and what relationship exists between the succession of a microbial community and the decomposition of organic materials in a garbage composting process.

This study analyzed the relationship between a continuous operation garbage treatment system's microbial community and the decomposition of organic matter and evaluated for effective microbes in the same garbage treatment system. Although many research articles have been published on the composting processes throughout the world, most of them employ batch processes and not continuous processes. Conversely, most real-world practical composting plants adopt a continuous operation system in which fresh feed is usually input once a day with continuous aeration and intermittent mixing.

To comprehensively analyze the microbial community, three different approaches were adopted in this study; 1) colony observation, 2) DNA analysis, and 3) the enzymatic activities of each colony.

In our experiment, the working volume was 10 L of leaf mold and 40 g/(day L) of garbage (the organic load) was input to the reactor every day at a fixed time. Dog food with 70 % moisture content was used as a model garbage substrate. The reactor's internal temperature, the total reactor's weight and the sampled residue's weight, moisture content and pH were measured before inputting garbage. Additionally, the reactor's internal temperature was measured six hours later. The internal temperature was about 55 ℃ at the highest without heating. The organic matter decomposition rate was about 50 % and the weight reduction rate was over 90 %, implying successful garbage decomposition was achieved.

The microbial composition and the number of the colonies seen on the medium plate changed every day and did not realize a "steady state." Thus an extremely efficient microbe does not exist. Only eleven microbes were isolated; yet many more microbes must exist in the system but were not counted due to a high dilution rate.

From our DNA analysis, the PCR-DGGE profile of the microbial community in the garbage residue showed that bands of isolated colonies were detected in the same positions as the bands of garbage residue, which contained all kinds of microbes. Nine microbes were identified using 16S rRNA genome from eleven isolated ones. The identified microbes were of different bacterial species and their characteristics were examined from the stand point of nutritional property and enzymatic activity.

The garbage decomposition process consists of two steps, solubilization and metabolization. Extracellular enzymes act during solubilization of solid garbage residue, and intracellular enzymes work when water-soluble substances are taken up into bacterial cells and metabolized. Protease and amylase activity were measured to assess extracellular enzymatic activity and dehydrogenase activity was measured to evaluate intracellular enzymatic activity. The enzyme activities of bacterial strains significantly differed by strain. Results from this study suggest complementary microbial activity and that *Bordetella trematum*, *Bacillus cereus*, *Bacillus subtills subtills* and *Streptomyces thermocarboxydus* effectively decompose garbage.

Keywords: garbage treatment process, continuous composting, microbial community, PCR-DGGE, colony observation

1. Introduction

Composting is regarded as one of the most environmentally friendly options used for managing different types of organic waste. Globally, immense research has focused on composting research for a long period. For instance, "The Practical Handbook of Compost Engineering" a 717-paged standard literature published in 1993, contains extensive information on composting from definition to odor management (Haug, 1993). Nevertheless, there are still so many unknowns about composting, especially information about the behavior of the microbial community.

This could be attributed to the complexity of the behavior of the microbial community. Following advances in molecular biological techniques, composting research took on new dimensions and novel methods employing DNA analysis such as PCR-DGGE (as was applied to this study) have been used in main stream research. Based on DGGE pattern bands, we identified the microbes in a composting system but not their function nor whether they were alive or dead. Therefore, we adopted additional approaches such as colony observation and enzymes' measurement. Colonies that appeared on the medium plate were classified based on color and shape and enumerated. We assumed that of the microbial community behavior could be visualized as a change in colonies' composition. Additionally, we attempted to identify colonies corresponding to DGGE pattern bands in order to indicate that colony observation is an effective tool for analysis of microbial community.

The composting process is a complex multistage biochemical reaction consisting of two major steps: solubilization and metabolization. Solubilization is a step by which microbes attempt to convert solid substrates into water-soluble state by excreting extracellular enzymes such as protease and amylase because they cannot directly digest solid organic substrates. In metabolization, water-soluble organics are easily taken up into the microbial cells and used as nutrients after processing by various intercellular enzymes. We measured enzymatic activities extracellular enzymes protease and amylase, and the intercellular enzyme dehydrogenase as an expression of the metabolic characteristic of each microbe.

One strength of this study was that we conducted the experiment in a continuously to match the processes in many real practical composting plants. These composting plants adopt continuous operation systems that input daily fresh feed and continuously aerate and intermittently mix it. There are many research articles on composting processes published worldwide; however, most of them addressed batch processes (e.g., Chang *et al*, 2010; Gao *et al*, 2010; He *et al*, 2013; Saffari *et al*, 2016; Zorpas *et al*, 2017; Manu *et al*, 2017; Cerda *et al*, 2018; Jain *et al*, 2018; Yu *et al*, 2018; Bian *et al*, 2019; Meng *et al*, 2019; Mali *et al*, 2020; Liu *et al*, 2020; Zhang *et al*, 2020; Li *et al*, 2020; Nafez *et al*, 2020; Wang *et al*, 2021; Meena *et al*, 2021; Assandri *et al*, 2021). Some studies focused on issues specific to continuous operations such as the concept of organic loading to a reactor (Maruchi *et al*, 2016) and the aeration conditions calculated based on material balance around a reactor (Matsuda, 2016). The behavior of a microbial community may differ between batches and continuous operations; e.g., batch operations are always under unsteady conditions whereas continuous operations can achieve steady state conditions if all their requirements are met. This highlights the need for research using continuous operations' experiments.

2. Materials and Method

2.1 The Experimental System

The experimental apparatus was a simple "static-type" garbage treatment system developed in our lab. It contained 10 L leaf mold used as a bulking agent covered by a nylon laundry net in a cardboard box. In our apparatus, temperature and aeration were not controlled, neither was leaf mold added during the two-weeks' experimental period. The photograph of this apparatus is shown in Fig. 1.

Dog food was used a model organic waste because it contains balanced and reliable amounts of meat, vegetables and small fish. Dried dog food (120 g) was crushed and mixed with water (280 g), and held overnight before being input at a rate of 40 g/(L. day) and a moisture content of 70% to the system. The model waste (i.e. dog food) was input to the system once a day at a specific time and manually mixed to uphold aerobic and well-mixed conditions in the reactor.

Figure 1. The experimental apparatus

2.2 Measurement of Physical Parameters

The following physical parameters were measured before the input of model waste into the reactor: The experimental system's total weight, the temperature in the reactor, and the water content and pH of a sampled residue. The reactor's temperature was measured at the center of the reactor (which was likely to be the highest temperature) and six hours after input. Three grams of the residue were sampled from the center of the reactor to assess water content, pH, and collect microbes.

2.3 Measurement of System Performance

In this study two indices were used to evaluate system performance: weight reduction by wet basis and organic decomposition by dry basis. Weight reduction by wet basis was calculated from the change in the reactor's total weight and the total model waste input on a wet basis; it took into consideration water loss due to evaporation. Organic decomposition by dry basis was the change in dry matter calculated from the total sample residue's weight and water content; it represented the decomposition of organic matter.

2.4 Analysis of the Number of Microbes and Microbial Growth

The plate dilution method was used to count the number of microbes in the residue. The microbes from a mixture of a 3 g sample reside and 27 g distilled water was inoculated in an agar medium. The mixture was homogenized for 10 minutes at 10,000 rpm. The solution was then diluted to a factor 10^7 to 10^9 before inoculation. The total colony count was an average of values from three agar plates with a trypticase-soy medium. The number of microbes was counted seven days after inoculation, and the real microbial density in the sample was calculated from the number of microbes in the colony number and the dilution rate.

2.5 DNA Analysis

DNA was extracted from the compost samples and colonies were isolated on culture plates. The DNA extraction method was similar to that employed when extracting soil DNA. It used NucleoSpin® Soil (Takara-Bio Corp.) as per manufacturer's recommendations. Extraction prioritized removing as many impurities as possible from the samples to ensure the lowest possible impurity level. The universal primers 357F-GC and 517R targeting the 16S rDNA were used for DNA PCR amplification. PCR product's concentration and purity were verified by measuring its UV intensity at a wavelength of 260 nm and 280 nm. The Denaturing Gradient Gel Electrophoresis (DGGE) method used was a standard one (Gel conc. 30∽70 %, 60 ℃, 80 V, 12∽14 hrs.).

2.6 Measurement of Enzymatic Activities

Firstly, an enzymes-containing solution was prepared from a 3 g sample residue and 27 g of distilled water. The mixture was homogenized for 10 minutes at 10,000 rpm. Then, the sample was separated by centrifugation at 10,000 rpm for 10 minutes and a 0.45 μm membrane filter was used to filtrate the supernatant liquid.

2.6.1 Protease

The protease activity in the sample solution was determined by assessing tyrosine production from a casein solution. The reaction occurred after using a 5 mL casein solution (conc. 0.6 %) and 1.0 mL of sample at 40 ℃ for 1 hr. After several post-treatment procedures, tyrosine production was measured at 750 nm absorbance and the difference between this value and a negative control was taken as the protease activity.

2.6.2 Amylase

The amylase activity was determined by the DNS method (Hemati *et al*, 2018) that uses starch as a substrate. After a reaction (at 40 ℃ for 24 hrs.) and several post-treatment procedures, glucose production from starch was measured at 500 nm absorbance, and the difference between this value and a negative control (not pure water but a trypticase-soy medium without glucose) was taken as the amylase activity.

2.6.3 Dehydrogenase

The dehydrogenase activity was measured according to the method used by Vargas-García *et al*, 2010. The triphenyl-formazan concentration of microbial cells was measured in the supernatant of cell culture at 482 nm absorbance. The microbial number was determined by OD_{610} measurement.

3. Results and Discussion

3.1 System Performance

The temperature and pH in the reactor over a course of 24 hours are shown in Fig. 2. There were two experimental runs and almost the same results were observed in the two experiments implying that system performance was repeatable. After day 2, the temperature in the reactor rose rapidly from 30 ℃ to more than 50 ℃ six hours after the input of fresh feed and decreased to the original value, while ambient temperature was more or less constant during the entire experimental period. This phenomenon was regularly recurred on a daily basis suggesting an established steady-state condition in the reactor. Since there was no artificial heating nor cooling, the temperature based on the microbial cell's heat generation by oxidation reaction was the most reliable index of the aerobic decomposition of organic matter. The pH in the reactor did not change drastically, but gradually decreased from pH 8 to pH 6.5, which were normal values of composting.

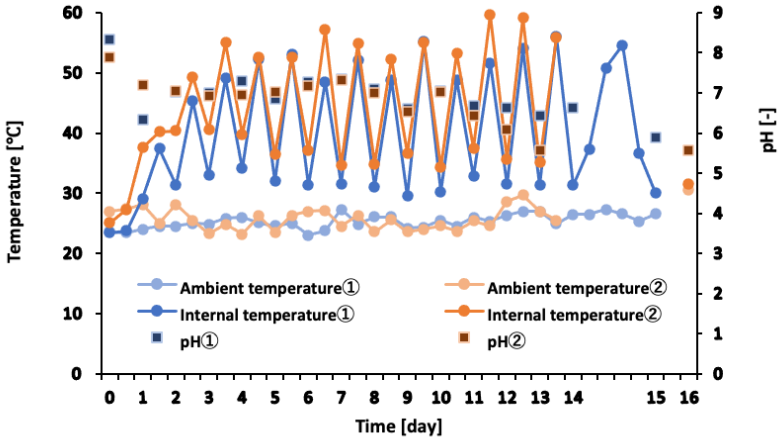


Figure 2. Temperature and pH change in the reactor

The system's optimal performance in which total input increased as fresh feed was input on a daily basis and the total weight of the reactor slightly decreased during the experimental period is shown in Fig 3. The difference between the weight of total input and that of the reactor represents the system's weight loss from water vaporization and organic material decomposition. The moisture content in the reactor gradually decreased from ca. 60 % to ca. 30 %, as shown in Fig. 3. Organic decomposition by dry basis was almost constant at ca. 50 % to ca. 55 % (data not shown). All these data suggest a successful composting process in this experiment based on a system's operation point of view.

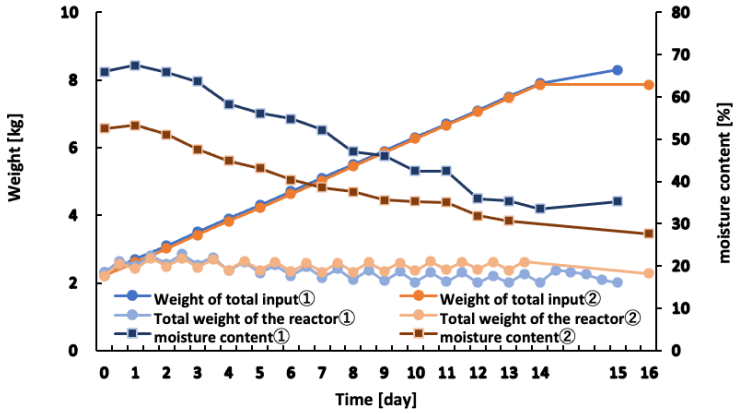


Figure 3. Changes in the weight of total input and the reactor, and moisture content

3.2 Microbial Ecosystem Observation

The total microbial number in the reactor over a time course is shown in Fig. 4. The number of microbes was measured as C.F.U. per gram (dry) of compost residue. The results of the two runs were almost the same: After day 2, the total number of microbes was more or less constant at 1.0×10^{11} CFU/g during the entire experimental period. There were eight types of microbes isolated from Run 1 (W1∽O1), and seven types from Run 2 (a∽g), based on color and shape as displayed in Table 1. Microbe colors were white (W), pink (P), beige (B), yellow (Y), and orange (O), and their shapes were circular, irregular, flat, or hemispherical. DNA from isolated microbes are shown in Fig. 5. Since the bands of Y1, W1', and W2 were the in same positions as those of d, b, and c, respectively, we concluded these colonies were comprised of the same microbial species. On the other hand, there were several bands observed in colony "a's" lane suggesting the coexistence of several kinds of microbes; however, it was impossible to further isolate these microbes precisely. Thus, we eliminated colony "a" from the isolated microbes.

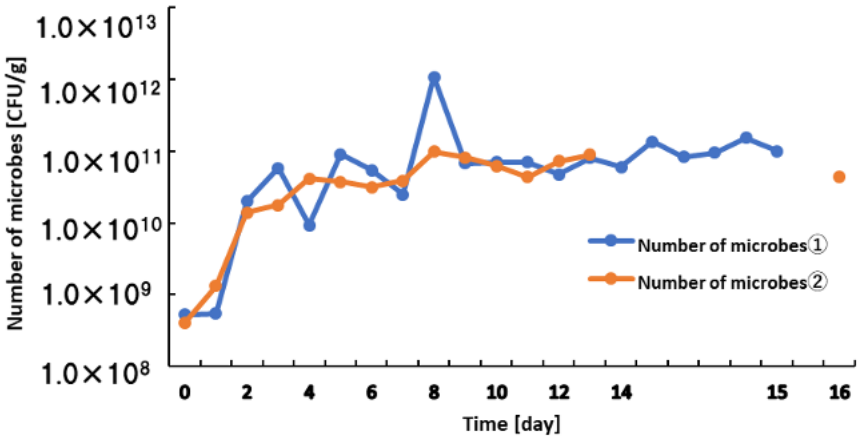
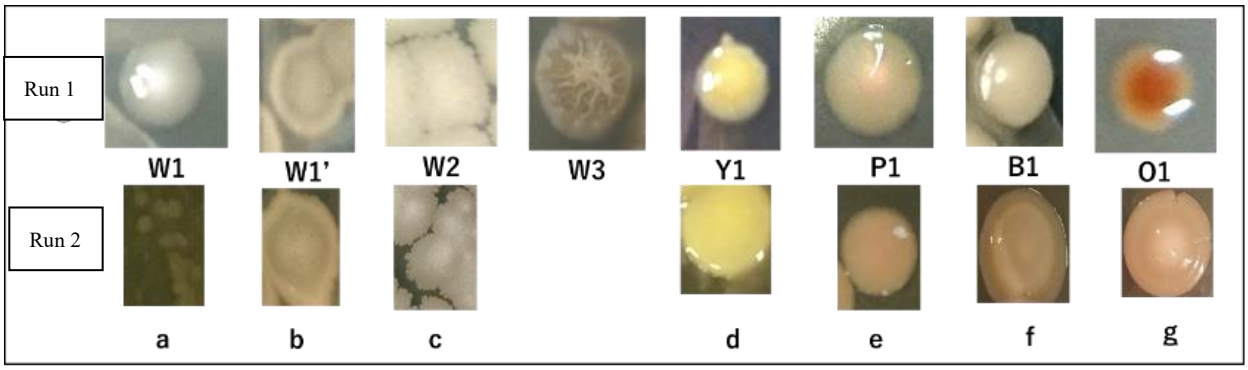


Figure 4. Changes in the total microbial number in the compost residue

Table 1. Variety of isolated microbes



The compost residue in the reactor was sampled twice a day (10:00 and 16:00) and a daily microbe isolation and count was done during the experimental period. The results of Runs 1 and 2 are shown in Figures 6 and 7, respectively. The isolated microbes were constantly changing, even when isolated on the same day, and no specific microbes was the dominant species in this system. This meant that no "steady state" was established in the microbial ecosystem of the composting process. Although the temperature in the reactor regularly changed as shown in Fig. 2, a change in the colony composition appeared to be due to other factors. It is also very unlikely that a specific microbe could dominate the composting process performance because the decomposition rate of organic matter was almost constant. This decomposition rate is shown in Fig. 3 (the decomposition rate on dry basis was 50 to 55%). The behavior of this microbial ecosystem, i.e. always changing, diverged from that of the total microbial number which was almost constant after day 2 as shown in Fig. 4. In other words, the microbial ecosystem in the composting process, especially in a continuous operation, is very stable from a macroscopic viewpoint but is never stable microscopically. This may be a common feature of microbial ecosystems comprised of so many types of microbes, such as intestinal, soil and water bodies' bacterial flora.

3.3 PCR-DGGE Analysis of Microbial Ecosystem

The PCR-DGGE pattern and colony composition change in Run 2 (the same as Fig. 7) is shown in Fig. 8 that also displays the PCR-DGGE pattern of each individual colony. From this figure, many kinds of microbes continued to exist in the composting process based on the band pattern of isolated colonies. The PCR-DGGE band pattern displayed the DNA of all microbes in the compost residue regardless of whether they were alive or dead or of their metabolic function. The intensity of the PCR-DGGE band changed slightly during the experimental period, but the band pattern largely did not change denoting a constant existence of these microbes. On the other hand, the colony's composition changed drastically as shown in Figs. 6 and 7. Differences between the intensity of the PCR-DGGE band and the colony's composition could be attributed to following: Only seven or eight major microbe types could be counted on a culture plate because of the sample residue large dilution rate (by a factor of more than 10^6) that drastically changed the colony ratio that was presented in percentage. From Fig. 8, microbes that appeared as isolated colonies on the culture media existed in the compost residue since all bands were seen in every lane during the experimental period. The colonies such as W1, W1', Y1, P1, and B1 were often observed in other experiments (data not shown). The nine major microbes identified by 16S rRNA gene analysis are shown in Table 2.

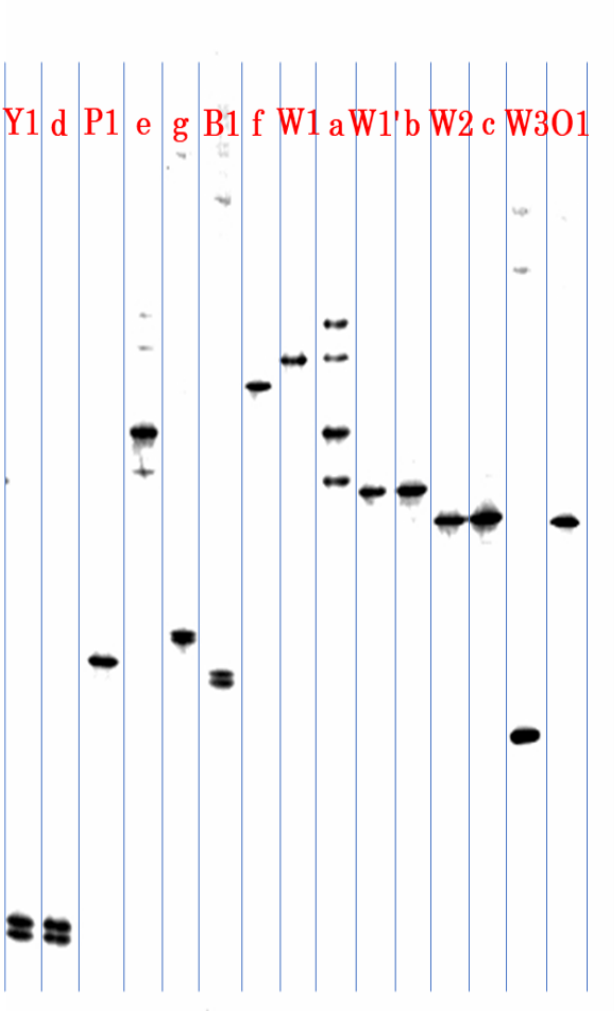


Figure 5. The PCR-DGGE band patterns of isolated microbes

Table 2. Identified microorganisms in the composting process

Colony	Bacterial species	Temperature
W1	*Cellulosimicrobium funkei*	Mesophilic (M)
W1’	*Bacillus cereus*	M
W2	*Bacillus subtilis subtilis*	M
W3	*Streptomyces thermocarboxydus*	Thermophilic (T)
Y1	*Cellulosimicrobium cellulans*	M
P1	*Alcaligenes faecalis faecalis*	M
CP1	*Paracoccus kondratievae*	M
B1	*Bordetella trematum*	M
CB1	*Proteus mirabills*	M

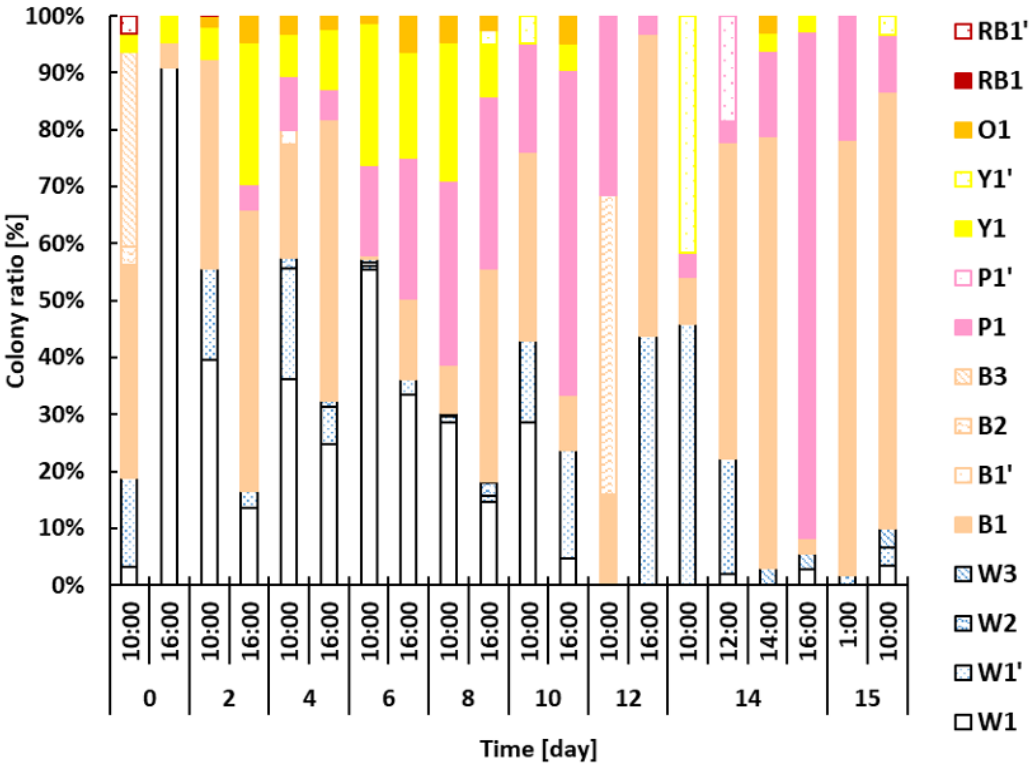


Figure 6. Change in the composition of isolated colonies in Run 1

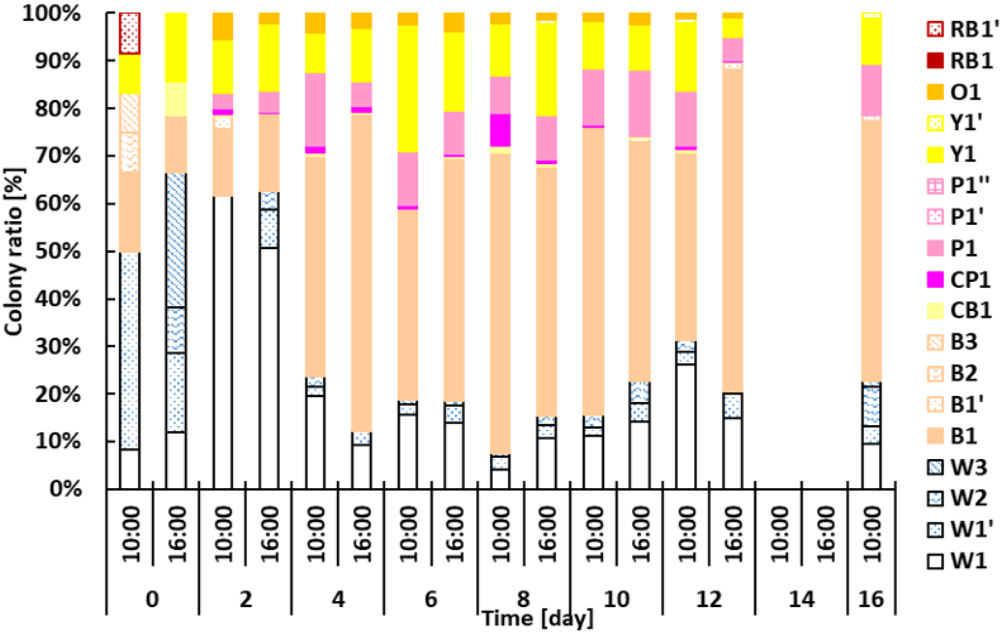


Figure 7. Change in the composition of isolated colonies in Run 2

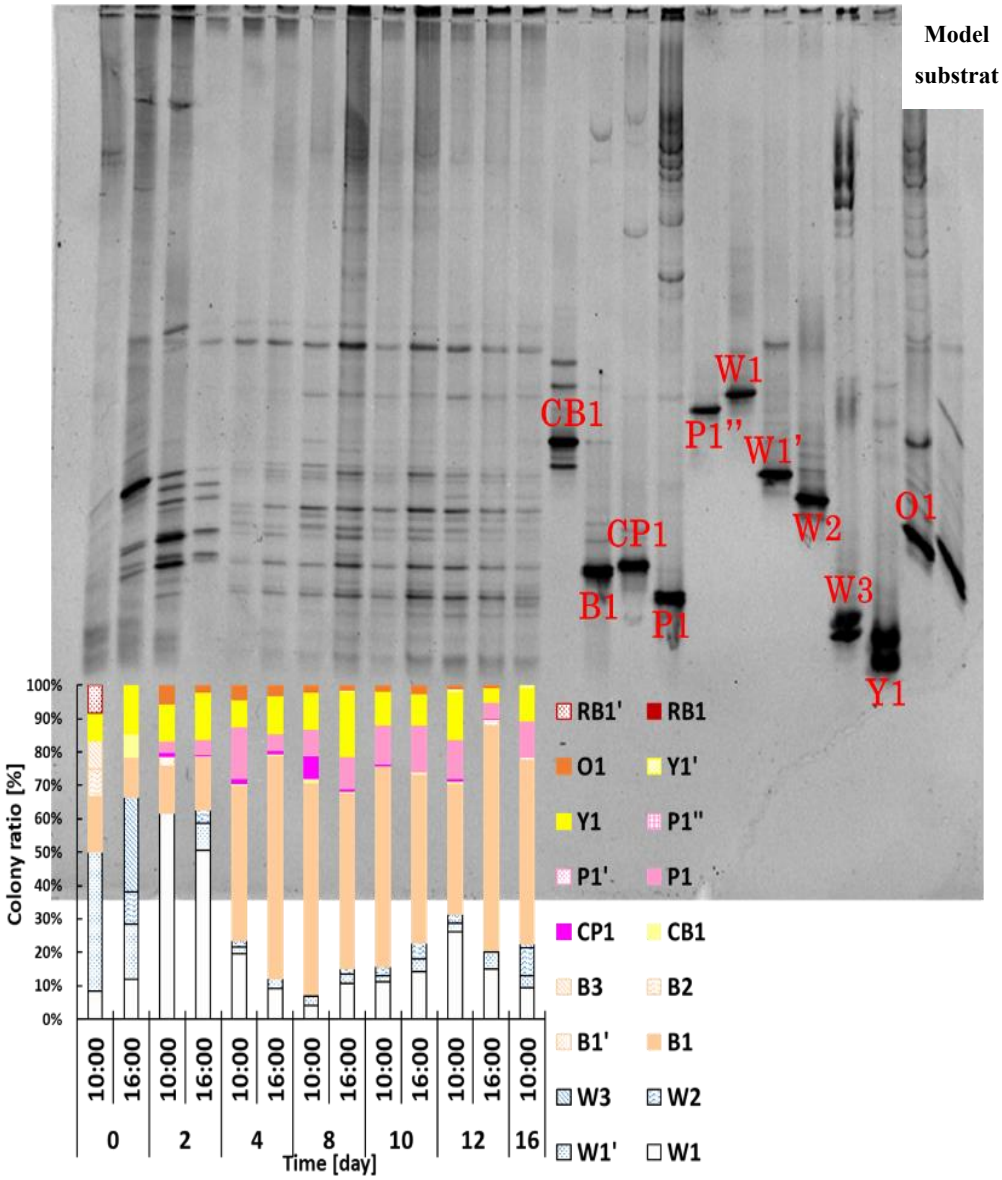


Figure 8. Run 2's PCR-DGGE pattern and changes in colony composition

3.4 Characterization of Microbes Based on Metabolic Function Analysis

The enzymatic activities of isolated colonies were measured and expressed as the enzymatic activity of a standardized microbial number based on OD value. Since the standard curve (OD vs Microbial number) was different for each colony, eight standard curves were plotted for the eight colonies identified (data not shown). The enzymes measured were protease, amylase, and dehydrogenase as described in 2.6. FDA (fluorescein diacetate, by Ryckeboer *et al.* 2003) was used as an index of hydrolytic activity: an important step in the solubilization process. The results from these measured activities were normalized to the highest value of each activity per unit time of cultivation (day) as a percentage. Normalization was done because each enzyme had a different unit of enzymatic activity and thus absolute values of their activities could not be directly compared. Furthermore, it took one or two days to measure enzymatic activity and OD. The amino acid decomposition test's results (Yamauchi 2018) were also shown here. Using this test we observed growth of isolated colonies on Murashige and Skoog (MS) medium that had a specific amino acid (six kinds, 2%) and sugar (glucose or sucrose, 3%) added to it. The amino acid solution was sterilized by filtration (0.22 μm) and the MS with sugar medium was treated using the normal autoclave method. The cultivation time was seven days at a temperature of 40 ℃. The results of this test are shown in Table 3. W1, W1', B1, and O1 did not grow on all culture media suggesting that they had strict auxotrophic conditions. Contrarily, W2 and W3 grew on many culture media signifying their survival in poor nutritional conditions and an ability to decompose various amino acids. The colonies P1 and Y1 showed the same distinct

characteristics, i.e. that they could grow without sugar. The radar chart shown in Fig. 9 displays the nutrient metabolism each colony. All colonies had different shapes denoting that the listed microbes had dissimilar nutritional requirements.

Table 3. The growth of isolated colonies on the specific cultures

Run	W1	W2	W3	W1'	B1	P1	Y1	O1	culture medium
1	-	+	+	-	-	-	-	-	MS + Glucose + : growth
2	-	+	+	-	-	-	-	-	MS + sucrose - : no growth
3	-	+	+	-	-	+	+	-	MS + L-alanine
4	-	+	+	-	-	-	-	-	MS + Glucose + L-alanine
5	-	+	+	-	-	-	-	-	MS + sucrose + L-alanine
6	-	-	-	-	-	-	-	-	MS + Glucose + L-phenylalanine
7	-	+	-	-	-	-	-	-	MS + sucrose + L-phenylalanine
8	-	-	-	-	-	-	-	-	MS + Glucose + L-cysteine
9	-	-	-	-	-	-	-	-	MS + sucrose + L-cysteine
10	-	+	-	-	-	-	-	-	MS + Glucose + glycine
11	-	+	-	-	-	-	-	-	MS + sucrose + glycine
12	-	-	-	-	-	-	-	-	MS + Glucose + L-glutamic acid
13	-	-	-	-	-	-	-	-	MS + sucrose + L-glutamic acid
14	-	+	+	-	-	+	+	-	MS + Glucose + L-lysine
15	-	+	+	-	-	+	+	-	MS + sucrose + L-lysine

The colony composition fluctuation is shown in Figs. 6 and 7. This fluctuation may be related to juxtaposing different microbial nutritional requirements to the system's ever changing nutritional concentrations during the composting process. Due to this, microbes that found the existent conditions optimal would grow faster and occupy dominant portions in the colony. Obviously, other physicochemical factors such as temperature, moisture content, and pH would also affect microbial activity. Nevertheless various microbial nutritional requirements would have an impact on microbial growth. From the radar chart in Fig. 9, the following considerations can be pointed out: The colonies Y1(*Cellulosimicrobium cellulans*), P1(*Alcaligenes faecalis faecalis*), and O1(not identified) detection by colony observation and PCR-DGGE denoted their crucial roles in the composting process but did not portray their high enzyme activities suggesting their possession of additional enzymatic activities or functions. Since the colony B1(*Bordetella trematum*) showed high dehydrogenase activity only, this microbe may have an important role in the metabolization process. An increase in the B1 colony after day 7 or later as water-soluble nutrients increased with solubilization progression over a few days, indicates their metabolization role. The colony W1'(*Bacillus cereus*) showed only a high protease activity signifying its role in protein decomposition. W2(*Bacillus subtilis subtilis*) showed generally high enzymatic activities for all evaluated enzymes except protease. W3(*Streptomyces thermocarboxydus*) also showed generally high enzymatic but it did not occupy a dominant portion in the colony probably because it was the only thermophilic bacterium identified in this study. This study's results indicate that the microorganisms complemented each other, and *Bordetella trematum*, *Bacillus cereus*, *Bacillus subtilis subtilis* and *Streptomyces thermocarboxydus* effectively decomposed garbage.

4. Conclusion

Microbial behavior in a "static type" garbage treatment system was analyzed using three different approaches, colony observation, DNA analysis, and metabolic characterization. The system's performance in two-weeks' continuous operation was satisfactory in terms of temperature change, weight reduction on both a wet and a dry basis, and pH change. A "steady state" was established based on macroscopic physical parameters and total microbial number. However, microbial composition and the number of colonies on the medium plate changed everyday and did not attain a "steady state". This implies that the treatment system was not completely efficient. The PCR-DGGE analysis illustrated that during the operation period many microbes constantly dwelt in the composting residue; however, fewer than ten colonies were found on the plate media. Bands of isolated colonies were identified from the PCR-DGGE band pattern and using the 16S rRNA genome, we identified nine microbes. The identified bacterial microbes were of different species, and they had distinct auxotrophic characteristics suggesting that these microorganisms complemented each other. This may provide information to

help clarify why macroscopic parameters were very stable while microbial colonies were always changing. We believe that this study's approach can help shed light on the "black box" of the composting process.

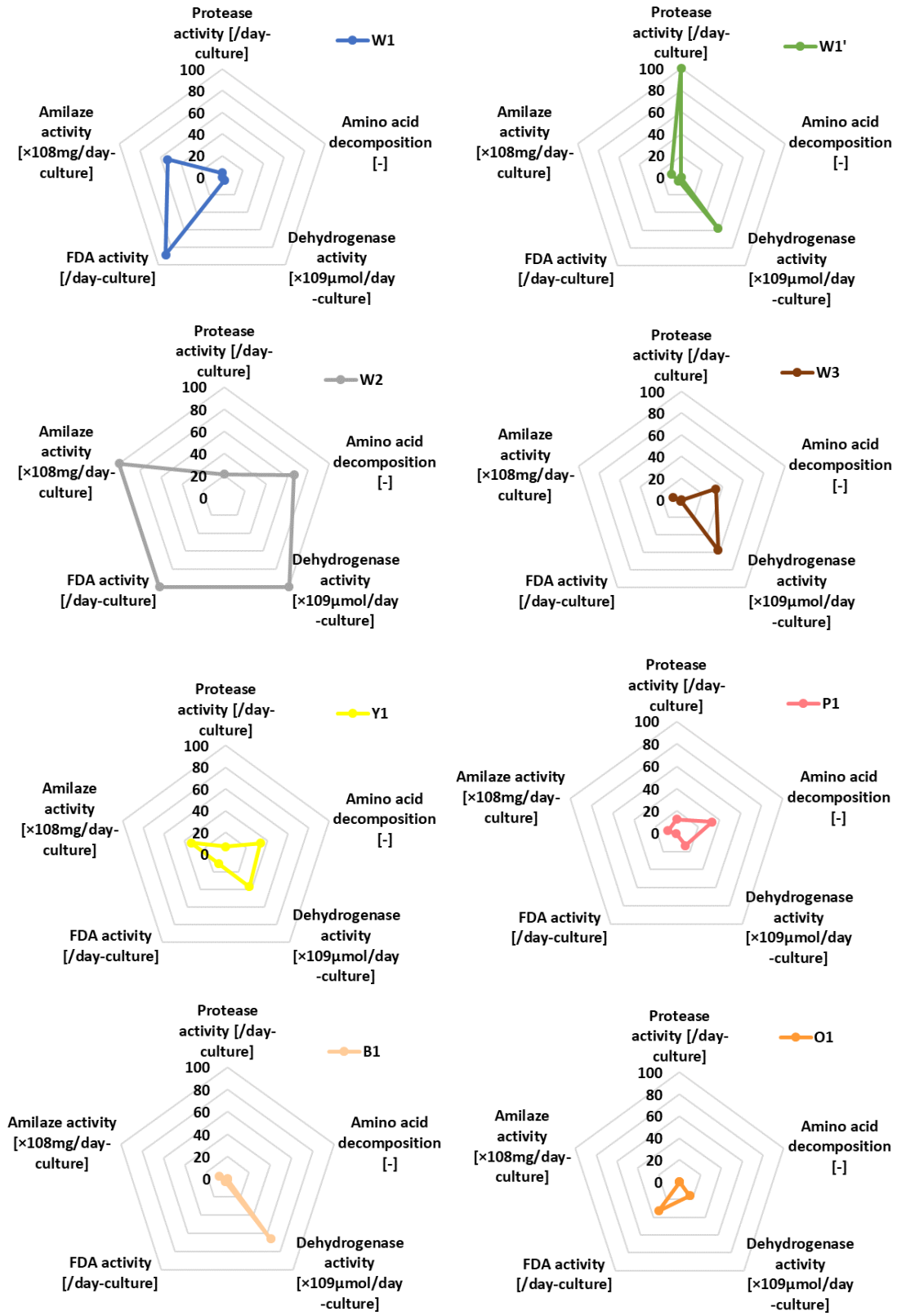


Figure 9. The radar chart of metabolic character of each isolated colony

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