Study on degradation of biodegradable plastics and other materials by aerobic thermophilic flora used for composting

Institute of Environmental Microbiology, Tadao 2-15-5, Machida-shi, Tokyo 194-0035, Japan

Sayaka Soeda, Takahiro Yoshii and Yasuhiro Yoshikawa*

Summary　Aerobic thermophilic flora (ATF) used for composting can rapidly reduce the volume of biosolids and domestic wastes through self-heating and produce pathogen-free compost. Therefore, it attracts attention as new fertilization methods to replace conventional organic fermented fertilizers. This study aimed to clarify degradation of biodegradable plastics (bioplastic) etc. by ATF and the bacteria that contribute to this process. Company M paper wrappers or cups, Company K polymer of 3HB and 3HH (PHBH) bags and City T poly butylene succinate (PBS) bags by ATF composting were efficiently biodegraded at 55ºC and 40-45% moisture content.

In the test of Company M papers, promising 5 predominant bacteria species were detected and among them, facultative anaerobic thermophile *Parageobacillus caldoxylosilyticus* was a high candidate. The closest relative bacterium (NR-135713.1) may be involved in the thermal biodegradation. In case of Company K PHBH bags, no literature could be found that any of the 5 predominant bacteria have PHBH esterase. However, *Ureibacillus suwonensis*, an obligate aerobic thermophile that degrades polyesters, has a cutinase, and an aerobic thermophile, *Ureibacillus thermosphaericus* has an esterase degrading polyhydroxyalkanoate. Close relatives of these aerobic thermophile (JX914499.1, LT631780.1 are former, and AP018335.1 is latter) may have PHBH esterase activity. In the test of City T PBS bags, 6 predominant bacteria were shown. Five of these were not found in the literature to have PBS esterase activity. However, *Geobacillus stearothermophilus*, a facultative anaerobic thermophile has polystyrene degrading enzymes and cellulase, which may have PBS esterase activity. Close relative of this bacterium (MF965113.1) is possible candidate.

There are few data on the specific esterase activity of PHBH and PBS studied in aerobic thermophiles. Because there are not so many research groups on aerobic thermophiles worldwide and the short period of time since the bioplastics started to be used internationally. In the future, it is necessary to isolate and culture candidate species of aerobic thermophiles that degrade bioplastics more efficiently, and to identify specific genes and measure the enzyme activities.

Key words: aerobic thermophile, composting, bioplastics, polybutylene succinate (PBS), polymer of 3HB and 3HH (PHBH)

*Correspondence to: Yoshikawa, Y.: y-yoshikawa@ous.ac.jp

Received 3 September 2024; Accepted 27 September 2024

Introduction

Environmental pollution by persistent plastics is a new and serious international problem. The United Nations Environment Programme (UNEP) reports that "plastic pollution is a global problem with serious environmental, social, economic, and health consequences, and each year, between 19 and 23 million tons of plastic waste enter aquatic ecosystems, causing habitat changes and reducing the ability of ecosystems to adapt to climate change", and proposes international legally binding regulations[1]. Stable materials that do not degrade in the environment, such as mass-produced plastics and the recently highlighted super persistent PFAS (perfluoroalkylated substances, e.g., 6-carbon PFHxA, 8-carbon PFOA, PFOS, etc.), are conversely a great threat to human life. As for PFAS, the U.S. Environmental Protection Agency (US-EPA) has conducted an extensive risk assessment of PFAS and reported it in the form of the Integrated Risk Information System (IRIS)[2].

Bioplastics are emerging as an alternative in this situation. There are reviews of microorganisms (bacteria and fungi) that biodegrade such plastics[3] and with respect to oxygen, it is stated that aerobic conditions (presence of oxygen) are generally more favorable for biodegradation of bioplastics when compared to anaerobic conditions, because many enzymes, such as cutinase and esterase, function best in the presence of oxygen[4]. In addition, the enzyme population of thermophilic bacteria is known to be more degradative than those of normal bacteria, and industrial applications are underway[5].

Bioplastics are materials that are decomposed by microorganisms into water and carbon dioxide. They are currently being developed by major manufacturers as bags for composting food waste, spoons, straws, and other cutlery (generic term for knives, forks, spoons, etc. for dining tables), packaging materials, agricultural films, medical supplies, toys, electrical products, and textiles. Therefore, it is expected that many bioplastics of various types will be available as raw materials for food waste composting projects in the future. This study is a start line investigation for a goal to find useful thermophilic biodegrading bacteria groups that can be effectively promote the use of bioplastic bags etc. and to propose to local government the composting facilities that can completely biodegrade these materials.

Materials and Methods

Bioplastics

Paper wrappers and paper cups from Company M coated by PBS; bags of polymer of PHBH (copolymerized polyester consisting of R-3-hydroxybutanoic acid (3HB) and R-3-hydroxyhexanoic acid (3HH)) from Company K; biodegradable PBS

PHBH Poly（3-hydroxybutyrate-co-3-hydroxyhexanoate）　　PBS polybutylene succinate

Fig. 1　Structural formula for bioplastics of PHBH and PBS. PHBH is a copolymer of two different monomers, 3HB and 3HH and this co-polymerization introduces flexibility to the structure compared to homopolymers like PHB (Polyhydroxybutyrate). The 3HB units provide rigidity and strength, while 3HH units introduce more flexibility and elasticity. PHBH is fully biodegradable in various environments, including soil and marine ecosystems, and used as an alternative to conventional plastics. PBS is synthesized from succinic acid and 1,4-butanediol. Its structure consists of ester bonds (-COO-) between the two monomers, giving it typical polyester properties. PBS is relatively flexible compared to some other biodegradable plastics, and is used in packaging films, agricultural mulch films, disposable products (e.g., cutlery, plates), and in certain medical applications due to its biodegradability and biocompatibility.

(polybutylene succinate) bags from City T were used as bioplastic sample (Fig. 1).

Aerobic thermophilic bacterial flora (ATF) as seed bacteria for compost

Aerobic thermophilic biodegrading bacterial flora (seed bacteria) were used to identify candidate bacterial groups that can effectively degrade the bioplastics. These are the seed bacteria used during the rapid composting of biosolid (old name: activated sludge) and domestic residues. In the case of MS plant compost (seed), the target is domestic residues such as food waste, while in the case of SG plant compost (seed), the target is digested sludge after methane is recovered. The fertilizer created by composting the target materials with ATF in each plant is used both as fertilizer and as seed bacteria. Thus, this composting is a sustainable reproduction system with complete self-contained thermal biodegradation.

Biodegradable samples

The test samples were paper wrappers and paper cups from Company M (coated with PBS), bags from Company K (PHBH) and PBS bags from City T (Fig. 2).

Thermal biodegradation test conditions

The treatment temperature was set at 55ºC to avoid excessive drying. Each compost (seed bacteria) was screened using a sieve with mesh size 2 mm before determination of moisture condition. The moisture content was determined as 'MAX water retention', which is the maximum moisture content at which water does not seep out: 40% for MS compost and 45% for SG compost. The test period was 9 weeks. This was because after two months, the test samples produced little CO_2 by biodegradation (end of decomposition).

Experimental procedure.

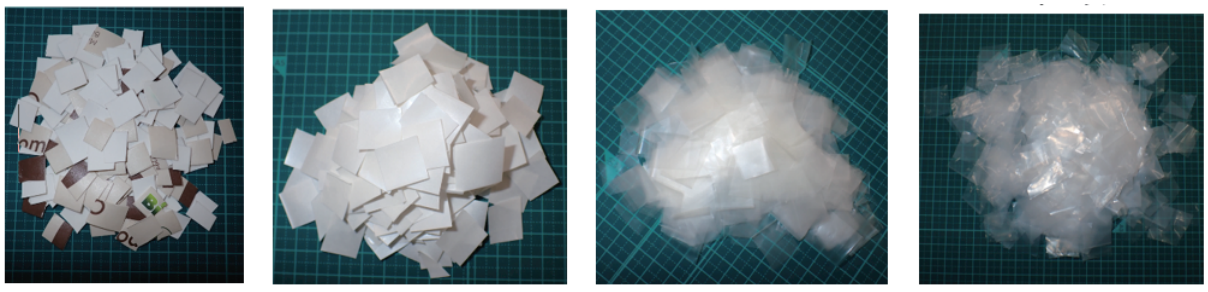
The compost used in the experiment was stored in a refrigerator for several months.

To achieve a state close to composting, the compost and water were mixed in a zippered polyester bag, the moisture content was adjusted to 30%, and the bag was placed in an incubator at 55ºC overnight for pre-culturing. Then, the test sample weight 0.5 g was added to 15 g of pre-cultured compost. The MS compost was watered to 40% and the SG compost to 45% and incubated in a container placed in an incubator at 55ºC. For bacterial flora analysis by DGGE (Denaturing Gradient Gel Electrophoresis) methods, sampling was carried out every 2 weeks.

The overall weight including a container was measured on days 3 and 7 after the start of the experiment, and weekly thereafter (weeks 2-9). The water content was calculated and replenished, assuming that the only weight change was water. The amount of CO_2 emissions was measured, and the degree of biodegradation was calculated. The experimental procedure is shown in Fig. 3.

Calculation of biodegradation rate

The biodegradation rate was calculated from the CO_2 emissions from the sample using the molecular formula and carbon ratio shown below. As a specif-



Company M, cups　Company M, wrappers　Company K, bags　City T, bags

Fig. 2　The test samples. These were crushed as uniformly as possible to 1~2 cm x 1~2 cm.

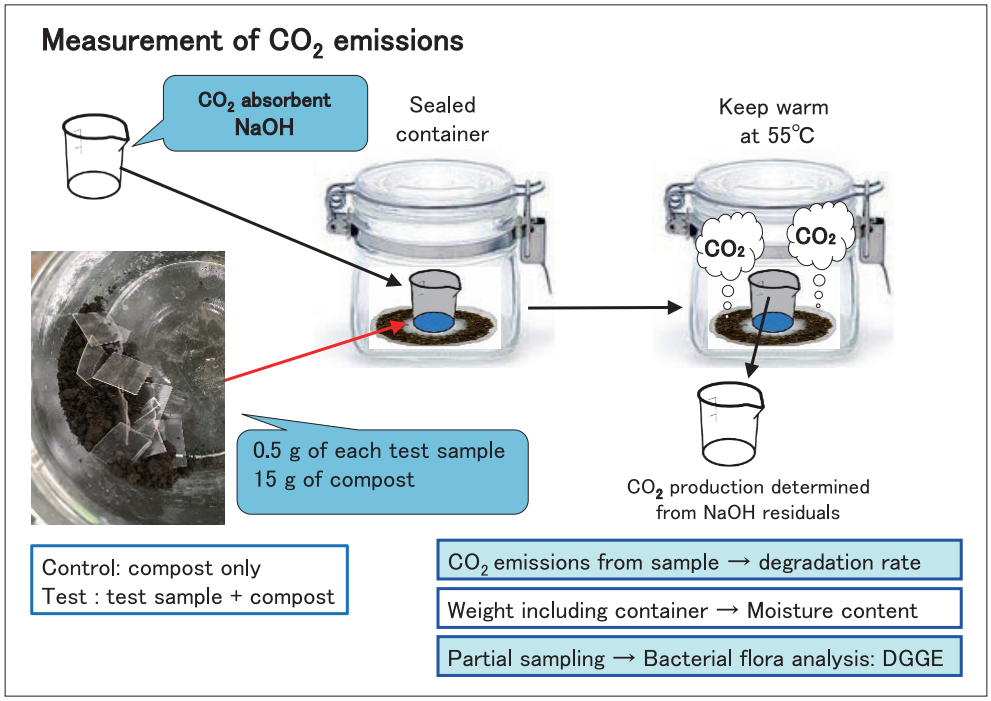


Fig. 3　Outline of the experiment procedures. The stored composts were pre-cultured and then mixed with the test samples to biodegrade the bioplastics by aerobic thermophilic flora while maintaining the pre-set temperature and moisture content. Thermal biodegradation was calculated from the amount of CO_2 gas produced. The dominant bacterial species active in the biodegradation process were sampled every 2 weeks and analyzed using the DGGE method.

ic example, the following calculation is made for a paper sample from Company M.

The degradation rate (Y%) of the sample was calculated from sample-derived CO_2 generation (X mg), and the molecular formula of cellulose $(C_6H_{10}O_5)$ n using the following formula.

1. 72 (carbon molecular weight: MW, 6 × 12) ÷ 162 (cellulose MW, 72 + 10 + 80) × 100 = 44.4 (carbon percentage %)
2. 0.5 (g, sample weight) × 0.444 (carbon ratio) × 44 (CO_2 MW, 12+32) ÷ 12 (C MW) × 1000 = 815 (mg of CO_2 produced at 100% degradation)
3. X (mg of CO_2 produced from sample) ÷ 815 × 100 = Y (% degradation)[6]

The paper sample from Company M is mostly cellulose $(C_6H_{10}O_5)$ n, and the weight of covered PBS seems to be almost negligible compared to the weight of the paper. Therefore, it is not considered in this experiment. Company K bags are PHBH $(C_4H_6O_2)x + (C_6H_{10}O_2)y$ (second component fraction 5 mol%: The mole percent of a copolymer is an indicator of the percentage of moles of each monomer in the copolymer). The exact second component fraction is unknown, but was calculated by adopting the values found in the literature of Company K. The PBS in the bag from City T has a carbon fraction of 56.3%.

Bacterial flora analysis (PCR-DGGE method)

Bacterial genome extraction was conducted using soil DNA extraction kit (ISOIL for Beads Beating) from Nippon Gene. PCR primers were used as follows; 357FGCN1: 5'-CGC CCG CGC CCC GCG CCC GGC CCG CCC CCG CCC CTA CGG GAG GCA G-3' and 907R-ex1: 5'-CCC GTC AAT TCM TTT GAG TTT-3'. TaKaRa ExTaq HS DNA polymerase was used, and thermal cycler is 30 cycles.

Electrophoresis conditions were 0.5 x TAE (Tris Acetate ETDA) Buffer, 100 V, 720 min using 7 M urea and 40% formamide as the 100% denaturing agents and denature gradient concentration 30-

70%. Electrophoresis was conducted in acrylamide gradient gel of 6-12%. The bands were cut out and PCR was performed using primers for PCR; 357F: 5'-CTC CTA CGG GAG GCA G-3' and 907R-ex1: 5'-CCC GTC AAT TCM TTT GAG TTT-3'

The PCR products were confirmed by agarose electrophoresis and purified with a kit (Wizad SV Gel and PCR Clean-Up System, Promega), after which sequencing analysis was outsourced (FASMAC). Homology search for the sequences was done with BLAST analysis at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Bacterial species with the highest homology (%) and E-Value closest to 0 were recorded.

Results

Biodegradation of Company M paper wrappers and cups with MS compost

Visual inspection

The result of biodegradation of Company M paper wrappers and cups with MS compost is shown in Fig. 4A. Both samples showed progressive biodegradation after the start of the test, which was difficult to visually check, and at the end of test, complete degradation was observed at 55ºC and 40% moisture content. Similar changes were also observed when SG compost was used, so visual inspection data using SG compost is omitted.

Trends in moisture content and CO_2 production

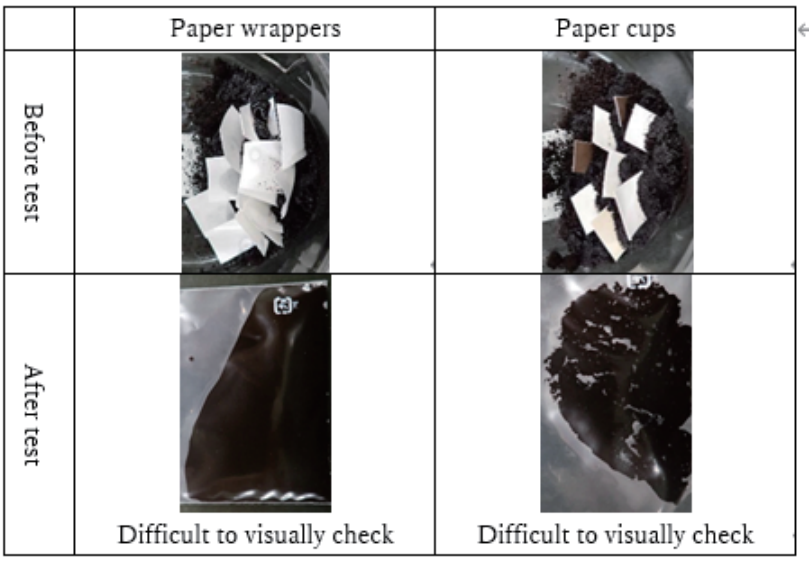
Moisture content changes are shown in Fig. 4B, which suggests that, at 55ºC, the test was slightly over-moistened at start. In contrast, moisture content was less than 20% at 5 and 7 weeks of test. Strict control of moisture was found to be relatively difficult in this small size model.

The trends in CO_2 production are shown in Fig. 4C. It was clear that gross CO_2 production was greatly influenced by the type of compost used as a base: Fig. 4C as paper products regarding MS compost and Fig. 8C as paper products regarding SG compost. However, it became clear that the CO_2 production of the test samples could be read off by measuring the difference from the control (compost only). Thus, we treated gross CO_2 emissions as using relative reference values. There was a decrease in moisture content in the second half of thermal biodegradation (weeks 5 and 7), but this did not seem to have a significant impact in terms of CO_2 emissions.

Biodegradation rates of paper wrappers and cups

The trends of biodegradation rate are shown in Fig. 5A. Thermal biodegradation rates were somewhat slow in weeks 1 and 2, however, increased rapidly from weeks 3 to 5. For paper cups, the rate

A



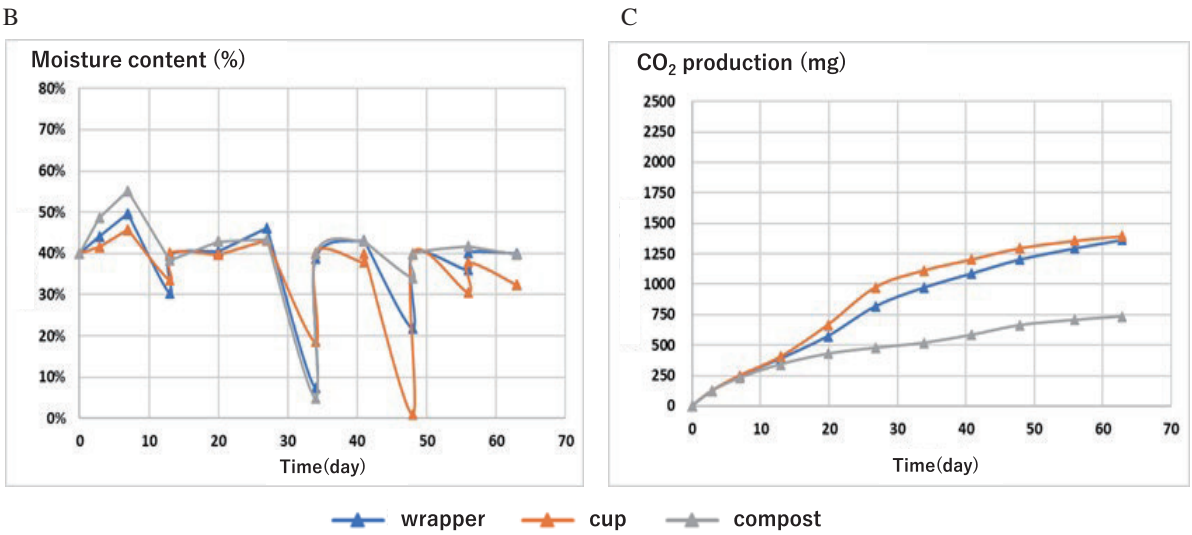


Fig. 4　Biodegradation of Company M paper wrappers and cups using MS compost. (A) The end of the test, the wrappers and cups are completely degraded and are difficult to visually check. (B) Vertical line indicates the moisture content and horizontal line indicates the number of days after the start of the test. Blue line is paper wrappers and orange line is paper cups. (C) The CO_2 production of the test samples (orange and blue lines) could be read off by measuring the difference from the control (compost only: gray line).

has plateaued after 6 weeks, while for paper wrappers, the rate has gradually increased.

In Fig. 5B, a weekly trend diagram of the biodegradation rate and the moisture content was showed. In the early stages of treatment (up to 4 weeks), the biodegradation rate of paper cups with low moisture content fluctuations (around 40%) was higher than that of paper wrappers. However, in the later stages of treatment, both paper wrappers and cups showed high biodegradation rates regard-

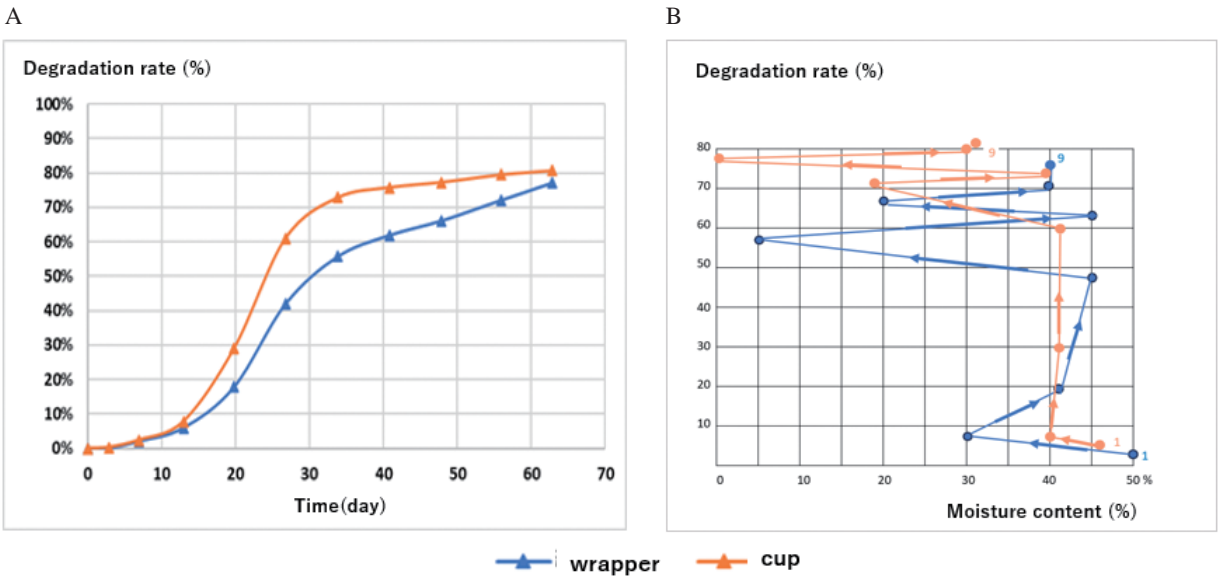


Fig. 5　Trend in biodegradation rate of Company M paper wrappers and cups by MS compost. (A) The ratio of the CO_2 production of the sample only (total CO_2 production minus the CO_2 production of the fertilizer) to the theoretical total CO_2 production was used as the biodegradation rate of the sample. (B) Trends in moisture content and biodegradation rate during the degradation process (weeks 1-9). Every week biodegradation rate is plotted on the vertical line (up to 80%) and the moisture content on the horizontal line (up to 50%).

less of the moisture content fluctuations. This suggests that a stable moisture content is important for the biodegradation of paper in the early stages and that in the later stages the effect of moisture content fluctuations on biodegradation is relatively small.

Biodegradation of Company K PHBH bags and City T PBS bags by MS compost

Visual inspection

The progress of biodegradation of Company K PHBH bags and City T PBS bags in MS compost were shown in Fig. 6A. Both the bags from Company K and City T were completely biodegraded under 55℃ and 40% moisture conditions, and it was difficult to check visually. Note that, similarly with SG compost, the PHBH bags from Company K and the PBS bags from City T were completely biodegraded, so the data are omitted from this paper.

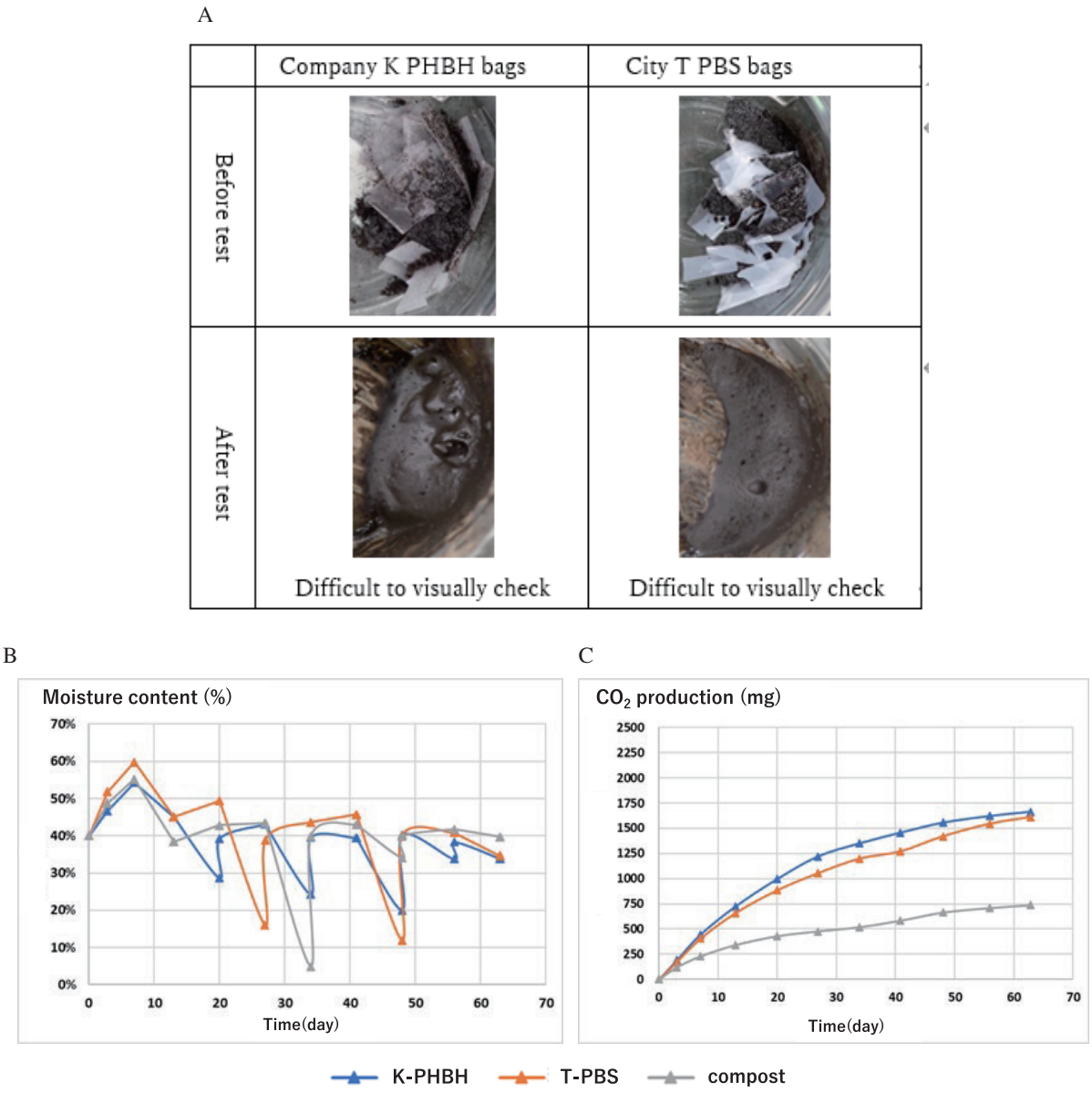


Fig. 6　Biodegradation of Company K PHBH bags and City T PBS bags with MS compost. (A) The end of the test, the Company K PHBH bags and City T PBS bags are completely degraded and are difficult to visually check. (B) Trend in moisture content of Company K PHBH bags (blue line) and City T PBS bags (orange line). Measurements were taken weekly from the start of the experiment until 9 weeks. (C) Trend in CO_2 production of Company K PHBH bags (blue line) and City T PBS bags (orange line). Gray line is MS compost only.

Changes in moisture content and CO_2 production

In trends of change in moisture content are shown in Fig. 6B. Overall, the test became overmoistened at 55ºC. It was particularly the case during the first two weeks of the early testing period. This may be due to the water produced by the vapor generated by the degradation of organic materials such as bioplastic bags. The CO_2 productions are shown in Fig. 6C. Productions of the CO_2 in biodegradation of Company K PHBH bags and City T PBS bags by MS compost tended to be higher than the case for Company M paper wrappers and cups.

Biodegradation rate

The biodegradation rates are shown in Fig. 7A. The degradation rate showed that under conditions of 40% moisture content at 55ºC, the combination of MS compost and either PHBH bags from Company K or PBS bags from City T tended to degrade relatively quickly than Company M paper wrappers and cups, and at a higher degradation rate. As can be seen in Fig. 7B, the degradation rate of the PHBH bags from Company K was particularly high, reaching 80% at week 5 and exceeding 90% at week 9, while the PBS bags from City T reached 70% at week 7 and were until 80% degraded at week 9. Regarding the PBS bags in City T, it is possible that the high fluctuations in moisture content may have had an impact.

Biodegradation of paper wrappers and cups from Company M and PHBH bags from Company K and PBS bags from City T using SG compost

Visual inspection

The results of visual inspection for biodegradation with SG compost were like those with MS compost. All biodegradations had progressed to the extent that it was difficult to see the test samples, so data were omitted.

Moisture content and CO_2 production

Trends in moisture content (Fig. 8A, B) and CO_2 production (Fig. 8C, D) during the biodegrading process of paper wrappers and cups from Company M, and PHBH bags from Company K and PBS bags from City T by SG compost are shown, respectively. The moisture content remained in the range of almost 30-60%, while the CO_2 production

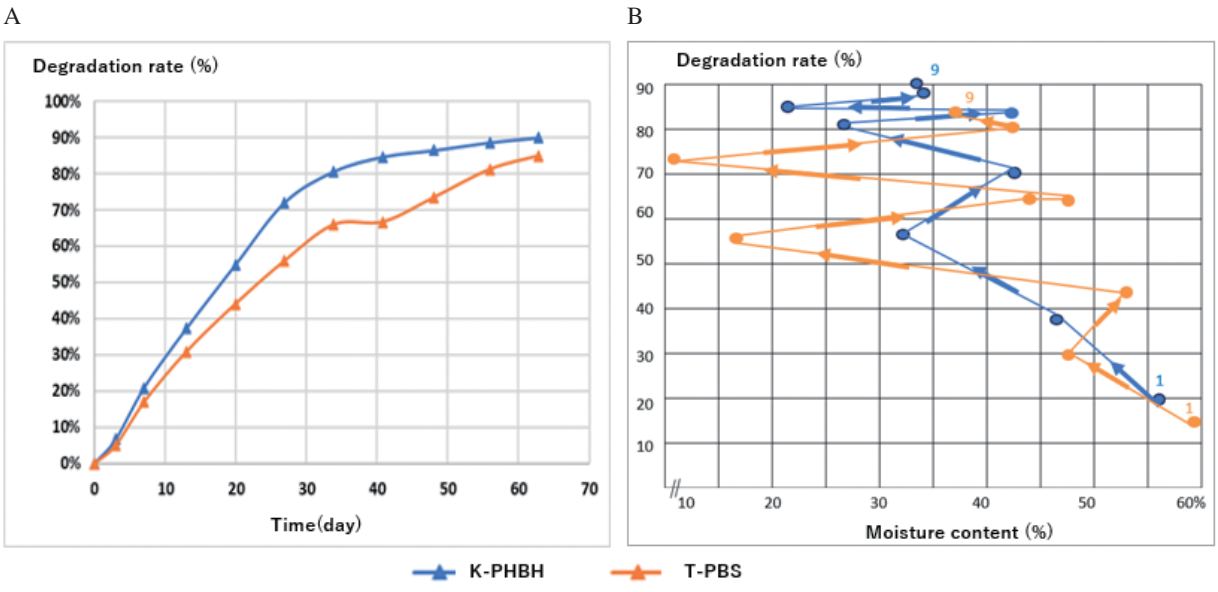


Fig. 7　Trend in biodegradation rates of Company K PHBH bags and City T PBS bags. (A) Blue line is Company K PHBH bags and orange line is City T PBS bags. Vertical line is degradation rate and horizontal line is days after test start. (B) Trend (weeks 1-9) in moisture content (horizontal line: up to 60%) and biodegradation rate (vertical line: up to 90%) of Company K PHBH bags (blue line) and City T PBS bags (orange line).

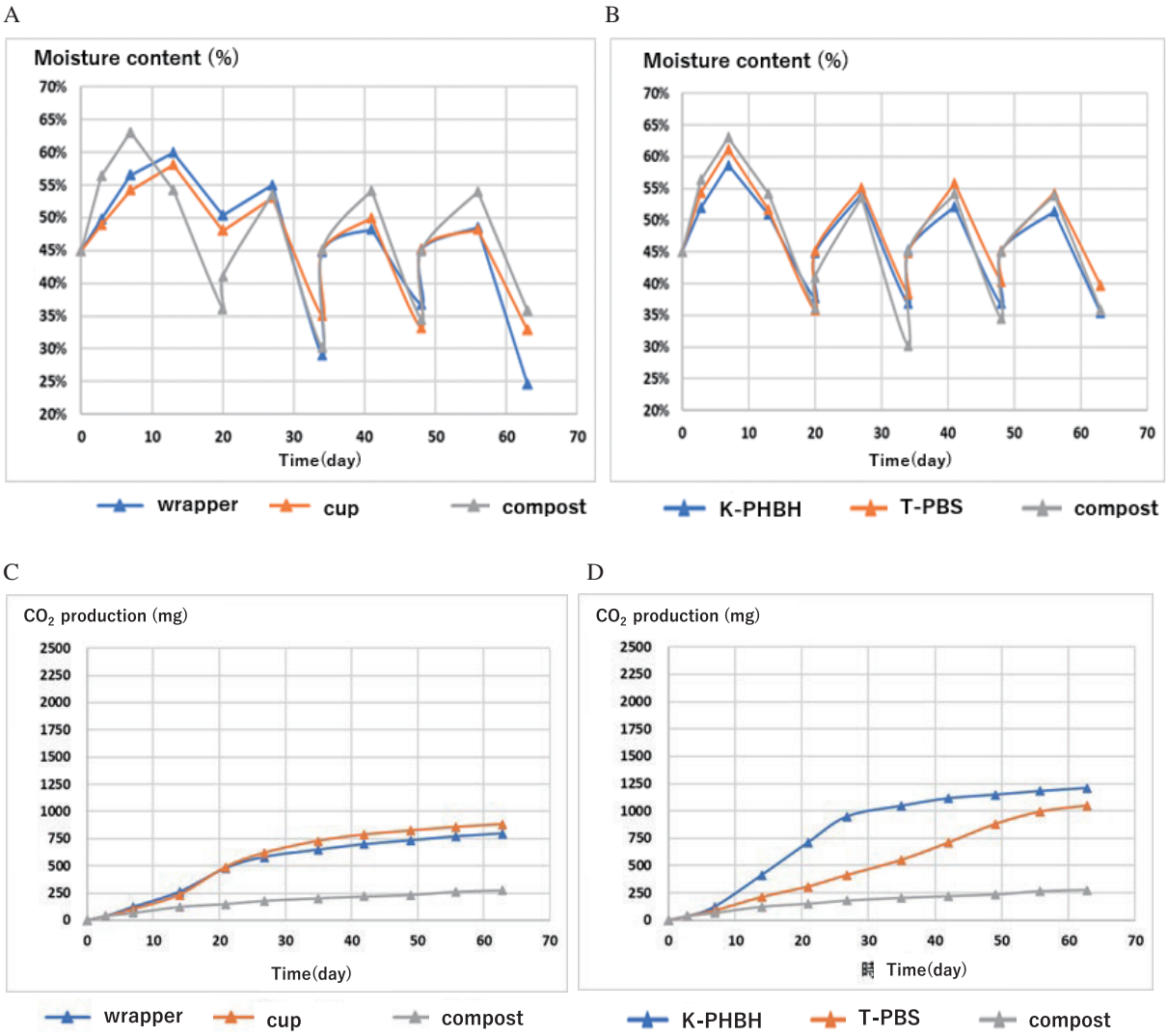


Fig. 8　Trends in moisture content and CO_2 production of Company M paper wrappers, paper cups, Company K PHBH bags and City T PBS bags with SG compost. (A) Change of moisture content. Blue line is Company M wrappers, orange line is Company M paper cups and gray line is compost only. (B) Change of moisture content. Blue line is Company K PHBH bags, orange line is City T PBS bags and gray line is compost only. (C) Change of CO_2 production of Company M paper wrappers (blue line), Company M paper cups (orange line) and compost only (gray line). (D) Change of CO_2 production of Company K PHBH bags (blue line), City T PBS bags (orange line) and compost only (gray line).

tended to be lower than that of MS compost. In both cases, biodegradation was carried out based on 55ºC and 45% moisture content.

Biodegradation rate

The trends in biodegradation rate of Company M paper wrappers and cups with SG compost are shown in Fig. 9A and 9B, respectively. The weekly trends in biodegradation rate and moisture contents of Company K PHBH bags and City T PBS bags with SG compost are shown in Fig. 9C and 9D, respectively.

In the combination of SG compost and paper wrappers/paper cups from Company M, as well as PHBH bags from Company K showed higher biodegradation rates compared to PBS bags from City T at 55ºC and 45% moisture content. Combination of Company K PHBH bags and SG compost showed the highest biodegradation rates like that of MS compost.

The trends in moisture content and biodegradation rate diagrams show that the SG compost has a higher moisture content compared to the MS compost. However, as far as thermal biodegradation is not necessarily related to the moisture content. The transition of degradation rates showed that PHBH from Company K tended to thermally degrade more easily, regardless of the kind of compost. This was followed by PBS from City T, paper cups from Company M and then paper wrappers. Comparing the degradation rate at 5 weeks with that at 9 weeks, Comparing the degradation rate at 5 weeks with that at 9 weeks, (I) SG compost-Company K/PHBH, (II) MS compost-Company K/PHBH, (III) MS compost-City T/PBS, (IV) MS compost-Company M paper cups, (V) SG compost-City T/PBS, (VI) SG compost-Company M/paper cups, (VII) MS compost-Company M paper wrappers and

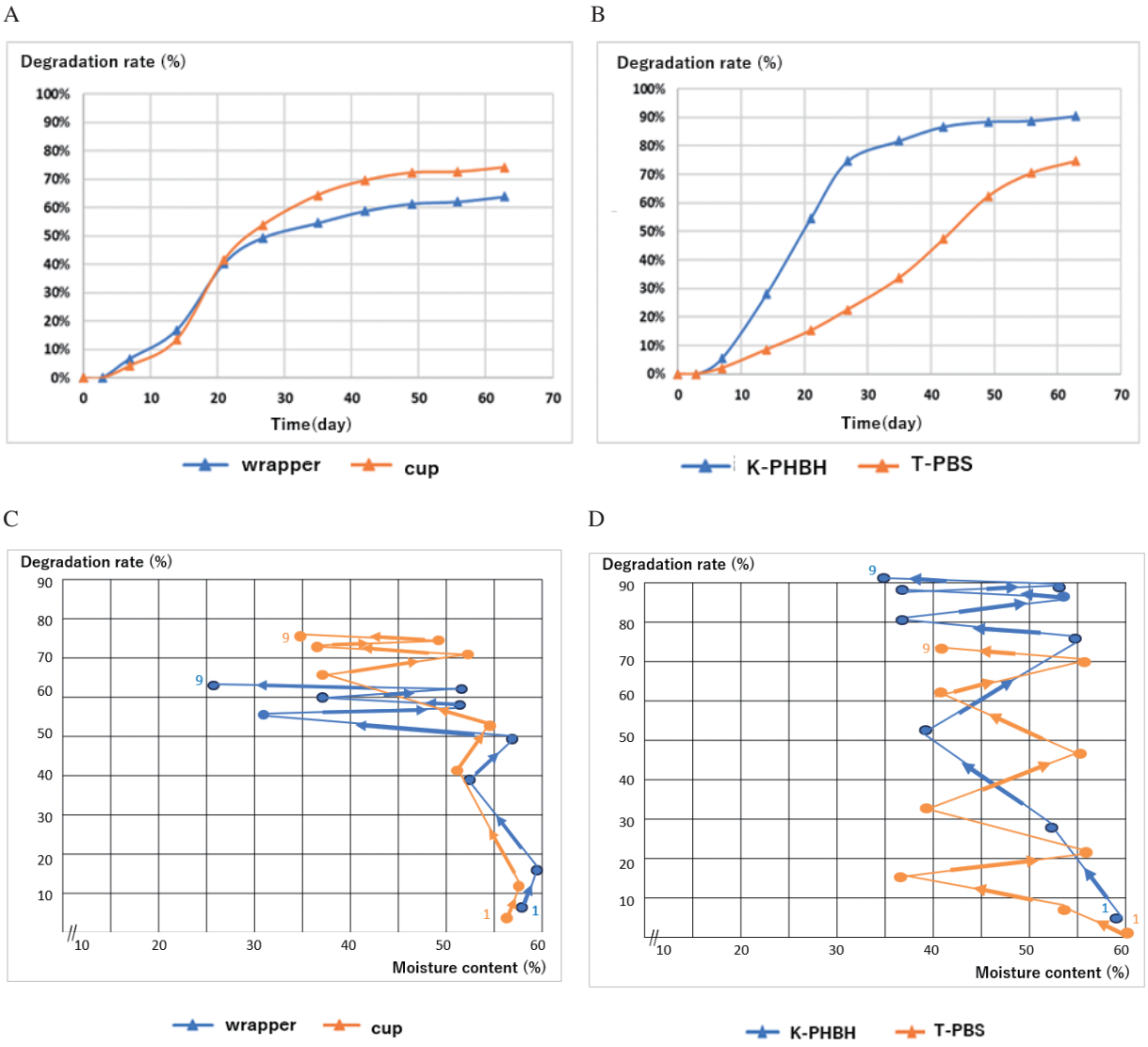


Fig. 9　Trends in biodegradation rate of Company M paper wrappers, paper cups, Company K PHBH bags and City T PBS bags treated with SG compost. (A) Change of biodegradation rate of Company M paper wrappers (blue line) and Company M paper cups (orange line). Vertical line is degradation rate and horizontal line is days after test start. (B) Change of biodegradation rate. Company K PHBH bags (blue line) and City T PBS bags (orange line). (C) Moisture content (horizontal line: up to 60%) and biodegradation rate (vertical line: up to 90%) of Company M paper wrappers (blue line) and Company M paper cups (orange line) treated with compost (weeks 1-9). (D) Moisture content and biodegradation rate (on the same scale as C) of Company K PHBH bags (blue line) and City T PBS bags (orange line).

(VIII) SG compost-Company M/paper wrappers were in that order.

Bacterial flora analysis of samples treated with MS compost

The main bacterial groups detected during biodegradation of samples from Company M paper wrappers and paper cups using MS compost are shown in Fig. 10A, and Company K PHPB bags and City T PBS bags using MS compost are shown in Fig. 10B. As seen from the data of biodegradation rate, Company K PHBH bags were degraded most rapidly and efficiently (80%, 40 days). This was followed by PBS bags from City T (70%, 50 days), paper cups from Company M (70%, 50 days) and paper wrappers from Company M (70%, 60 days).

The bands not seen in the compost itself but seen during biodegradation of samples were as follows. Two bands in Company K PHBH bags, 2 bands in City T PBS bags, 4 bands in Company M paper cups, and 4 bands in Company M wrappers.

Bands with a 'dash' symbol in the number were predicted to be homologous, as they were in the same position as bands with the same number. On the other hand, if the most closely related candidate bacteria were different in the homology analysis, they were analyzed as different candidate bacteria, even if the number was a dash.

Bacterial flora analysis of samples treated with SG compos

The biodegradation rate by SG compost showed a high degradation rate in Company K PHBH bags (80%, 25 days), followed by Company M paper cups and paper wrappers (50%, 35 days), and then

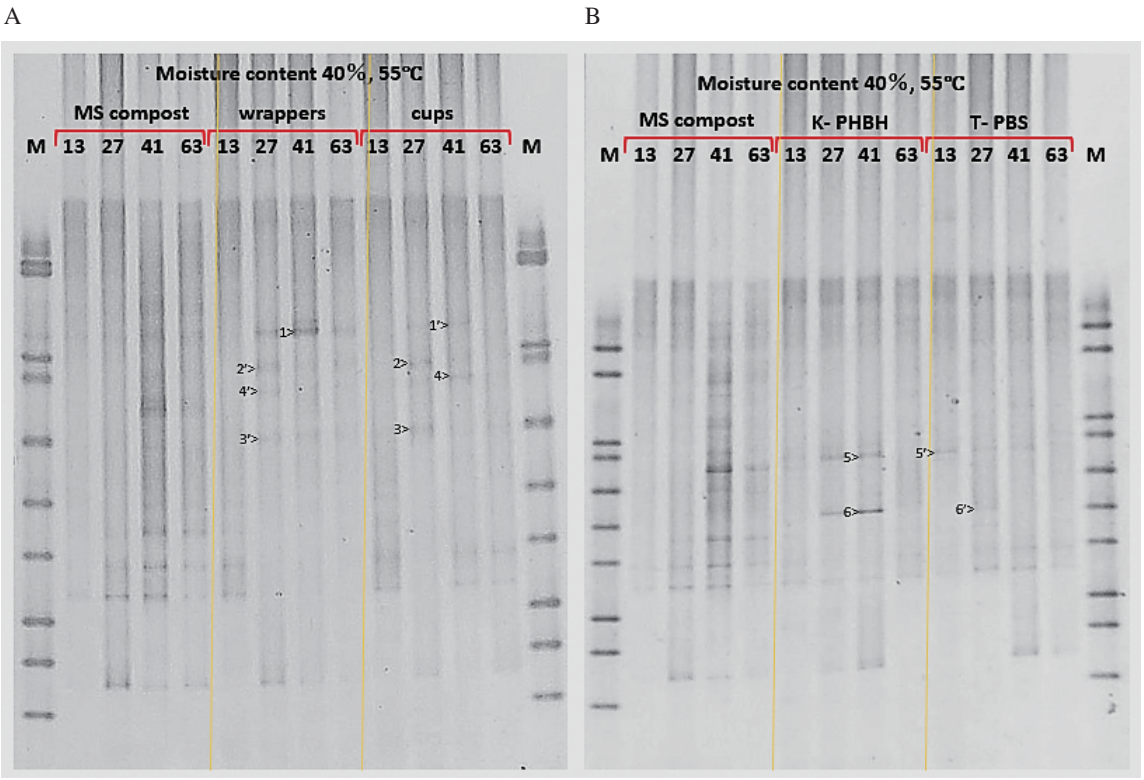


Fig. 10 DGGE analysis of Company M paper wrappers, Company M paper cups, Company K PHBH bags and City T PBS bags treated with MS compost. (A) DGGE image of dominant bacteria during biodegradation (55℃, 40%) in Company M paper wrappers and Company M paper cups. (B)DGGE image in Company K PHBH bags and City T PBS bags. Numbers (13~63) in the DGGE gel images are the number of days after the start of the test. The DNA markers for DGGE (DGGE Marker II: 10 fragments, www. nippon-gene.com) in the lane M are separated not by molecular weight but are separated according to the degree of denaturation by denaturing agent concentration (30-70%).

City T PBS bags (50%, 50 days). The prominent bacterial of the biodegradation process treated with SG compost are shown in Fig. 11A (Company M paper wrappers and cups) and Fig. 11B (Company K PHBH bags and City T PBS bags). The bands that were not found in the compost itself but were found during biodegradation of samples were as follows. Five bands in Company K PHBH bags, 5 in Company M paper cups, 5 in Company M paper wrappers and 4 in City T PBS bags.

List of bacterial species closely related to the bacterial groups predominantly detected during biodegradation of the samples.

The following rules were used to describe the bacterial species. Numbers in colored column are bacteria species for which the sequencing analysis results were not clear. Thus, reliability is relatively low, as light blue is slightly unclear and purple is quite unclear.

Bacterial groups are classified according to their optimum growth temperature as follows. Cryogenic bacteria that can grow at temperatures below 20ºC. Meso-thermophilic bacteria (light green column) that grow at temperature of 20-40ºC. Thermophilic bacteria (pink column) can grow at temperatures above 55ºC. Highly thermophilic bacteria are at a growth limit of 75ºC or higher and hyper thermophilic bacteria are at 90ºC or higher.

Bacteria classification according to oxygen conditions are as follows. Obligate aerobic: bacteria cannot grow in the absence of oxygen (orange column). Aerobic: bacteria growth is usually optimal in the presence of 18-21% oxygen (light orange column). Facultative anaerobic: bacteria can grow in the presence or absence of oxygen (light violet

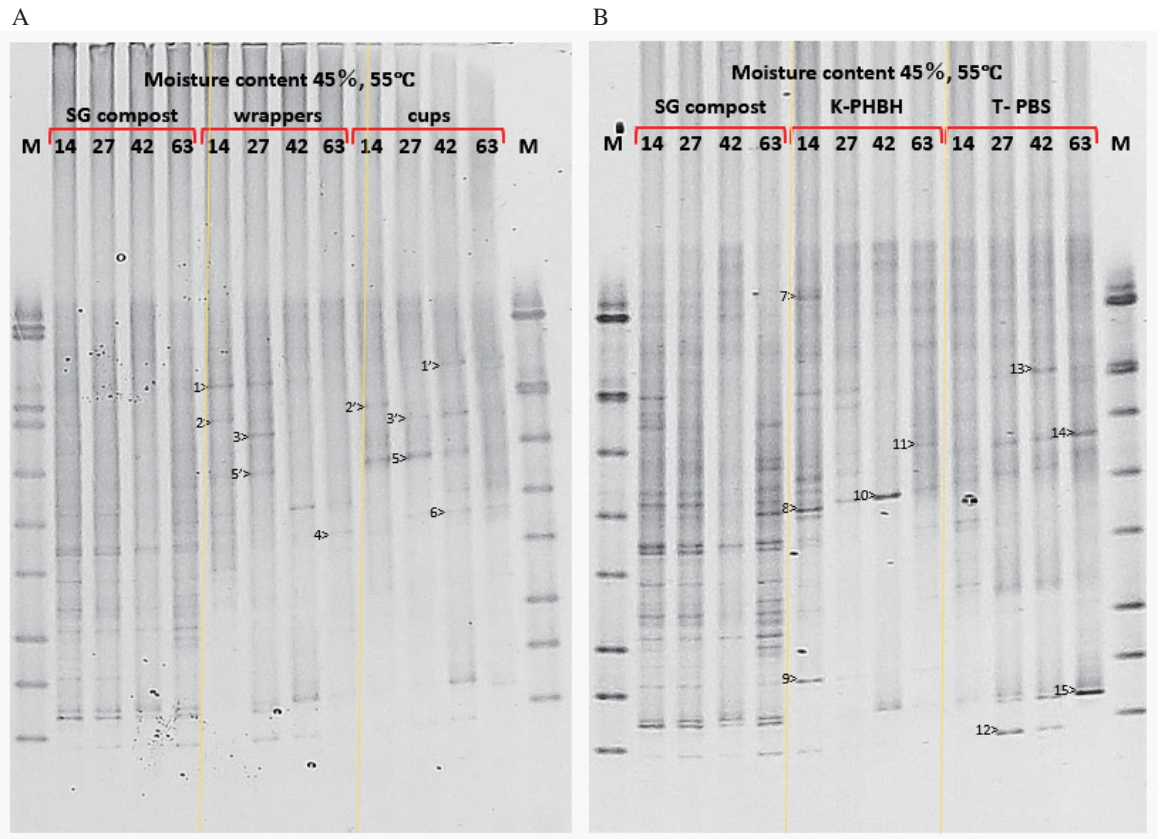


Fig. 11　DGGE analysis of Company M paper wrappers, Company M paper cups, Company K PHBH bags and City T PBS bags treated with SG compost. (A) DGGE image of dominant bacteria during biodegradation at 55ºC, 45% moisture content in Company M paper wrappers and Company M paper cups. (B) DGGE image in Company K PHBH bags and City T PBS bags. Numbers (14~63) in the DGGE gel images are the number of days after the start of the test.

column). Anaerobic: bacteria can grow only less than 1% oxygen (light blue column). Obligate anaerobic: bacteria cannot grow in the presence of oxygen (violet column).

Company M paper wrappers in combination with MS or SG compost

The main bacterial groups found in the combination of Company M paper wrappers and MS compost (55ºC, 40%) or SG compost (55ºC, 45%), but not in the compost alone, during the biodegradation process were as follows (Table 1).

Company M paper cups in combination with MS or SG compost

The main bacterial groups found in the combination of Company M paper cups and MS compost (55ºC, 40%) or SG compost (55ºC, 45%), but not in the compost alone, during the biodegradation process were as follows (Table 2).

Company K-PHBH in combination with MS or SG compost

The main bacterial groups that were not present in the compost alone but were found or more predominant (thicker bands) in the biodegradation process in the combination of K PHBH bags and MS

Table 1　Predominant bacterial analogues found in the biodegradation of Company M paper wrappers by MS or SG compost.

Compost	No.	Sequence ID	Closest relative(s)	Homology (%)	Identities	Habitat	Temperature preference	Air preference	Comment/ Degradation
MS	1	MH298782.1	*Acetivibrio saccincola*	99	506/507	Biogas- ferments	Thermophilic	Anaerobic	Cellulose
	2'	KC313899.1	*Bacterium ADC-6-7*	98	499/508	Biogas- ferments	-	-	-
		CP046244.1	*Moorella glycerini*	87	456/523	Springs	Thermophilic	Anaerobic	Glycerol
	3'	NR_036959.1	*Halocella cellulosilytica*	92	491/534	Lake/marsh	Mesophilic	Obligate anaerobic	Cellulose
	4'	LN881574.1	*Tepidimicrobium xylanilyticum*	96	483/503	-	Thermophilic	Anaerobic	Xylan
SG	1	MH298782.1	*Acetivibrio saccincola*	98	497/505	Biogas- ferments	Thermophilic	Anaerobic	Cellulose
	2	KC313893.1	Bacterium ADC-6-1	88	473/540	-	-	-	-
		MT122842.1	*Parageobacillus caldoxylosilyticus*	85	469/552	Rhizosphere	Thermophilic	Tolerant anaerobic	Xylose
	3	KC313898.1	Bacterium ADC-6-6	96	494/515	Biogas- ferments	-	-	-
		NR_135713.1	*Natranaerobaculum magadiense*	87	433/500	-	Thermophilic	Anaerobic	-
	4	CP019699.1	*Novibacillus thermophilus*	96	534/555	Sediment	Thermophilic	Tolerant anaerobic	In compost
	5'	NR_036959.1	*Halocella cellulosilytica*	92	494/538	Lake/marsh	Mesophilic	Obligate anaerobic	Cellulose

Table 2　Predominant bacterial analogues found in the biodegradation of Company M paper cups by MS or SG compost.

Compost	No.	Sequence ID	Closest relative(s)	Homology (%)	Identities	Habitat	Temperature preference	Air preference	Comment/ Degradation
MS	1'	MH298782.1	*Acetivibrio saccincola*	99	506/507	Biogas- ferments	Thermophilic	Anaerobic	Cellulose
	2	KC313899.1	*Bacterium ADC-6-7*	98	499/508	Biogas- ferments	-	-	-
		CP046244.1	*Moorella glycerini*	87	456/523	Springs	Thermophilic	Anaerobic	Glycerol
	3	NR_036959.1	*Halocella cellulosilytica*	92	491/534	Lake/marsh	Mesophilic	Obligate anaerobic	Cellulose
	4	LN881574.1	*Tepidimicrobium xylanilyticum*	96	483/503	-	Thermophilic	Anaerobic	Xylan
SG	1'	MH298782.1	*Acetivibrio saccincola*	98	497/505	Biogas- ferments	Thermophilic	Anaerobic	Cellulose
	2'	KC313893.1	Bacterium ADC-6-1	88	473/540	-	-	-	-
		MT122842.1	*Parageobacillus caldoxylosilyticus*	85	469/552	Rhizosphere	Thermophilic	Tolerant anaerobic	Xylose
	3'	KC313898.1	Bacterium ADC-6-6	96	494/515	Biogas- ferments	-	-	-
		NR_135713.1	*Natranaerobaculum magadiense*	87	433/500	-	Thermophilic	Anaerobic	-
	5	NR_036959.1	*Halocella cellulosilytica*	92	494/538	Lake/marsh	Mesophilic	Obligate anaerobic	Cellulose
	6	MN602556.1	*Capillibacterium thermochitinicola*	97	495/511	Insect	Thermophilic	Obligate anaerobic	Chitin

Table 3　Predominant bacterial analogues found in the biodegradation of Company K PHBH bags by MS or SG compost.

Compost	No.	Sequence ID	Closest relative(s)	Homology (%)	Identities	Habitat	Temperature preference	Air preference	Comment/ Degradation
MS	5	CP068564.1	*Keratinibaculum paraultunense*	78	389/500	Wastewater	Thermophilic	Anaerobic	Keratin
	6	MN713613.1	*Alkalispirillum mobile*	93	492/531	Anaerobic digester	Mesophilic	Aerobic	Sulphur oxidation
SG	7	JX914499.1	*Ureibacillus suwonensis*	97	517/534	Compost	Thermophilic	Obligate aerobic	Cellulose/ Chitin
	8	LT631780.1	*Ureibacillus suwonensis*	98	524/533	Compost	Thermophilic	Obligate aerobic	Cellulose/ Chitin
	9	AB562468.1	*Saccharomonospora viridis*	98	492/504	Soil	Thermophilic	Aerobic	In compost
	10	MN713613.1	*Alkalispirillum mobile*	92	492/534	Anaerobic digester	Mesophilic	Aerobic	Sulphur oxidation
	11	AP018335.1	*Ureibacillus thermosphaericus*	93	502/537	Soil	Thermophilic	Aerobic	Poly lactic acid (PLA) polymer

Table 4　Predominant bacterial analogues found in the biodegradation of City T PBS bags by MS or SG compost.

Compost	No.	Sequence ID	Closest relative(s)	Homology (%)	Identities	Habitat	Temperature preference	Air preference	Comment/ Degradation
MS	5'	CP068564.1	*Keratinibaculum paraultunense*	78	389/500	Wastewater	Thermophilic	Anaerobic	Keratin
	6'	MN713613.1	*Alkalispirillum mobile*	93	492/531	Anaerobic digester	Mesophilic	Aerobic	Sulphur oxidation
SG	12	NR_074379.1	*Sphaerobacter thermophilus*	96	497/517	In compost	Thermophilic	Obligate aerobic	In compost
	13	KC313893.1	Bacterium ADC-6-1	83	445/534	-	-	-	-
		MF965113.1	*Geobacillus stearothermophilus*	84	386/460	In compost	Thermophilic	Obligate anaerobic	Starch
	14	KP010251.1	*Aeribacillus pallidus*	93	509/545	Food_ Fermentation	Thermophilic	Aerobic	Starch/ Triptyline
	15	NR_173523.1	*Aggregatilinea lenta*	85	435/511	Biosolid	Mesophilic	Obligate anaerobic	-

compost (55ºC, 40%) or SG compost (55ºC, 45%) were as follows (Table 3).

City T PBS in combination with MS or SG compost

The main bacterial groups that were not present in the compost alone but were found or more predominant (thicker bands) in the biodegradation process in the combination of City T PBS and MS compost (55ºC, 40%) or SG compost (55ºC, 45%) were as follows (Table 4).

Discussion

Predominant bacterial groups found in the biodegradation process of paper wrappers and paper cups from Company M

Abbreviations of the bacteria species were denoted as follows, M for bacteria　predominant in samples treated with MS compost, and S for bacteria in SG compost. The predominantly detected bacteria, denoted as Nos or No, are analogues of candidate bacteria.

The closely relative candidate bacteria of predominant group of which were not found in the compost alone but were detected only in the test sample were as follows. The cellulose-degrading anaerobic bacteria *Acetivibrio saccincola* is also known as *Herbivorax saccincola*. It has no spores, has cellulosomes and degrades polysaccharides (closely relatives are Nos. M01, S01, M01', S01'). *Halocella cellulosilytica* of the family Halobacteriaceae, an obligate anaerobe with cellulase activity in high salt conditions (Nos. M03', S05', M03, S05). Tepidimicrobium family, *Tepidimicrobium xylanilyticum*[7] is anaerobes and capable of degrading xylan, side chains consisted of heterosaccharides attached to the main chain of β 1-4 linked xylose. It produces short chain fatty acids, such as acetic acid, propionic acid and butyric acid. In this case, a group of bacteria (Nos. M04', M04) was detected as an analogue. In addition, *Parageobacillus caldoxylosilyticus*[8] is a facultative anaerobic bacteria belong to Bacillariophyceae and has xylan degrading enzymes. A group of closely related bacteria (Nos. S02, S02') was detected. Closely related

bacteria (No. S06) of *Capillibacterium thermochitinicola* which is an obligate anaerobe Bacteroidetes, marine Flexuosa family that degrades chitin consisting of a nitrogen-containing polysaccharide polymer similar to cellulose-like structure.

As described above total five groups of bacteria were detected. Of these, the most promising bacteria was considered to be analogs of *Parageobacillus caldoxylosilyticus*, a facultative anaerobic thermophilic bacterium with xylan degrading enzymes that can grow even in the presence of oxygen. None of the above five bacteria groups, however, have been reported to have esterase activity degrading polybutylene succinate (PBS) at present.

On the other hand, the closely relative group (Nos.M02', M02) was detected homology to *Moorella glycerini* which is anaerobic thermophilic bacteria of Clostridium class and Thermoanaerobacteraceae family. It contributes to decomposition of organic matter in anaerobic environment. The closely relative group (Nos. S03, S03') was detected homology to *Natranaerobaculum magadiense* which is salt-facultative bacteria of Clostridium class. And the group (No. S04) a relative of *Novibacillus thermophilus* which is a salt-facultative bacterium of the Clostridium class.

These three bacteria groups were detected only in the test sample degradation process, although they were not reported to be capable of degrading cellulose, xylan, or chitin. It is possible that these bacteria either supported the activity of the former five groups as so-called satellite bacteria or grew by using the biodegradation products of the former groups which have degradation enzymes.

Predominant bacterial groups found in the biodegradation process of Company K's biodegradable plastic bags (PHBH)

The predominant bacterial groups found in the biodegradation process of Company K PHBH bags were the following five species. *Keratinibaculum paraultunense* (homologous relative, No. M05) is an anaerobic thermophilic bacterium of the Actinobacteriaceae, belonging Dermatophyta family and has a keratinolytic enzyme. *Saccharomonospora viridis* (homologous relative, No. S09) is an aerobic thermophilic Actinobacteria, family Pseudonocardiaceae. *Alkalispirillum mobile* (homologous relative, Nos. S10, M06) is an aerobic meso-thermophilic bacterium of the alpha-proteobacterial class, family Phyllobacteriaceae. *Ureibacillus suwonensis* (homologous relative, Nos. S07, S08) and *Ureibacillus thermosphaericus* (homologous relative, No. S11) are obligate aerobic thermophilic bacteria of Bacillus phylum, Paenibacillaceae. Above five group bacteria species have not been reported to produce PHBH esterase.

Among them, however, *Ureibacillus suwonensis* can degrade polyesters and has a cutinolytic enzyme. *Ureibacillus thermosphaericus*[9] has an esterase that degrades polyhydroxyalkanoate PHAs which is a different polymer. Thus, the possibility in the future remains that homologous relative of these two aerobic thermophilic bacteria have PHBH esterase activity.

Predominant bacterial species found during biodegradation of biodegradable City T PBS bags

Several bacterial species have been identified that may have esterase activity to biodegrade butylene succinate polymer (polybutylene succinate: PBS). One notable example is the bacterium of the genus Thermobifida[10]. The cutinase of this bacterium has been extensively studied, showing the ability to degrade a variety of synthetic polyesters, including PBS. The cutinase is an enzyme that hydrolyses the ester bonds of synthetic polymers, making it effective in the biodegradation of substances such as PBS. The other example is the novel Bacillus strain NR4[11]. This strain showed a wide range of degrading activity against polycaprolactone (PCL) and poly (butylene adipate-co-terephthalate) (PBAT) blends. The strain exhibited robust esterase activity under a range of conditions, highlighting its potential for biopolymer degradation. Among the six groups of bacteria found to predominate in the thermal biodegradation of compost-treated samples in the present study, no reports of PBS esterase activity have been made for *Sphaerobacter thermophilus*

(homologous relative, No. S12) of the family Spherobacteriaceae, which is a biophilic aerobic thermophile. *Aggregatilinea lenta* (homologous relative, No. S15), a facultative anaerobic mesothermal bacterium of the family Chloroflexibacteriaceae, is a bacterium with no known PBS esterase production. Two other bacteria, *Keratinibaculum paraultunense* (homologous relative, Nos. M05, M05') of the anaerobic thermophilic Actinobacteria, Dermatophilidae, and the aerobic mesophilic alpha-proteobacteria, Philobacteriaceae, *Alkalispirillum mobile* (homologous relative, Nos. S10, M06, M06') have been reported to have neither PHBH nor PBS esterase.

On the other hand, *Aeribacillus pallidus* (homologous relative, No. S14), a thermophilic bacterium of the Bacillariophyceae and the family Allibacillaceae, has esterases that degrade 2-naphthyl caprylate and 2-naphthyl myristate and others in high temperature environments, but there is no literature on the presence of PBS esterase. The facultative anaerobic thermophilic bacterium *Geobacillus stearothermophilus* (homologous relative, No. S13)[12, 13, 14, 15] has a diverse range of esterases and has been reported to have polystyrene degrading enzymes, lipase and cellulase, and may possess PBS esterase.

There are still not so many research groups on aerobic thermophiles worldwide. In addition, the biodegradable plastics targeted in this study have been in use internationally for a short period of time. For this reason, there are few data available on the specific esterase activity of PHBH and PBS studied in aerobic thermophiles. Even in the present study, we had to limit ourselves to a case of analogous esterase potential for a group of closely related bacteria. In the future, it will be necessary to isolate and cultivate candidate species of aerobic thermophilic bacteria that degrade these biodegradable plastics and papers more efficiently, and to identify specific genes and measure their activities for industrialization.

Acknowledgments

We thank Company M, Company K and City T for their assistance in providing test materials.

References

1）UNEP: Revised draft text of the international legally binding instrument on plastic pollution, including in the marine environment. https://wedocs.unep.org/bitstream/handle/20.500.11822/44526/RevisedZeroDraftText.pdf. 2023.

2）US-EPA: IRIS Toxicological review of perfluorohexanoic acid [PFHxA, CASRN 307-24-4] and related salt. https://cfpub.epa.gov/ncea/iris/iris_documents/documents/toxreviews/0704tr.pdf. 2023.

3）Pathak, V. M., Navneet: Review on the current status of polymer degradation: a microbial approach. Bioresour Bioprocess 2017; 4: 1-31.

4）Wilson, D. B: Evidence for a novel mechanism of microbial cellulose degradation. Cellulose 2009; 16: 723-727.

5）Xiao, M., Li, Y-J., Bayer, E. A., Kosugi, A., Cui, Q., Feng, Y: Cellulosomal hemicellulases: Indispensable players for ensuring effective lignocellulose bioconversion. Green Carbon 2024; 2: 57-69.

6）ISO 14855-1(JISK 6953-1): Determination of the ultimate aerobic biodegradability of plastic materials under controlled composting conditions -Method by analysis of evolved carbon dioxide, Part 1: General method. https://www.iso.org/standard/72046.html. 2012.

7）Phitsuwan, P., Morag, E., Tachaapaikoon, C., Pason, P., Kosugi, A., Ratanakhanokchai, K: Behavior and supportive evidence of a large xylanase-containing multienzyme complex of *Tepidimicrobium xylanilyticum* BT14. Biol Res 2012; 7: 5934-5949.

8）Yunitsyna, O., Sinelnikov, I., Kisil, O., Bolotova, K., Aksenov, A., Simonsen, G: Isolation of thermophilic enzyme-producing parageobacillus bacteria from chipped woody waste. Biol Res 2019; 14: 1452-1465.

9）Samoylova, Y. V., Sorokina, K. N., Romanenko, M. V., Parmon, V. N: Cloning, expression and characterization of the esterase estUT1 from *Ureibacillus thermosphaericus* which belongs to a new lipase family XVIII. Extremophiles 2018; 22: 271–285.

10）Liang, X., Zou, H: Biotechnological application of cutinase: A powerful tool in synthetic biology. Syn Biol 2023; 1: 54-64.

11）Nawaz, A., Hasan, F., Shah, A. A: Degradation of poly(ε -caprolactone) by a newly isolated *Brevundimonas sp*. strain MRL-AN1 from soil. FEMS Mi-

crobiol Lett 2015; 362: 1-7.

12）Henke, E., Bornscheuer, U. T: Esterases from *Bacillus subtilis* and *B. stearothermophilus* share high sequence homology but differ substantially in their properties. Appl Microbiol Biotechnol 2002; 60: 320-326.

13）Zhou, J-F., Gao, P-K., Dai, X-H., Cui, X-Y., Tian, H-M., Xie, J-J., Li, G-Q., Ma, T: Heavy hydrocarbon degradation of crude oil by a novel thermophilic *Geobacillus stearothermophilus* strain A-2. Internatl Biodet Biodegr 2018; 126: 224-230.

14）Tekedar, H. C., Sanli, G.S: Molecular cloning, over expression and characterization of thermoalkalophilic esterases isolated from *Geobacillus* sp. Extremophiles 2011; 15: 203-211.

15）Xing, R-Z., Zhao, Z-Q., Zhao, W-Q., Chen, Z., Chen, J-F., Zhou S-G: Biodegradation of polystyrene by *Geobacillus stearothermophilus*. Huan Jing Ke Xue 2021; 42: 3056-3062.