Chapter 4
Bacterial and Biochemical Properties of Newly Invented Aerobic, High-Temperature Compost

Takahiro Yoshii, Toshiyuki Moriya, and Tairo Oshima

Abstract Compost is the environmentally safe and the most economical way to treat organic waste. Due to heat emitted by fermentation processes, the compost inside is hot, and therefore, a variety of thermophilic bacteria thrive in compost. The thermophiles play an important role in the degradation of biopolymers in compost. This chapter deals with microbial community in a newly developed aerobic, high-temperature compost, where temperature reaches 100°C or even higher. Physical, chemical, biochemical, and microbial analyses of the high-temperature compost are presented. The technical problems associated with the biochemical and microbial analyses are also discussed.

Keywords Extreme thermophiles • *Calditerricola satsumensis* • *Calditerricola yamamurae* • DGGE • Real-time PCR • Zymogram • *Thermus thermophilus*

4.1 Introduction

Composting is a classical way to treat organic wastes produced from house kitchens, restaurants, agriculture fields, cattle farming, and food industries. Composting has many advantages over burning. Composting is economical, safe, and environmentally favorable. To burn organic wastes, a large amount of oil is needed. Thus, we can reduce emissions of CO_2 if organic waste is decomposed by composting instead of burning. Composting does not require sophisticated equipment, whereas burning requires expensive combustion furnaces.

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Composting is environmentally safe since composting does not produce harmful substances such as nitrogen oxides (NOx), sulfur oxides (SOx), and dioxins. The final products can be used as fertilizers and soil conditioners and thus contribute to improvements of production of food resources. Composting kills pathogenic microbes and viruses. Composting processes also kill seeds of weeds. Since burning is now generally prohibited in many countries, composting becomes more important for decomposing organic wastes than before.

Heat emitted from fermentation processes of microorganisms causes the inside of compost piles to become hot, and compost is one of the important environments for isolating thermophiles (Finstein and Morris 1975). According to the literature, the inside temperature reaches up to 75–80°C (Golueke 1972; Saiki et al. 1978). The isolation of many moderate thermophiles belonging to the genera *Geobacillus, Bacillus*, and *Clostridium* and related species has been reported. Methanogens, including thermophilic methanogens, which belong to Archaea have also been isolated from the traditional compost.

Recently the Sanyu Company in the city of Kagoshima, Japan, invented an aerobic, high-temperature compost process in which the internal temperature often exceeds 95°C or even 100°C. This high-temperature composting rapidly degrades wastes from cattle farming including bones, skins, or even dead bodies of animals (Oshima and Moriya 2008). In this chapter, the bacterial and biochemical nature of the high-temperature composting will be described.

4.2 Compost and Thermophiles

Composting can be considered to be microbial degradation of organic materials by aerobic respiration. Due to heat emitted from microbial respiration, the compost pile is hot inside; usually the temperature of the central portion of the compost pile reaches up to 80°C. It is well known that thermophiles play important roles in thermogenic composting. In the past, compost was the main resource for isolation of thermophilic bacteria (Finstein and Morris 1975).

The best known thermophile in compost is *Geobacillus stearothermophilus* (formally *Bacillus stearothermophilus*) which is a moderate thermopile and can grow up to 75°C. *G. stearothermophilus* is an aerobic, gram-positive, spore-forming rod. This thermophile had been the most extensively studied species until extreme thermophiles such as *Thermus* species were isolated from hydrothermal environments such as hot springs in 1960s.

Microbial flora changes during the composting processes, and the change has been investigated for a long time. When compost fermentation starts by mixing the organic waste with matured compost soil, temperature raises with time, and within a few days the temperature inside reaches the maximum. Along with the temperature change, population of mesophiles decreases and that of thermophiles increases.

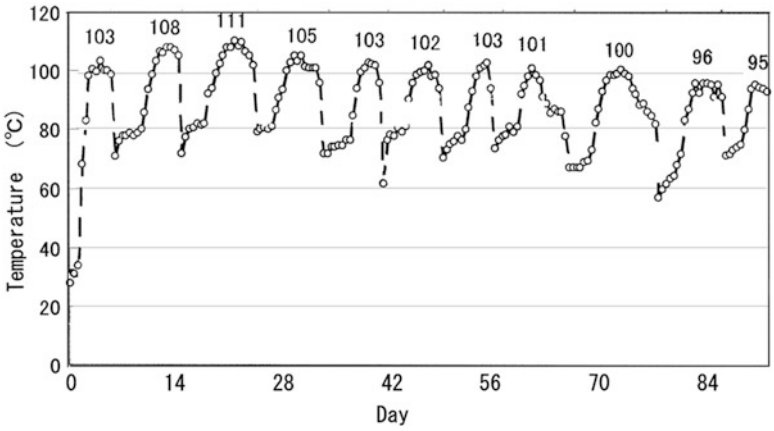

Fig. 4.1 An example of temperature change of an aerobic, high-temperature compost. Temperature at 50 cm below the center of the top surface was measured

4.3 High-Temperature Compost

Since burning was restricted due to environmental issues, organic waste from city life has been treated by composting in many cities. Thus, the size of a compost pile was enlarged. The inside temperatures of these large-scale compost are higher than those of the traditional compost. We have analyzed the aerobic high-temperature compost developed by Sanyu Company in Kagoshima city in Japan (Oshima and Moriya 2008).

A typical example of temperature changes of an aerobic high-temperature compost is shown in Fig. 4.1. In this experiment, temperature was measured at 50 cm below the top surface of a composting pile with an alcohol thermometer. The inside temperature reaches up to 90–110°C within a few days after the raw materials had been mixed with the matured compost soil as shown in Fig. 4.1. The temperature starts to decrease after 5–6 days to about 80–90°C. The compost is thoroughly stirred using a shovel car every week. By this procedure, a large amount of water vapor escapes and compost cools down.

After the turning, the temperature rises again within a day or so. Often 100°C or higher temperature is recorded in the first 3–4 weeks. The temperature at about 5cm below the surface or 5cm above the floor is around 65–70°C, and thus, most part of the compost pile is hotter than 70°C.

The aerobic, high-temperature composting has three unique advantages over the traditional compost: firstly, since inside temperature is higher than 70°C, unfavorable pathogenic microorganisms can be killed quickly; secondly, degradation rates of organic wastes is faster than those of the traditional compost, for instance, an animal body, pig or even beef including their bones, disappears within a few weeks; and thirdly, auxiliary materials such as wood tips or husk are not necessary to add

Fig. 4.2 An example of high-temperature compost pile

to the high-temperature compost, which are needed to adjust water content of compost piles in the traditional composting. The water content of the aerobic, high-temperature compost is controlled by changing the ratio of organic waste to be decomposed and the matured compost soil used as inoculum.

The water content is one of the most crucial factors to operate the aerobic, high-temperature compost. Usually organic waste or active sludge of which water content is around 80% is mixed well with the roughly equal amount of the matured compost soil of which water content is around 15%; the water content of the resulted mixture is around 50%. A typical procedure is as follows: matured compost (91.3 t) and sewage sludge cake (76.1 t) which is dewatered by adding calcium hydroxide are mixed thoroughly, and the mixture is stacked to make a pile of 5×3×8 m (width×height×length). Figure 4.2 illustrates a compost pile at a factory of Sanyu Company.

The fermentation processes start immediately after mixing, and the temperature starts to elevate as already shown in Fig. 4.1. Water content decreases gradually during the composting, especially at a time of stirring every week. The water content of the final product is around 15%, and the final product can be used as inoculum to decompose new organic waste. It takes 6–10 weeks to reduce the water content to 15%. Aeration is another crucial factor. Air is continuously supplied from the bottom of a compost pile through pipes with small holes which are buried on the ground floor. Air compressors are used, and the flow rate should be adjusted to keep the possible highest temperature inside of the pile.

Carbon and nitrogen content decreases rapidly in the first half of the composting process. Total carbon content of the starting mixture is about 20–25 mg/g wet weight of compost and drops to 15–20 mg/g in the final stage. Total nitrogen content also

gets reduced during the composting process. The ratio of total C to total N is almost constant throughout the fermentation and is around 8.5–10. Most of nitrogen in the original waste can be outgassed in the form of NH_3. Nitrate is not detected, but nitrate is formed rapidly when the matured compost is mixed with garden soil or farming soil. The pH is around 8 and is almost constant during the composting process.

4.4 Bacterial Community of the High-Temperature Compost

At first, microbial flora of the final products is analyzed by using viable count techniques. Some typical results of bacterial populations of the final product are shown in Table 4.1. In traditional compost, usually the final product contains around 10^8 culturable aerobic mesophiles plus 10^4–10^5 culturable moderate thermophiles per g wet weight of compost. Mesophiles are defined as microorganisms which form colonies below 55°C, and thermophiles are those which form colonies at 55°C.

As shown in Table 4.1, the aerobic high-temperature compost contains about 1×10^7 culturable mesophiles and about 3×10^6 spores per g wet weight of the compost. Thermophiles detected are about 3×10^6 per gram. The ratio of thermophiles to mesophiles is about 1:3, and this value is about 100–1,000 times larger than that of the traditional compost.

Taking into account that the inside of the compost pile is hot, we expected that the detected mesophiles would be mostly spore formers and vegetative cells would not be present in the course of active degradation of waste. Thus, these mesophiles can scarcely contribute to the decomposition of organic substances. Assuming that culturable thermophiles are 1% of the whole thermophiles in compost, it can be assumed that the aerobic high-temperature compost contains around 10^8–10^9 thermophile cells per gram.

We investigated some of the thermophiles isolated from the compost. It was found that the majority of the isolates belong to genera *Geobacillus*, *Bacillus*, *Saccharococcus*, and *Thermaerobacter* and the related genera as expected.

Table 4.1 Bacterial colony-forming units (CFU) per g wet weight of the final products of different compost factories

Sample taken from	Mesophiles	Spore-forming mesophiles	Thermophiles
Factory in Osaka	3.0×10^7	1.6×10^6	2.9×10^6
Factory in Kohchi	7.7×10^6	7.2×10^6	3.9×10^6
Factory in Saga	3.3×10^9	1.7×10^7	5.5×10^6
Factory in Hokuto	6.3×10^6	5.1×10^6	4.8×10^6

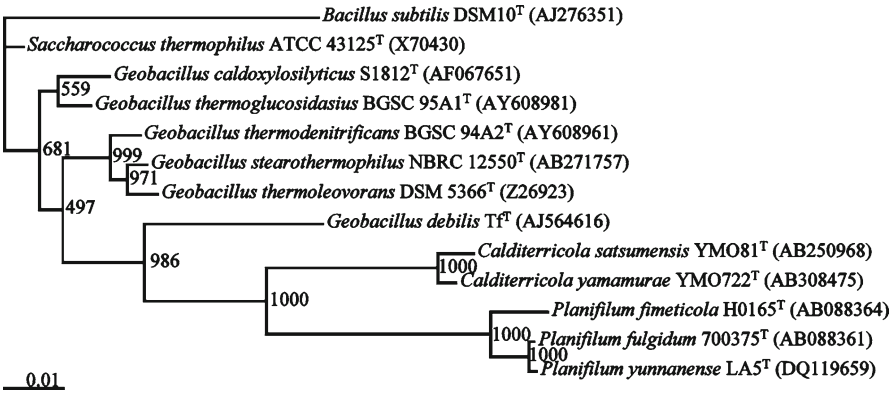

Fig. 4.3 Phylogenetic tree constructed with 16S rDNA gene sequences (1,409 nt) using Phylip neighbor-joining analysis and the Kimura two-parameter mode. *Bar*, 1 substitution in 100 nt. *Numbers* given at the nodes represent bootstrap percentage values (1,000 analysis). The dendrogram was rooted using the sequence of *Bacillus subtilis* as an out-group

4.5 *Calditerricola satsumensis* YMO81 and *C. yamamurae* YMO722

In the course of the analyses of bacteria in the aerobic, high-temperature compost, we could isolate new extreme thermophiles. We named the new strains to be *Calditerricola satsumensis* YMO81^T and *C. yamamurae* YMO722^T (Moriya et al. 2011). The highest growth temperature of *C. satsumensis* is 82°C. They are the first extreme thermophiles isolated from compost. The new isolates are long rod (0.2×3 μm), gram stain negative, nonspore forming, and nonmotile. The G+C content of the chromosomal DNA of *C. satsumensis* YMO81 was 70%. Although BLAST search of 16S rRNA gene sequences shows that the closest species to the isolates is *Planifilum yunnanense* strain LA5^T (Yan et al. 2007), but the homology was only 91%, suggesting that the isolates belong to a new genus. We proposed a new genus *Calditerricola* under the family Bacillaceae. DNA-DNA hybridization homology between *C. satsumensis* YMO81^T and *C. yamamurae* YMO722^T was only 51%, indicating that they are different species to each other. An example of the phylogenetic analyses on these new isolates is illustrated in Fig. 4.3.

Both strains were deposited at the Japan Collection of Microorganisms, RIKEN Bioresource Center (JCM); the German Collection of Microorganisms and Cell Cultures (DSMZ); and the American Type Culture Collection (ATCC). *C. satsumensis* is weakly halophilic and 2% NaCl is required for the best growth. The grow rate of the new isolates is fast; doubling time of the isolates is about 30 min under optimum conditions. In the stationary growth phase, the isolates die gradually at 80°C or even at 4°C. These isolates could not grow in rich media such as Lysogeny broth or Soybean-casein digest medium.

Colony formation can be observed after overnight incubation at 75°C on a gellan gum plate. However, the colony-forming efficiency of the isolates is quite low. The rate was much improved by the addition of $FeSO_4$ and $VOSO_4$ (100 μg/l and 2 mg/l, respectively). Even in the presence of $FeSO_4$ and $VOSO_4$, one colony can be formed from 100 cells. The reasons why the colony formation rate is so low, and why some metal ions improved the viability, are not yet understood. The low viability rate probably suggests that *Calditerricola* species belong to "unculturable bacteria." Since *C. satsumensis* and *C. yamamurae* die rapidly at high temperature and also at ambient temperature, it is problematic to store them. The authors tested many different methods to store them in a laboratory. Storage at −80°C with glycerol is one of the simplest ways to store most of bacteria. *Calditerricola* species die under the conditions, and therefore, this method is not applicable. Second method is freeze-drying. In this method, the cells are suspended in a selected dispersion medium. We tested many dispersion media. The best dispersion medium for *C. satsumensis* and *C. yamamurae* consists of (g/100ml of 0.1 mM phosphate buffer, pH 7.0) sodium glutamate, 3; ribitol (=adonitol), 1.5; and cysteine hydrochloride, 0.05. Though usually the cells suspended in the dispersion solution are freeze-dried, it is advisable to disperse a drop of the cell suspension on a piece of small filter paper strip in an ampoule and then dried under vacuum without prior freezing. This modified method is called liquid-drying (or simply L-dry) method (Malik 1990) and improved the rate of viability of *Calditerricola* species. A slightly different way we adopted successfully is to dry a small drop of the cell suspension (without the filter paper) directly under vacuum without freezing. These microorganisms seem to be highly sensitive to freezing. To our experience, L-dry method on a paper strip is the best for long-term storage of *C. satsumensis* and *C. yamamurae*.

Recently we found that Microbank™ Bacterial and Fungal Preservation System (Pro-Lab Diagnostics Inc., Round Rock, TX) is the best for short-time storage of the thermophile cells. This system consists of beads and a special cryopreservation solution in a small plastic tube. To make a storage ampoule, pour a small amount of the cell suspension or culture medium into the plastic tube and mix well with the "cryopreservation solution" and then remove and discard the liquid by using a sterilized micro-tip. The remaining beads in the plastic tube are stored at −80°C. The cells bind to the beads. To revive the cells, pick up a bead with a set of sterilized tweezers and drop it into a fresh culture medium at 75–80°C in advance. It is the easiest and quickest way, and the survival rate is high. By these procedures, *Calditerricola* species can be stored for at least 6 months. This method can be applied for many thermophiles other than *C. satsumensis* as well as for mesophiles.

C. satsumensis YMO81 required the presence of ten essential amino acids for growth (Moriya et al. 2011); the essential amino acids are glutamine, methionine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, tryptophan, and valine. In the compost, many species produce and secrete proteolytic enzymes as described in the latter part of this chapter, and these amino acids are easily available.

Polyamines are noncyclic (viz., nucleic acid bases are excluded), aliphatic organic compounds which contain two or more amino nitrogen atoms in a molecule.

Polyamines are essential for cell proliferation and play important roles in a variety of biological reactions such as DNA, RNA, and protein biosyntheses. Especially polyamines are important for life at high temperature, since they interact with acidic cell components such as nucleic acids and membrane lipids (polyamines interact with phospholipids) (Terui et al. 2005; Grosjean and Oshima 2007).

It is known that most of extremely thermophilic organisms produce unusual polyamines in addition to the standard polyamines, especially long-chain polyamines such as caldohexamine and caldopentamine and branched polyamines such as tetrakis (3-aminopropyl)ammonium (Oshima 2007, 2010; Morimoto et al. 2010). *C. satsumensis* YMO81^T and *C. yamamurae* YMO722^T produce spermidine, spermine, and a unique branched polyamine, N^4-aminopropylspermine $(NH_2(CH_2)_3)_2N(CH_2)_4NH(CH_2)_3NH_2$ as the major polyamine components. Small amounts of 1,3-diaminopropane, putrescine, and agmatine are also found. The intracellular concentration of the branched polyamine, N^4-aminopropylspermine, increases when the growth temperature is raised. In contrast, this unique polyamine is not detected from the cells grown at lower temperatures such as 60°C. The finding suggests that N^4-aminopropylspermine is one of the key substances for life of *Calditerricola* species at high temperatures.

4.6 Analyses on Microbial Flora Using DGGE Technique

Culture-dependent investigations on microbial community of a compost have a great disadvantage. Unfortunately the culturable bacteria in natural environment such as soil and compost are thought to be only 0.1–10% (Rozak and Colwell 1978; Torsvik et al. 1990; Van Elsas et al. 1997). Majority of bacteria in soil and compost have been neglected by classical studies based on isolation of bacteria and characterization of the isolates. However, introduction of sophisticated DNA technology in microbial ecology has opened a new era for the studies on microbial structure of environment. Now we can analyze the microbial population of compost without isolation and cultivation.

DGGE technique (Muyzer et al. 1993; Amann et al. 1995; Head et al. 1998; Kowalchuk et al. 1997; Heuer et al. 1997; van Wintzingerode et al. 1997; Felske et al. 1998; Nakatsu et al. 2000; Duineveld et al. 2001) is now widely used for investigation of microbial flora in environment. This consists of isolation of DNA from microbial habitats, use of PCR for amplification of 16S rDNA with suitable primers, and use of denaturing gradient gel electrophoresis for separation of the PCR products. Though the method is powerful for tracking microbial populations in space and time and it has been used by many investigators, there are many problems to be solved in such studies in future (van Wintzingerode et al. 1997).

DNA isolation from compost is one example of the DGGE-associated problems (Griffiths et al. 2000; Pan et al. 2010; Inceoglu et al. 2010). We temporarily selected ISOIL kit (Nippon Gene Co., LTD., Tokyo, Japan) for the isolation of DNA.

We perform the isolation according to the manufacturer's instructions. However, contamination of small amount of humic acid is unavoidable. In future, we have to compare the procedures with and without using a bead beater. Also purification procedures to remove the contaminated humic acid are another subject to be studied in future.

The techniques that we have used for analyzing the structure of bacterial community in the compost are briefly described. RNA was extracted from each sample using PowerSoil RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA), and reverse-transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). PCR for denatured gradient gel electrophoresis (DGGE) was carried out using TaKaRa Ex Taq Hot Start Version (Takara Bio, Kyoto, Japan) and primers, 357F-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and 907R (5′-CCC GTC AAT TCM TTT GAG TTT-3′), that were designed to amplify bacterial 16S rRNA genes. Thermal cycler conditions consisted of the following 30 cycles: denaturation at 93°C for 30 s, annealing at 65°C (reducing 5°C every 10 cycles) for 30 s, and extension at 72°C for 30 s. PCR products were detected with ethidium bromide after 1.5% agarose gel electrophoresis. DGGE was performed according to Muyzer et al. using the DCode universal mutation detection system (Bio-Rad, Hercules, CA). Denaturing gradient gel, 1-mm thickness and 160 × 160 mm, was prepared in 0.5 × Tris acetate-EDTA (TAE) buffer. The concentration gradient used was 6–12% polyacrylamide and 2.1–4.9 M urea with 12–28% formamide. Electrophoresis was carried out at 100 V for 780 min in 0.5 × TAE buffer at 60°C. SYBR Green I (Invitrogen Japan, Tokyo, Japan) was used to stain the gel after electrophoresis. Separated DGGE bands were excised using a plastic tip and washed with sterile water. The PCR re-amplification was performed using the same primer set without GC-clump. The sequences of each DGGE band were determined by FASMAC Co., Ltd. (Kanagawa, Japan). The sequence similarities were phylogenetically analyzed using BLAST program provided by the National Center for Biotechnology Information (NCBI) and the DNA Data Bank of Japan (DDBJ).

DGGE band patterns of 16S rRNA gene based on DNA had continued to change during composting process (Fig. 4.4a, Table 4.2). As the temperature of compost exceeded 80°C from 40°C, the most remarkable difference was observed between band patterns of the fermentation start (1st day) and the 5th day. The sequences of two of the predominant bands in 5 days can be assigned to *C. satsumensis* and were detected in each sample till 25 days (Fig. 4.4a; bands 5 and 6). The sequence of the major band in days 15 and that of 20 were almost identical to the partial 16S rRNA sequence of *Thermus thermophilus* (Fig. 4.4a, band 11). The bands found in late period (days 25–38) could be attributed to *Sphaerobacter thermophilus* (Fig. 4.4a, band 17), *Saccharomonospora viridis* (Fig. 4.4a, band 18), and a species belonging to the genus Planifilum (Fig. 4.4a; bands 15 and 16). Members of the genus *Bacillus* were dominant in the latter period of composting (Fig. 4.4a; bands 20 and 21). The bands related to the genus *Thermaerobacter* (the closest species is *T. composti* or *T. marianensis*) were presented in every sample throughout the composting (Fig. 4.4a, band 9).

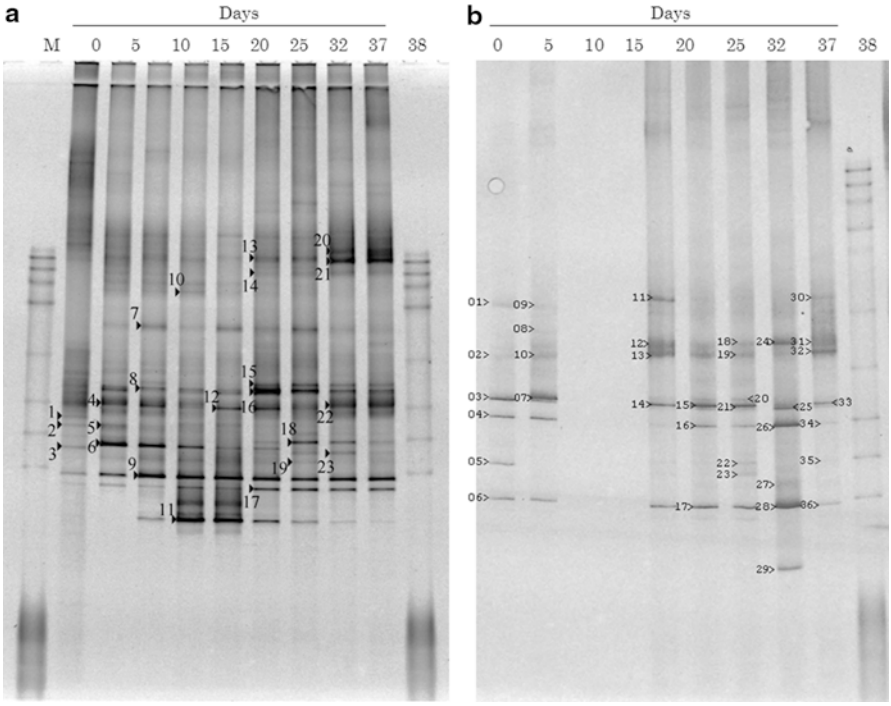

Fig. 4.4 DGGE band profiles of 16S rRNA genes extracted from an aerobic, high-temperature compost. The *arrows* on DGGE gel indicate the excised bands in order to determine the base sequences (see Tables 4.2 and 4.3). *Gel A* DNA-based profiles, *Gel B* RNA-based profiles, *lane M* DGGE marker II (Nippon Gene Co., LTD., Tokyo, Japan)

One of the serious problems associated with DGGE analyses of microbial community is viability of the microorganisms. DNAs in dead cells can be extracted and are undistinguishable from those of living cells. One possible way to solve this problem is to extract and analyze RNAs instead of DNAs. We carried out the RNA analyses of the aerobic, high-temperature compost. In our preliminary experiments, *E. coli* could not survive for 5–10 min after they had poured into the actively fermented compost, which was, however, detected after 24 h in the compost, suggesting that DNAs in dead cells can be stable up to 24 h at 70–90°C. RNA disappeared much faster than DNA.

The results of DGGE fingerprints based on RNA were greatly different from those of DNAs (Fig. 4.4b, Table 4.3). The band was not detected from compost samples in days 10 and 15. A DNA band assigned to *C. satsumensis* was found only in the early stages of composting (Fig. 4.4b, band 05). The dominant bands in almost all compost samples had high similarity to the genus *Planifilum* (Fig. 4.4b; bands 02, 03, 04, 07, 08, 14, 15, 16, 21, 24, 26, 33, and 34) and *Thermaerobacter* (Fig. 4.4b; bands 06, 17, 27, 28, and 36).

Table 4.2 Species identification based on base sequences of bands detected on DGGE shown in Fig. 4.4a

Band	Name	Similarity (%)
1	*Barnesiella intestinihominis* JCM 15079	87
2	*Barnesiella viscericola*	87
3	*Methylocaldum szegediense* OR2	88
4	*Planifilum yunnanense* LA5	96
5	*Calditerricola satsumensis*	100
6	*Calditerricola satsumensis*	100
7	*Bacillus* sp. R-7336	99
8	*Planifilum fimeticola* H0165	95
9	*Thermaerobacter marianensis* DSM 12885	99
10	*Anoxybacillus toebii* NS1-1	99
11	*Thermus thermophilus* HB8	100
12	*Planifilum yunnanense* LA5	99
13	*Bacillus* sp. R-7336	96
14	*Bacillus* sp. R-6491	99
15	*Planifilum fimeticola* H0165	95
16	*Planifilum fimeticola* H0165	96
17	*Sphaerobacter thermophilus* DSM 20745	100
18	*Saccharomonospora viridis* DSM 43017	100
19	*Thermanaeromonas toyohensis* ToBE	87
20	*Bacillus bataviensis* CCGE2104	98
21	*Bacillus* sp. R-6491	99
22	*Planifilum yunnanense* LA5	100
23	*Actinomadura* sp. IMMIB L-889	100

4.7 Abundance Ratio of *C. satsumensis* in the Composting Process

For the determination of relative abundance of *C. satsumensis* in the compost microbial community, we conducted a quantitative PCR using SYBR premix Ex Taq GC (Takara Bio) and Thermal Cycler Dice Real-Time System (TP800, Takara Bio). Universal primers, EU350F (5′-TAC GGG AGG CAG CAG-3′) and EU500R (5′-CCG CGG CTG CTG GCA C-3′), were used in order to estimate amounts of 16S rDNA of eubacteria. The specific forward primer for *C. satsumensis* was designed on the base sequences of 16S rRNA genes, YMO16F (5′-TTT TCG CGT GAA GCC TTC G-3′), and EU500R primer was used as the reverse primer. PCR program was run as follows: the first denaturation at 98°C for 2 min, followed by 40 cycles of 10 s at 98°C and 1 min at 68°C. The quantitative analyses of DNAs were carried out using the recorded melting curves of DNAs according to the instructions of the manufacturer. Abundance ratio of *C. satsumensis* per whole eubacterial cells is shown in Fig. 4.5. The abundance ratio was below 0.1% at the start of compost. It increased

Table 4.3 Species identifications based on base sequences of bands detected on DGGE shown in Fig. 4.4b

Band	Name	Similarity (%)
01	*Anoxybacillus toebii*	99
02	*Planifilum yunnanense*	95
03	*Planifilum yunnanense*	99
04	*Planifilum yunnanense*	99
05	*Calditerricola satsumensis*	99
06	*Thermaerobacter subterraneus*	98
07	*Planifilum fimeticola*	96
08	*Planifilum fimeticola*	95
09	*Anoxybacillus toebii*	94
10	*Planifilum fimeticola*	91
11	*Bacillus thermoamylovorans*	92
12	*Bacillus thermoamylovorans*	97
13	*Bacillus thermoamylovorans*	97
14	*Planifilum fimeticola*	95
15	*Planifilum fimeticola*	95
16	*Planifilum yunnanense*	99
17	*Aerothermobacter marianas*	97
18	*Planifilum fimeticola*	90
19	*Planifilum fimeticola*	90
20	*Planifilum fimeticola*	90
21	*Planifilum fimeticola*	95
22	*Thermaerobacter subterraneus*	85
23	*Thermaerobacter composti*	91
24	*Planifilum yunnanense*	95
25	*Planifilum yunnanense*	91
26	*Planifilum yunnanense*	95
27	*Thermaerobacter subterraneus*	95
28	*Aerothermobacter marianas*	98
29	*Thermus thermophilus*	99
30	*Bacillus subtilis*	96
31	*Bacillus subtilis*	98
32	*Bacillus subtilis*	97
33	*Planifilum fimeticola*	96
34	*Planifilum yunnanense*	99
35	*Saccharomonospora viridis*	95
36	*Aerothermobacter marianas*	98

to 23% rapidly within the first 5 days. The predominance of *C. satsumensis* continued until the day 15, the period in which the temperature of the compost is highest. Then the abundance ratio decreased till the end of composting process and returned to the low level (<0.1%) which is identical to the figure at the start. There is some inconsistency between the analyses based on RNAs (cDNA) and DNAs. RNA analyses indicated that the abundance ratio at the start is the highest (>7%), and then it fell down to less than 1% immediately by 5th day.

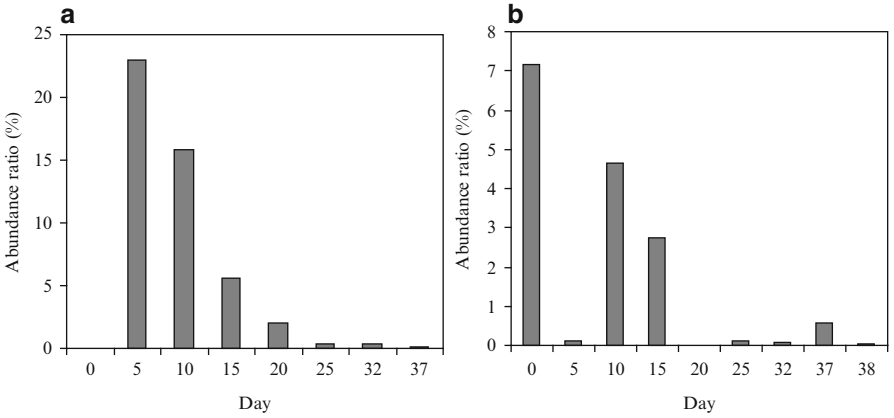

Fig. 4.5 Changes of the relative abundance *C. satsumensis* during the composting process. The relative abundance of *C. satsumensis* in each sample is represented as the ratio of *C. satsumensis* 16S rDNA/total amount of eubacteria 16S rDNAs. Each 16S rDNA amount was determined using DNA (**a**) or RNA (**b**) extracted from compost

As mentioned, DGGE is a powerful method to analyze the microbial community in environment. However, the method is able to detect only 20–40 major species at a time. Often *C. satsumensis* could not be detected in the final stages of high-temperature composting, though the bacterium flourishes instantly and becomes the most abundant species in the compost when the final compost is mixed with new organic waste. In contrast, often DGGE detects DNA from dead or dormant cells, and it is difficult to distinguish actively growing cells from dead cells. Other problems to be solved in future include that often a band seen on DGGE plates does not represent a single species. Improvement on the sensitivity as well as the accuracy of band separation would be desirable (Sekiguchi et al. 2001).

4.8 Population Changes of *Thermus thermophilus* and *Thermaerobacter composti* in the Composting Process

T. thermophilus (Oshima and Imahori 1974) and *T. composti* are extremely thermophilic bacteria growing at temperatures above 75°C and have been isolated from compost soils (Beffa et al. 1996; Yabe et al. 2009). The 16S rDNA genes which are highly homologous to those of *T. thermophilus* or *T. composti* were detected on the DNA-based DGGE (Fig. 4.4a, band 11 or 9). We analyzed the abundance of these bacteria using a quantitative real-time PCR. Specific primers for *T. thermophilus* were designed TTH-F (5′-GCC TAA GAC ATG CAA GTC GT-3′) and TTH-R (5′-CAA AGC CCT TTG GAC AC-3′). To perform the quantitative PCR of *T. composti* 16S rDNA, TN457R (5′-TCC TCA CCC CCG ACC TTC-3′) were designed as a reverse primer and was used with EU350F. The cells of *T. thermophilus* accounted for more than 40% of the total cells in the compost between days 15 and 20, and it is the most dominant organism at this stage. However, the bacterial

population decreased greatly during the late phase of composting process to less than 5%. The abundance ratio of *T. composti* drifted about in a range of 1–4% except the early phase. The highest value of the abundance ratio of *T. composti* was 4% at around the day 20. The findings suggest that these two thermophilic eubacteria as well as *Calditerricola* species play important roles in the aerobic, high-temperature compost.

4.9 Analyses on Hydrolytic Enzyme Activities

To analyze the enzymatic activities of the compost, we performed the activity staining method (zymogram analyses). The enzymes were extracted from compost directly into a buffer solution. Then, the enzyme proteins were collected by ammonium sulfate precipitation (70% saturation) and concentrated in a small volume. The zymogram for gelatinases (collagenases) was carried out according to Nakamura et al. (2004) with slight modifications. Each enzyme sample was loaded on a 10% polyacrylamide gel plate containing 0.8% gelatin. After the electrophoresis, the gel plate was placed in Tris-HCl buffer and incubated at 70°C for 3 h. After the incubation, the gel was stained by Coomassie Brilliant Blue and was destained using a mixture consisting of 10% acetate, 15% methanol, and 75% distilled water. The hydrolysis of gelatin results in the formation of the clear zones on the gel. It seems that the change of gelatinase activity is related to the change of the microbial community structure during composting. Two major gelatinases were detected; one with larger molecular weight was detected only in the first half of the compost processes, whereas the smaller enzyme was constantly produced and secreted into compost soil throughout the entire composting process (Fig. 4.6a).

The carboxymethyl cellulase (CMCase) and xylanase activities were also measured in similar manner using carboxymethyl cellulose (CMC) and xylan instead of gelatin in the zymography gel plates. Congo red was used for staining residual CMC and xylan. The amylase activity in the compost was determined using soluble starch instead of gelatin. The undegraded starch was stained by iodine solution.

The determination of esterase activity in the compost samples was carried out using a polyacrylamide gel without gelatin (Miller and Karn 1980). After the electrophoresis, the gel was incubated in Tris-HCl buffer containing α-naphthyl acetate and fast blue RR at 70°C for 30 min. The esterases were visible as colored bands resulting from the formation of diazo dye complex.

The amylase activities were detected in the early and late stages of the composting process, and low-molecular-weight amylases were found only in the late phase (Fig. 4.6b). CMCase activities were more abundant only in the late (days 25–37) than early stages (days 0–20) (Fig. 4.6c). Thermostable xylanases of different molecular weight were found in all samples except day 15 (Fig. 4.6d). The high esterase activities were found in middle phase of composting process as opposed to other enzymes (Fig. 4.6e).

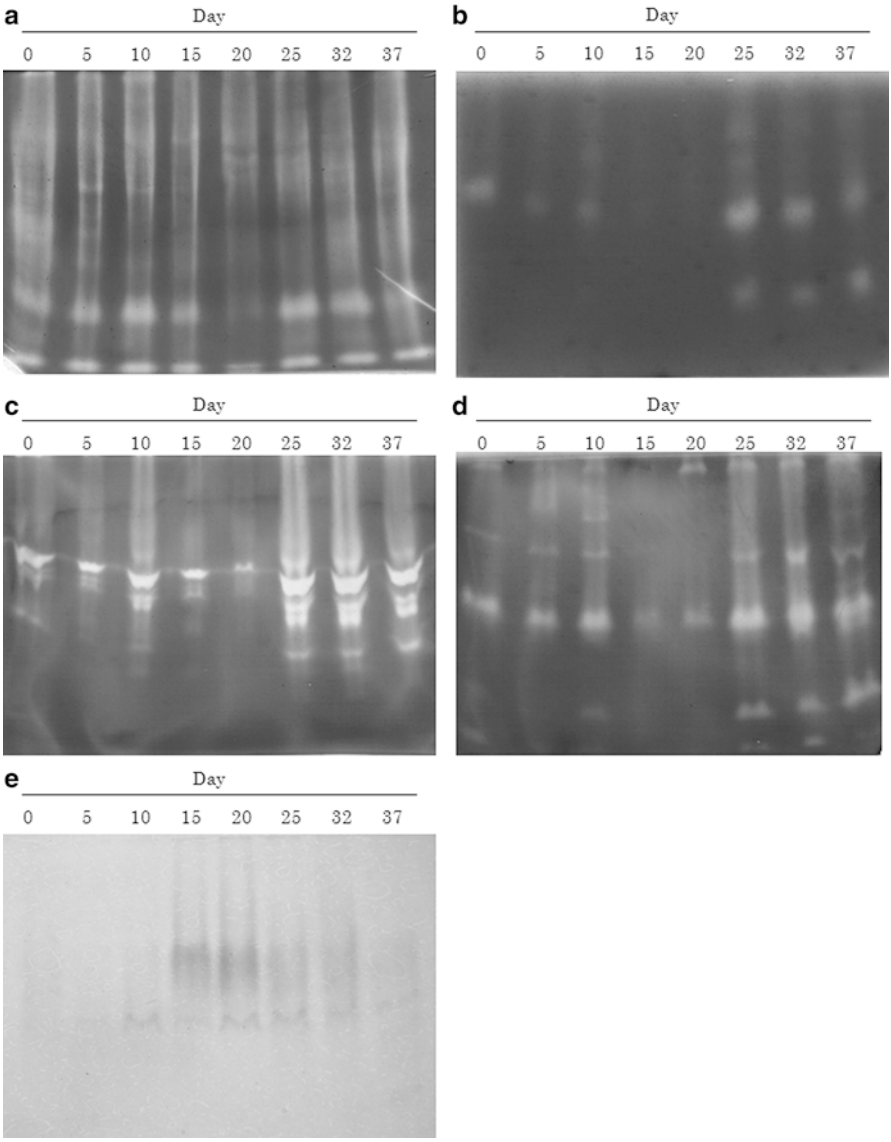

Fig. 4.6 The zymogram of gelatinases (**a**), amylases (**b**), carboxymethyl cellulases (**c**), xylanases (**d**), or esterases (**e**) extracted directly from the aerobic, high-temperature compost

As mentioned above, *T. thermophilus* is one of the most dominant species in the middle phase of the high-temperature composting. In this context, it is noteworthy that two papers reported the production of lipolytic enzymes by *T. thermophilus* isolated from compost (Beffa et al. 1996; Fuciños et al. 2005).

4.10 Conclusions

Newly developed aerobic, high-temperature compost seems to be an interesting process from viewpoint of pure science as well as applied research. Often temperature exceeds 100°C inside. Organic wastes including so-to-speak "hard to digest waste" such as skins, bones, and straw are digested within relatively short period. Temperature changes during the compost process suggest that both mesophiles and thermophiles equally play crucial roles in the compost.

New extremely thermophilic bacteria (eubacteria), *Calditerricola satsumensis* and *C. yamamurae*, were isolated from an aerobic, high-temperature compost. They are different from the representative thermophiles of traditional composts such as *Geobacillus stearothermophilus* in that they are gram stain negative and nonspore formers. Their cellular polyamine compositions entirely differ from those of *Bacillus* and *Geobacillus* species. Though they can be easily isolated from high-temperature composts which were stored for long time such as several months and can grow actively under suitable conditions, these new isolates cannot survive at high temperature as well as ambient temperature, suggesting that the isolates are deeply adapted to the environment of the compost.

DGGE is a powerful tool to analyze structure of bacterial community in compost, though there are many technical problems to be solved in future. Our DGGE analyses implied that the bacterial community of the aerobic, high-temperature compost varies dynamically during the fermentation process. The whole bacterial community of the compost can be regarded as a "super organism" (Wilson and Sober 1989).

The aerobic, high-temperature compost seems to be a promising resource for hunting enzymes of industrial interest. Hydrolytic enzymes for collagen, keratin, cellulose, starch, and so on were detected, and they are heat resistant without exception.

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