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Effects of Continuous Thermophilic Composting (CTC) on Bacterial Community in the Active Composting Process

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Abstract The method of continuous thermophilic composting (CTC) remarkably shortened the active composting cycle and enhanced the compost stability. Effects of CTC on the quantities of bacteria, with a comparison to the traditional composting (TC) method, were explored by plate count with incubation at 30, 40 and 50°C, respectively, and by quantitative PCR targeting the universal bacterial 16S rRNA genes and the *Bacillus* 16S rRNA genes. The comparison of cultivatable or uncultivatable bacterial numbers indicated that CTC might have increased the biomass of bacteria, especially *Bacillus* spp., during the composting. Denaturing gradient gel electrophoresis (DGGE) analysis was employed to investigate the effects of CTC on bacterial diversity, and a community dominated by fewer species was detected in a typical CTC run. The analysis of sequence and phylogeny based on DGGE indicated that the continuously high temperature had changed the structure of bacterial community and strengthened the mainstay role of the thermophilic and spore-forming *Bacillus* spp. in CTC run.

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Introduction

Composting is a self-heating, aerobic, bio-decomposition process of organic waste that has advantages over other disposal strategies since it reduces waste volume by 40–50%, kills pathogens by the heat generated during the thermophilic phase, and provides a final product that can be used as a soil conditioner or fertilizer [12, 16, 41]. Continuous thermophilic composting (CTC), which was first applied to a 55-gal rotating drum composting system by Schulze [29], was demonstrated to be more effective than traditional composting (TC) since it produced a more stable product and shortened the process [42]. It is known that the decomposition of organic substrates in compost is mainly carried out by microorganisms. Therefore, it is very important to perform comprehensive studies on the structure and the dynamic variation of microbial communities for the optimization of CTC method.

A typical TC process is usually divided into four different phases, mesophilic (S), thermophilic (T), cooling (C), and maturing (M) according to the temperatures in compost, and mesophilic microorganisms dominate the S phase, thermophilic bacteria (including actinobacteria) and fungi dominate the T phase, and revived mesophilic microorganisms dominate the C or M phase [28]. Since the temperature is higher than 50°C throughout the whole active composting process in a CTC pile, the S phase may be very short or missing, and these conditions are likely to select for different microbial population than those found during TC process. Although many studies have been conducted to investigate the microbial communities in

various composts [5, 9, 11, 28, 35], only a few studies have focused on the effects of temperature on bacterial species diversity in continuous thermophilic [26, 32, 34] or isothermal [22] composting.

Nowadays, cultivation-independent approaches, such as denaturing gradient gel electrophoresis (DGGE), clone library, terminal restriction fragment length polymorphism (T-RFLP), quantitative PCR (qPCR), and microarray have been used for investigating microbial communities in compost [9, 11, 35]. Among these, DGGE has proven to be an effective technique to detect microbial community shifts and in identifying the phylogenetic affiliations of microbial populations [1, 36, 39]. qPCR, a fast, reliable, sensitive, and convenient method to enumerate various cultivable or uncultivable bacteria, has recently been applied and optimized to quantify bacteria from various environments [15, 25].

In the present study, several forced aeration static pile composting systems were employed to treat organic waste with TC and CTC methods. To investigate the effects of continuous high temperature on the bacterial community in CTC piles, the authors assessed the diversity and the dynamics variation of bacterial community by DGGE analyses and phylogenetic analysis of 16S rRNA gene sequences, estimated the cell numbers of cultivable bacteria with the plate count method and further quantified the total numbers of total bacteria and *Bacillus* spp. by qPCR analysis, respectively.

Materials and Methods

Composting and Sampling

Solid wastes were collected from a municipal transfer station near the campus of Hunan University in Changsha, China, and most of the inorganic materials, such as glass, and inert components, such as plastics, were discarded. Then, the source materials for composting, consisting mainly of vegetables, food, leaves, grass, and small branches, were shredded to a size of 1–3 cm. The moisture ratio of the materials was adjusted to about 60% by spreading them on the ground and natural air drying for 12 h.

The composting experiment was designed as described previously [42] and included packing 20 kg (wet weight, about 28 L) of source materials into a bench-scale composting reactor (30 cm in diameter, 45 cm in depth). The reactors were placed in an incubator and sequentially operated under TC or CTC conditions (TC, run A; CTC, runs B–E) for 60 days. In run A, the incubator temperature was maintained at 30°C. The incubator temperature was maintained at 50°C during the first 30 days in runs B and C, while it was maintained at 40°C on the first day and then raised to 50°C during the remaining 29 days of thermophilic composting in runs D and E. Air from a compressor was supplied at a flow rate of 0.25 L/[min·kg (wet weight)] to maintain aerobic conditions throughout the experimental runs, and a higher flow rate was used to reduce the temperature to 60°C in runs C and E when the composting pile temperature exceeded 60°C. Each composting run was conducted in triplicate, and three subsamples of each sample were tested. The compost was turned manually every 24 h, and samples with a total weight of 50 g (wet weight) were collected daily at five random locations (10 g from each location) in each pile immediately after windrow turning and subjected to physicochemical properties analyses or stored at −20°C for other uses.

C/N ratio, germination index (GI), specific oxygen uptake rate (SOUR), dissolved organic carbon (DOC), and dehydrogenase activity (DA) were monitored daily to determine the average composting cycle [42].

DNA Extraction and Purification

Total DNA was extracted from each 1 g of compost sample (wet weight) with protease K and cetyl trimethylammonium bromide as described by Yang et al. [44]. After purification with a TIANquick Midi Purification Kit (TianGen, Beijing, China), the DNA was dissolved in 200 μL sterilized Milli-Q water (DNA concentrations ranged from about 100 to 200 ng/μL), and 5 μL of DNA was used for agarose gel electrophoresis.

PCR Amplification

Universal bacterial 16S rRNA gene primers 341 F (5′-CCT ACG GGA GGC AGC AG-3′) with GC rich clamp (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G-3′) [14, 24, 43] and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) [24, 43] was used for direct amplification of partial 16S rDNA fragments from total DNA. Each 50 μL PCR reaction mixture contained about 150 ng of template DNA, 5 μL of 10× buffer with 15 mmol/L $MgCl_2$ (TianGen, Beijing, China), 20 nmol each dNTP mixture (TianGen, Beijing, China), 5 pmol each primer (Sangon, Shanghai, China), 2.5 U *Taq* DNA polymerase (TianGen, Beijing, China), 10 μg bovine serum albumin (BSA) V (Sangon, Shanghai, China), and 39.5 μL of sterilized Milli-Q water. PCR amplification was run on a MyCycler (Bio-Rad, Hercules, CA, USA) using the following cycling conditions: 5 min at 94°C; 35 cycles with each cycle consisting of 40 s at 94°C, 45 s at 55°C, and 40 s at 72°C; followed by a final 7-min extension at 72°C. Amplification products were stored at −20°C before being analyzed.

Each 5 μL PCR product was visualized on 1.5% agarose gel under the Gel Doc XR System (Bio-Rad, Hercules, CA,

USA) after being mixed with 0.5 μL of 100× SYBR™ Green I and run at 10 V/cm for 25 min. The left 45 μL PCR product was purified by a TIANquick Midi Purification Kit (TianGen, Beijing, China) and applied to DGGE analysis within 4 h.

DGGE Analysis

The DGGE was carried out using a DCode™ Universal Detection System instrument and gradient former model 475 according to the manufacturer's instructions (Bio-Rad). The denaturant solution was prepared as described by Muyzer et al. [24], and electrophoresis was performed in 8% acrylamide gels with a denaturing gradient of 45–75%. Gels were run in 1× TAE buffer at 60°C for 14 h at 100 V. Gels were stained with 1× SYBR™ Green I and visualized under UV light with the Gel Doc XR System (Bio-Rad). Bands were recognized by the program QuantityOne V4.63 (Bio-Rad), and some weak bands that could not be recognized by the program were identified manually under UV light.

Identification and Phylogenetic Analysis of DGGE Bands

All recognized bands, even those co-migrating in different lanes, were excised from DGGE gels under UV light, and a bead beating method was applied to extract DNA from the gel slices [24]. The purified DNA was amplified using the primer pair GC341F/907R. After purification with a kit (TianGen), the products were ligated into the pGM-T easy vector (TianGen) and resulting plasmids were electroporated into competent *Escherichia coli* DH5α cells using a MicroPulser Electroporator (Bio-Rad). For each band, three different clones were selected for sequencing with the help of penbritin and blue-white spot screening [3]. Plasmid DNA was obtained from transformant using alkali lysis method [6], and the insert was amplified using the primer pair GC341F/907R for re-DGGE to affirm that the insert was the targeted band. Each affirmed clone was sequenced in a single run (Sangon). Few mutative bases were detected among the three independently sequenced clones, and we aligned the three sequences and determined a consensus sequence of each band. Twenty-five distinct bands, designated as A to Y in the DGGE profile, were identified from the SYBR™ Green I stained denaturing gradient gels, and phylogenetic identity was determined by comparing the partial 16S rRNA gene sequences of the clones with sequences in GenBank using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was constructed with MegAlign in Lasergene 7.0 (DNASTAR, Madison, WI, USA) using ClustalV method. Sequences with a similarity of >97% were considered to belong to the same species [20].

Plate Count of Bacteria

The cultivation-dependent method of plate count was used to estimate the number of viable bacteria presenting at the two composting piles. About 1 g of compost sample (wet weight) was suspended in 90 mL of sterile sodium chloride solution and was sequentially shaken at 200 rpm for 1 h at 35°C. Appropriate serial dilutions of the suspensions were made in sterile sodium chloride solution. Aliquots (0.1 mL) of each dilution were spread with the medium reported by Reasoner and Geldreich [27], which contained 0.5 g of yeast extract, 0.5 g of Difco Proteose Peptone no. 3 (BD, Franklin Lakes, NJ, USA), 0.5 g of Casamino Acids (BD), 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of sodium pyruvate, and 15 g of agar per liter of distilled water. The pH was adjusted to 7.2 with crystalline K_2HPO_4 or KH_2PO_4, and then the medium was sterilized at 121°C for 15 min. Nine plates were used per dilution, and every three plates of the same dilution were cultured at 30, 40 and 50°C, respectively.

qPCR Analysis

Real-time PCR was performed on an iCycler iQ5 thermocycler (Bio-Rad) to determine the 16S rRNA gene copy numbers of total bacteria and *Bacillus* spp. in the compost.

The amplifications were carried out in a total volume of 25 μL. The qPCR mix for amplifying bacterial 16S rRNA gene contained 2 ng of compost microbial DNA, 4 pmol universal bacterial primers (341F and 517R 5′-ATT ACC GCG GCT GCT GG-3′) [23], 5 μg BSA, 10 μL of 2.5× RealMasterMix and 1.5 μL of 20× SYBR solution (TianGen). The two-step Amp+Melt protocol was as follows: (1) amplification step—denaturing at 95°C for 4 min, 40 cycles of 30 s at 94°C and 30 s at 55°C, 1 min at 95°C, and 1 min at 55°C, and (2) melting curve analysis step—79 cycles of 30 s starting from 55°C to 94°C. In the present study, *Bacillus* specific primers, BacF265 (5′-GGC TCA CCA AGG CAA CGA T-3′) and BacR525 (5′-GGC TGC TGG CAC GTA GTT AG-3′), were designed for amplifying 16S rRNA gene of *Bacillus* spp. with AlleleID 6 (PREMIER Biosoft International, Palo Alto, CA, USA) based on the 16S rRNA gene sequences of *Bacillus subtillis*. The qPCR mix contained 3 ng of compost microbial DNA, 4 pmol primers (BacF265 and BacR525), 5 μg BSA V, 10 μL of 2.5× RealMasterMix, and 1.5 μL of 20× SYBR solution (TianGen). The two-step Amp+Melt protocol was as follows: (1) amplification step—denaturing at 95°C for 4 min, 40 cycles of 30 s at 94°C and 30 s at 57°C, 1 min at 95°C, and 1 min at 57°C, and (2) melting curve analysis step—75 cycles of 30 s starting from 57°C to 94°C. The fluorescent signal was measured at the end of each annealing/extension step. A negative control without

the corresponding template DNA was included in every qPCR assay for each primer and probe set. All experiments were done in triplicate. Data analysis was carried out with the iQ5 Optical System Software (version 1.0.1384.0 CR) (Bio-Rad). The parameter C_t (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected. Before qPCR, primer pairs 341F/517R and BacF265/BacR525 were tested by PCR and determined not to produce non-specific product.

16S rRNA gene fragments of *Bacillus subtilis* were PCR amplified with primer pair of 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) [18, 19] and 1492R (5′-GTT ACC TTG TTA CGA CTT-3′) [30, 46]. The PCR products were cloned into the Target Clone™ vector (Toyobo, Osaka, Japan). The plasmids were extracted in the range of 10^8–10^9 copies per microliter, serially diluted, and used as templates in qPCR for standard curves generation, as previous reports described [17, 45].

Statistical Analysis

To estimate the succession of bacterial communities during the active composting process, the data of band position on the DGGE pattern was analyzed by performing the hierarchical cluster analysis with the SPSS version 16 (SPSS Inc., Chicago, IL, USA). The Ward method was used in the analysis. The program was also used for analyzing the correlation and the significance of cell numbers from the plate count or the 16S rRNA gene copies from the qPCR.

Nucleotide Sequence Accession Numbers

The retrieved 25 distinct 16S rRNA sequences (bands A to Y) of the 16S rRNA gene clones have been deposited orderly in the GenBank database under accession numbers FJ810808–FJ810832.

Results and Discussion

Composting and Sampling

Run B showed the shortest average composting cycle of 14 days as determined by C/N ratio, GI, SOUR, DOC, and DA, while runs A, C, D, and E matured in 27, 16, 18, and 19 days, respectively (Table 1). Therefore, two typical runs, designated as A and B from the TC runs A and CTC runs B, respectively, were selected for sampling. The sample taken from the source materials was designated “0”; the other samples were designated based on run and sampling time (e.g., “A3” indicates a sample taken from run A on day 3). Thirteen samples were selected from runs A and B (Table 2).

Table 1 Composting cycles (days) of the five runs determined by C/N ratio, GI, SOUR, DOC and DA [42, 43]

	A	B	C	D	E
C/N ratio	19	14	16	17	18
GI	26	14	15	17	19
SOUR	27	15	17	18	19
DOC	27	14	16	17	18
DA	32	13	16	18	19
Composting cycle	27	14	16	17	19

DGGE Analysis

Total DNA with a maximum length of 23 kb was extracted and sequentially purified, and partial 16S rRNA gene fragments of about 620 bp (including the GC rich clamp) were PCR amplified without unexpected products. Due to the detection limit of DGGE analysis, only dominant species in the community could be detected. Furthermore, it is not always possible to separate DNA fragments despite sequence variation [7, 38]. Therefore, it is necessary to perform the DGGE by loading the same volume of PCR product that amplified from equal-purified DNA and sequence all the interested bands in the gel, and each 45 μL of specific PCR product was subjected to DGGE in the present study. No obvious differences between the banding patterns of the triplicate samples were observed, which indicated good reproducibility of DGGE analysis in this study, and a representative gel images and one of its repetition are shown in Fig. 1. All the bands of interest in the gel were sequenced, and 25 distinct bands, designated as A to Y, were finally identified from the DGGE profile (Fig. 1a). Then, each distinct band could be considered as a dominant bacteria strain in the communities.

The peak temperatures of 55.4 and 69.6°C appeared on day 5 in run A and on day 5 in run B (Table 2), respectively. In the DGGE profile, the band numbers in run A increased from seven on day 0 to 12 on day 5 when the peak temperature appeared. The result indicated an increase of the detectable bacteria species in the pile with the rising temperature. In run B, only seven, nine, and 11 distinct bands were identified from B1, B3, and B5, respectively. The extremely high temperature apparently suppressed the bacteria after the peak temperature phase in run B since only eight and nine bands were identified from lanes B8 and B10, respectively, while 13, 16, and 11 bands were identified from lanes A8, A13, and A20 from the M phase in run A, respectively. Five bands (B, G, H, L, and T) were not detected in any CTC samples by DGGE analysis.

Table 2 Summaries of the samples

Sample name	Source	Temperature (°C) [42, 43]	pH [42, 43]	TOC/% [42, 43]	TKN/% [42, 43]	DOC/g/kg [42, 43]
0	Source material	30.0	6.83	46.26	0.92	43.15
A3	Traditional compost run A	43.9	6.22	42.78	0.91	38.24
A5[a]		55.4	6.58	38.13	0.90	30.49
A8		47.9	6.91	30.06	0.87	17.89
A13		37.4	7.25	20.94	0.83	8.45
A20		33.0	7.21	18.45	0.81	6.18
A28		30.1	7.06	18.00	0.79	5.44
B1	Continuous thermophilic compost run B	52.1	6.46	44.25	0.92	42.07
B3		65.6	7.75	40.25	0.91	35.18
B5[a]		69.6	8.09	34.07	0.89	26.88
B8		57.2	7.85	22.41	0.84	12.65
B10		52.1	7.75	19.07	0.82	7.29
B14		50.0	7.55	18.06	0.79	5.02
B28		50.0	7.21	18.01	0.78	4.98

TOC total organic carbon; *TKN* totoal Kjeldahl nitrogen; *DOC* dissolved organic carbon

[a] The samples of peak temperature

The results above indicated that the continuous high temperature in run B observably suppressed the growth of some bacteria and therefore induced a decrease in diversity of abundant bacteria during the active composting process.

Similar numbers of bands, 14, 15, and 13, were detected from matured compost of A28, B14, and B28, respectively. However, the relative abundant diversity of bacterial communities in matured compost from runs A and B did not necessarily mean a high activity of bacteria since lower values of SOUR and DA in these samples were observed in our previous report [42], compared with the samples from active composting process. The quantitative analysis also showed the small biomass in matured compost from either run A or B (Fig. 4). Therefore, we surmised that the relative abundant diversity of bacterial communities in matured compost should be ascribed to the decrease of most dominant bacteria in the T phase, following a decrease of the available nutritive materials in the piles.

Statistics Analysis

No obvious differences between the banding patterns of the triplicate samples were observed, which indicated good reproducibility of DGGE analysis in this study. Therefore, the data of band position in a representative gel images (Fig. 1a) and one of its repetition (Fig. 1b) was used for cluster analysis. All the samples from runs A and B were grouped into two clusters A and B on the basis of the band position on DGGE pattern (Fig. 2). Cluster A consisted of almost all the samples in the phases C and M, except A8, and matured compost in both runs, while the cluster B consisted of all the other samples, which indicated that the peak temperature would have brought a tremendous change to the structure of bacterial community, especially in run B.

In cluster A, samples B8, B10, B14, and B28 showed high similarity to each other, especially B14 and B28, and samples A13 and A20 showed high similarity to each other but showed a distant relationship with A28. We speculated that many bacteria were killed by the extremely high temperature on day 5 and therefore less bacterial species revived in run B compared with the samples from the C and M phases in run A. Although matured compost samples A28 and B14 adopted similar numbers of dominant bacteria, they showed low similarity to each other, indicating very different bacterial community formed in the two matured compost due to different temperature change curves in the two piles. That samples A3, A5, and A8 showed high similarity to each other in cluster B indicated good succession of bacterial species in run A. However, sample 0 showed higher similarity to B1 and B3 other than A3. Sample B5 showed low similarity to any other samples from runs A and B, which suggested a unique bacterial community selected by the peak temperature of 72°C.

Phylogenetic Analysis

A phylogenetic tree (Fig. 3) was constructed by the program MegAlign in Lasergene version 7.0 (DNASTAR, USA) using the ClustalV method. Based on the phylogenetic tree details, most of microorganisms could be divided into three groups of the *Proteobacteria* (*Pseudomonodales* and *Burkholseriales*), the *Actinomycetales*, and the *Bacillales*. Band Q, with a sequence 97% identical to *Sphingobacterium thalpophilum*, showed distant genetic relationship with any other bands on the phylogenetic tree.

Bands B, H, L, and T, which were only detected from the samples in run A, showed high similarity to some

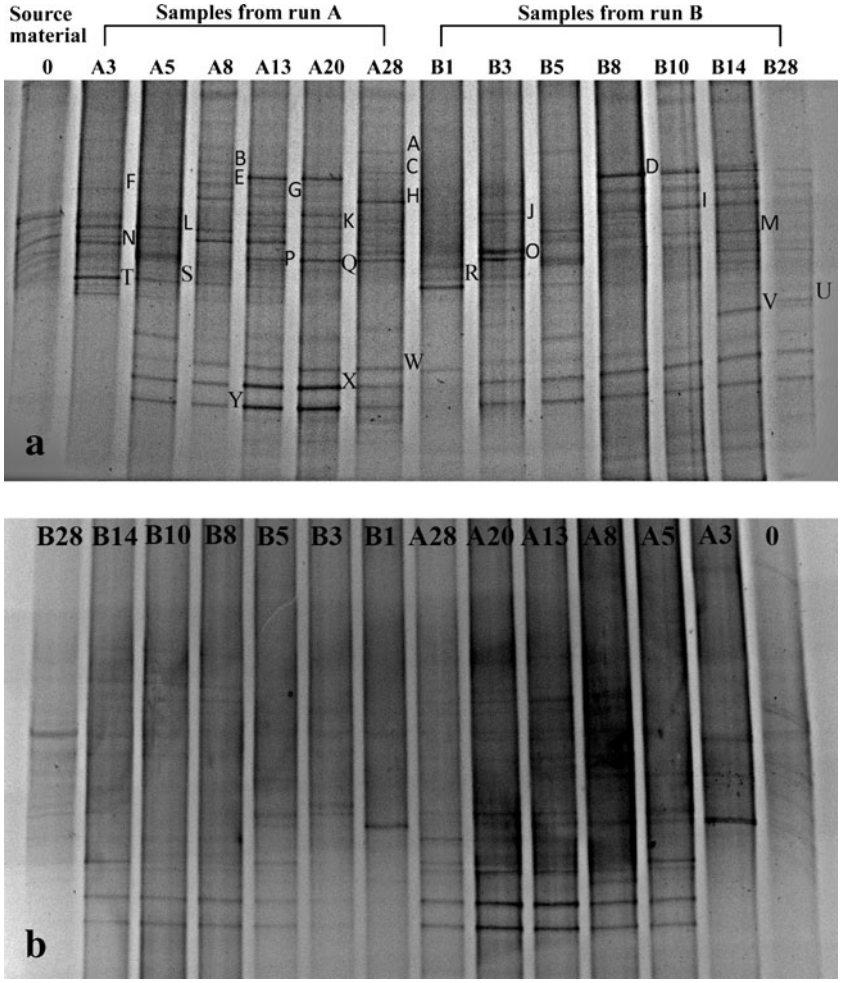

Figure 1 **a** DGGE pattern of amplified bacterial 16S rDNA fragments. *Lane 0* is the source material sampled on 0 day, *lanes A3*, *A5*, *A8*, *A13*, *A20* and *A28* are samples from third, fifth, eighth, 13th, 20th, and 28th day in run A, respectively, and *lanes B1*, *B3*, *B5*, *B8*, *B10*, *B14* and *B28* are samples from first, third, fifth, eighth, tenth, 14th, and 28th day in run B, respectively. *Letters A to Y* stand for the excised and sequenced distinct bands on the DGGE pattern. **b** Repetition of DGGE pattern for statistics analysis

reported mesophiles of *Alcaligenes faecalis* [10, 13], *Pseudomonas mendocina* [21, 28], *Streptomyces violaceoruber* [28], and *Pseudomonas* sp. [4], respectively. In contrast, the other bands could be detected from both runs, which revealed the thermostability or thermophily of their corresponding strains. Fifteen bands appeared in three or more samples from run B, and ten of their corresponding sequences showed high similarity to strains in genus *Bacillus*, most of which are facultative or obligate thermophiles. However, only one band of E that was detected from three samples in run A showed a sequence identity of 97% to a mesophilic species of *Cellulosimicrobium cellulans*.

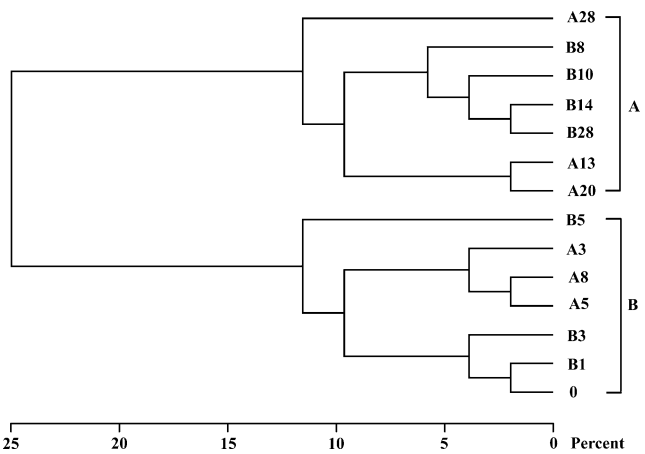

Figure 2 Dendrogram of hierarchical cluster analysis of samples from CTC and TC runs on the basis of band position on the DGGE pattern

Four sequences corresponding to bands E, G, I, and L were >97% identical to species from the order *Actinomycetales* and were grouped into an independent cluster on the phylogenetic tree. Bands E (>97% identical to *C. cellulans*) and G (>97% identical to *Micromonaspora* sp.) appeared mainly in the samples in the later composting period in runs A and B on the DGGE pattern, indicating their functions of degrading recalcitrant matters and maturing compost. Band I, >97% identical to *Thermocrispum municipale*, was detected in all the samples in run B except B5, which indicated its important role in degrading organic matter in run B. Bacteria in the other big cluster on the phylogenetic tree were >97% identical to the phylum of *Proteobacteria*, especially the genus of *Pseudomonas*. These strains were absent in most

Figure 3 Phylogenetic tree of sequenced 16S rDNA fragments excised from the DGGE pattern constructed by Lasergene V7.0 using ClustalV method

samples from run B, while they were detected in several samples in run A, indicating that they are less important in run B. The biggest cluster on the phylogenetic tree was mainly composed of several bands whose corresponding sequences were >97% identical to *Bacillus* spp., and the other two bands in this cluster were >97% identical to *Paenibacillus* spp., species close to *Bacillus* spp. It was not a surprise to detect so many species of *Bacillus* bacteria dominating the run B because these bacteria can be found everywhere and have been commonly detected as the predominant species in compost. Moreover, most of *Bacillus* spp. have been known as facultative or obligate thermophiles [33].

Quantitative Analysis of Bacteria

The result that the organic materials in run B were degraded faster than that in the run A (Table 2) suggested a more enormous bacteria biomass in run B. Two methods, plate count and qPCR, were performed to investigate the numbers change of total bacteria and *Bacillus* spp. during the composting processes in the two runs. Temperatures of 30, 40, and 50°C were selected for total bacteria cultivation since different bacteria, mesophiles, facultative, or obligate thermophiles might grow in different phases during the composting process (Fig. 4a–c). Figure 5 shows the numbers of 16S rDNA copies of total bacteria and *Bacillus* spp. enumerated by qPCR.

The cultivatable bacteria numbers in both runs at three temperature ranged from 0.5×10^8 to 25×10^8 CFU/g (wet weight), consisting with that reported by Strom [32]. Similar bacteria numbers were counted from the same samples at 30 and 40°C in run A. Fewer bacteria could grow on the medium at 50°C, especially those from A3 and A8.In run B, the maximum number of cultivatable bacteria per sample was typically counted at 50°C, especially the samples B1, B3, B5, B8, and B10, while the fewest

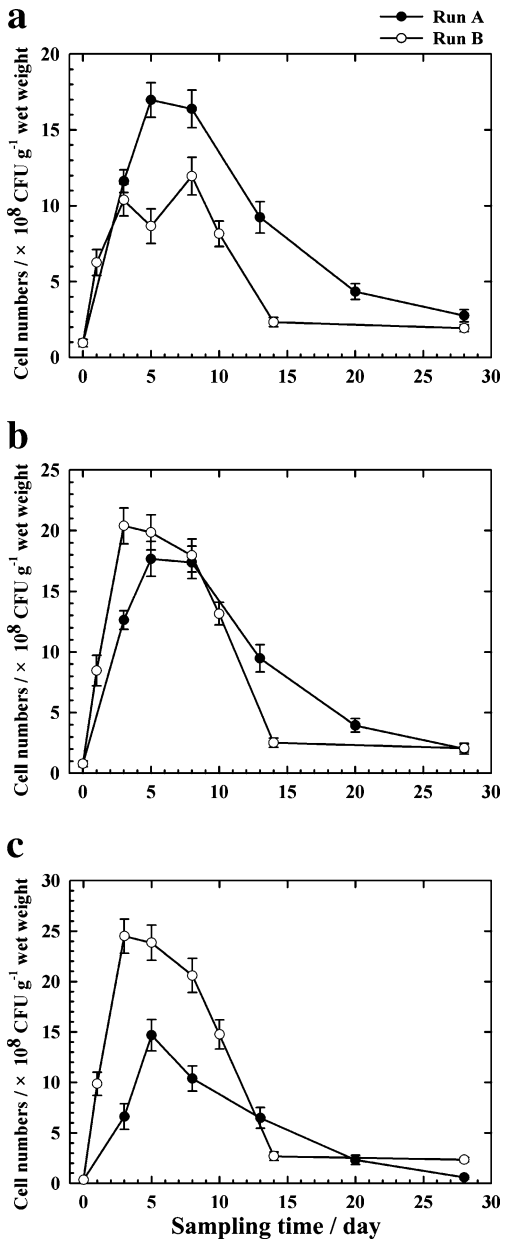

Figure 4 Bacterial colonies numbers counted from the culture plates at 30°C (**a**), 40°C (**b**), and 50°C (**c**) in runs A and B, respectively

colonies were cultured at 30°C. The results indicated the dominance of thermophiles in run B and also suggested that low culture temperature must have inhibited the growth of cultivatable thermophiles and led to lower numbers in plate count. That fewer colonies were counted from B5 than those from B3 and B8 at 30°C might indicate the predominance of the obligate thermophiles in B5 and the suppression of extremely high temperature on most bacteria.

The plate count showed that the bacteria numbers of the same sample from different culture temperatures differed from each other, and the bacteria numbers at the same culture temperature showed some notable differences between the samples from similar phases in runs A and B. For example, much more bacteria ($P<0.01$), $(16.97\pm1.15)\times10^8$ vs. $(8.65\pm1.14)\times10^8$ CFU/g (wet weight), were counted from A5 than those from B5 at 30°C, and more bacteria were counted from B5 at 50°C than that at 30°C. However, similar bacteria numbers were numerated from the two samples at 40°C. The results indicated that a CTC method would have remarkably changed the proportion of mesophiles and thermophiles in compost by evidently decreasing the mesophiles in the peak temperature phase and post-peak temperature phase (Fig. 4a). Anyway, more bacteria (the sum of cultured bacteria at 30, 40, and 50°C) were counted from run B than that from run A in the similar phases and, therefore, might have invited a faster degradation of organic matters in run B [8]. This could also be supported by the lower DOC concentrations in samples from run B, compared with the samples from run A in the same day (Table 2), since the DOC concentration reflected the directly usable substances for microorganisms. However, similar low bacteria numbers at the three temperatures were summed from A28, B14, and B28, suggesting that the matured compost from run B was as stable as that from run A.

A SYBR Green I™ based qPCR was employed to investigate the changes of total bacteria population in runs A and B (Fig. 5) since several studies have reported that only 0.1–10% of the microorganisms in the environment were cultivable [2, 37, 40]. The DGGE analysis in the present study also showed the dominance of *Bacillus* spp. in most samples from both runs. Therefore, qPCR was also employed to quantify *Bacillus* spp. in the samples (Fig. 4d). The qPCR results clearly showed that *Bacillus* spp. possessed greater importance to run B than that to run A since about one half of the detected 16S rDNA fragments came from *Bacillus* spp. in run B while the proportion in run A was less than one third. However, the facts that

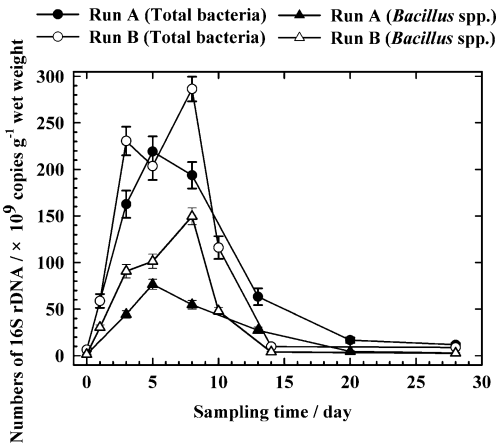

Figure 5 16S rRNA gene copy numbers of total bacteria and *Bacillus* spp. determined by qPCR in runs A and B

bacteria may have multiple copies of 16S rRNA gene, such as that many *Bacillus* spp. have been shown to possess upwards of ten copies of the rRNA gene [31], and that this varies from organism to organism make it difficult to determine the actual numbers of bacteria with qPCR results.

The most bacteria in run A were detected on day 5, while it occurred on day 8 after a decrease on day 5 in run B. Furthermore, the qPCR detected less total bacteria in B5 than that in A5, which suggested the suppression of extremely high temperature to some bacteria in run B. More *Bacillus* cells were detected from samples in run B than that in run A during the initial 10 days of composting, and the results indicated that the CTC method favored the growth of *Bacillus* strains. Considering the larger proportion of thermophiles in the bacterial community in run B (Fig. 4a–c) and the higher activity of thermophiles [22], the quantify analysis showed the main reason that, compared with TC method, the CTC method could accelerate the degradation of organic matter and the maturation of compost, though the CTC method reduces the size of bacterial community.

Effects of CTC on Bacterial Community

The DGGE analysis showed that the CTC method reduced the diversity of dominant bacteria in compost, compared with the TC method. Combined results from the DGGE analysis and the phylogenetic analysis indicated that the continuously high temperature remarkably changed the structure of bacterial community and strengthened the mainstay role of the thermophilic and spore-forming *Bacillus* spp. in run B. Both the plate count and the qPCR analysis in the present study indicated that more bacteria (the sum of mesophiles and thermophiles) might reside in the CTC pile than those in the TC pile during active composting. Therefore, the CTC method accelerated the degradation of organic matter and shortened the composting cycle. Since the qPCR also detected more *Bacillus* cells in run B and a faster degradation of organic materials in run B, the present study indicated that more thermophilic *Bacillus* spp. in compost could benefited the maturation of compost.

In sum, the present study deeply investigated the effects of continuously high temperature on bacterial communities and showed the key role of *Bacillus* spp. in continuously high temperature composting.

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