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Research Article

Bacterial Community Dynamics and Influencing Variables during Composting with Different Initial Raw Materials (Pig Manure and Tea Waste)

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Abstract

This research was carried out to determine the structure dynamics and shaping factors of bacterial community during agricultural waste composting with pig manure or tea waste. Bacterial community structure was determined by Denaturing Gradient Gel Electrophoresis (DGGE). Redundancy analysis was used to estimate the relationship between bacterial community structure and environmental parameters. Results showed that *Firmicutes, Proteobacteria, Acidobacteria*, and *Bacteroidetes* were the dominant bacterial phyla and varied in samples with different initial materials. *Anoxybacillus toebii, Keratinibaculum paraultunense, Peptoniphilus methioninivoras*, were remarkable observed during the cooling and maturation stages of pig manure piles. While *Clostridium bytyricum, Halanaerobium salsuginis* were widely distributed during the whole composting process for tea waste. Redundancy analysis indicated that EC, pH, TN, and TOC showed predominant influence on the bacterial community structure. Significant amounts of the variation (47.0%) of community structure were explained by those parameters. These parameters might be the most responsive ones influencing the succession of bacterial communities during agricultural waste composting for pig manure and tea waste.

Keywords: Composting; Bacterial Community; Redundancy Analysis; Shaping Factor; Initial Raw Material

Introduction

Composting has been considered as a resourceful treatment technology to stabilize agricultural solid wastes [1-5]. Under controlled conditions, the biodegradable components in solid waste were utilized and transformed by the widespread microorganisms in nature, including bacterial and fungal communities. Bacterial communities play crucial roles during agricultural waste composting process. They are important drivers for the organic matter decomposition and stabilization during composting [6, 7].

Physico-chemical factors have various influences on the bacterial community dynamics during agricultural waste composting [7]. Previous study found that the properties of the raw material causes differences in microbiological parameters [8]. Many researchers found that different raw materials in composting system had an important impact on bacterial populations. Bacterial populations adapting to the physiological and biochemical properties of available carbon substrates will have higher biological activity [9, 10]. Inoculation of *Pichia kudriavzevii* RB1 accelerated composting process and changed the microbial community structure [11]. The multistage inoculation could influence the duration of high pile temperature and diversity of bacterial community during municipal solid waste composting [12].

Many studies suggested that the abundance and diversity of bacterial population are influenced by their initial composition originating from different raw material [12, 13], and inoculation with different bacteria [14-16] or fungi [13, 14]. Different raw materials induced different physico-chemical parameters and different initial microbial communities, thus affecting microbial community dynamics during the composting process, especially in the early stage [11]. The different microbial community structure will react to composting process [11, 12]. It is of great importance to determine the interaction between raw material and microorganism (especially bacterial community). Up to now, it is lack and incomplete that the environmental factors have been analyzed with the changes of microbial community structure to separate out their relative importance.

Thus, pig manure and tea waste were used in this study as the main initial materials for composting, respectively. The changes of physico-chemical parameters with different starting materials were determined. The structure dynamics of bacterial communities in different piles was characterized by PCR-Denaturing Gradient Gel Electrophoresis (DGGE). The relationship between changes in physico-chemical factors and structure changes of bacterial community were determined by Redundancy analysis. Variation partitioning analysis was also conducted to relate changeable environment to the succession in community structure, and present statistical analysis for mining potential relations. It is necessary to determine the dynamic changes and shaping factors of bacterial communities during composting system with different starting materials, and to provide theoretical guidance and assistance for the composting of agricultural wastes.

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Materials and Methods

Composting piles set-up

Pig Manure (PM) and Tea Waste (TW) were used as the main difficult-degradable materials for different treatments, respectively. They were gathered from the countryside of Changsha, Hunan, China. These materials were chopped into about 15 mm pieces after air-dried. The discarded vegetables were gathered from food market around Changsha. Vegetable waste was cut into about 15 mm pieces and supplied as easy decomposable materials. The properties and homogenized ratios of those wastes are shown in Table 1.

Agricultural wastes were packed loosely in the containers with good heat preservation (length × width × height: 0.50 × 0.40 × 0.45 m). Moisture was adjusted by adding ultrapure water at around 50-60%. The composting piles were turned when the pile temperature exceeds 50°C to avoid the possible anaerobic environment.

Sample collection and Physico-chemical parameter determination

Sub-samples were collected on days 0, 5, 10, 15, 20, 30, 40 and 50, respectively. Samples for determination of physico-chemical parameters was stored at 4°C. Other portions for DNA extraction was immediately stored at -20°C until using. Piles temperature was measured with a thermometer. Composting samples were mixed with ultrapure water at the ratio of 1:10 (weight/volume), shaken at 120 rpm for 1 h and centrifuged at 8, 000 rpm for 10 min to get the filtrate [17, 18]. The pH and EC in the filtrate were measured with the EC meter. The moisture content was determined by drying samples at 105 ± 2°C for 5h. Total Organic Carbon (TOC) and Total Nitrogen (TN) were determined by aerobic combustion and Kjeldahl method, respectively. TOC/TN value was taken as the C/N ratio.

DNA extraction and PCR-DGGE

Total DNA of composting samples were extracted by following the methods mentioned previously [2] and stored at -20°C until using. Bacterial 16S rDNA genes were amplified by using primers 341F/907R (C C T A C G G G A G G C A G C A G/C C G T C A A T T C C T T T R A G T T T) [14]. The primer 341F had an additional GC clamp at its 5' to reinforce the DNA strands [7]. Amplification reactions (50 µL) were prepared with 1 µL of extracted DNA, 25 µL of 2 × Power Taq PCR MasterMix (Bioteke, Beijing), 1 µL of each primer (10 µM), and 22 µLof sterile ultrapure water. The PCR were performed on the MyCycler (Bio-Rad, USA) with procedure: 3 min at 94°C; 40s at 94°C, 40 s at 55°C, 40 s at 72°C for 35 cycles; 7 min at 72°C and ended at 4°C.

The amplified products were electrophoretically separated on the Dcode™ Universal Mutation Detection System (Bio-Rad, USA). 30 µL of purified PCR product was loaded into the 8% polyacrylamide gel (denaturing gradient of 35-70%) and electrophoresised was carried out in the 1 × TAE buffer at 60°C (90 V, 12 h). After the electrophoresis, the DGGE gel was stained by 50 mL of 1 × TAE containing 5µL of SYBR™ Green I nucleic acid dye for 10 min and then was scanned by the Gel Doc2000 UV Transilluminator (Bio-Rad, USA).

Bands sequencing

Based on the DGGE isolation result, representative bands were excised and purified by using the DNA Fragment Purification Kit (Takara, Japan). The purified genes were spliced into the pGEM-T Easy Vectors and transferred into *Escherichia coli* (DH5α) competent strain. Positive clones were identified and further cultured by ampicillin resistance and blue-white colony appearance. The cloned plasmid DNA was sequenced by IIIumina Miseq platform. Phylogenetic analysis was performed with NCBI search data and MEGA 6.0 [19]. Twenty-six bacterial gene sequences were distinguished and detected in the DGGE profile. Nucleotide sequence information for Band1 to Band26 were deposited in GenBank with accession numbers were MN209868 to MN209893, respectively.

Data analysis

All physico-chemical parameters were measured by 3 parallels. Bacterial DGGE profile was analyzed after subtracting the background noise by Quantity One 4.6.3 (Bio-Rad, USA) and the relative fluorescence intensity of bands were quantified [7]. The matrix of bacterial species structure was constructed by the percentage of the peak of band intensity in the different lanes. To eliminate the interference caused by different dimension in different data, the matrix of environmental factor was constructed by SPSS software conducting Z-standardization. The correlation between the structure of bacterial species and physico-chemical parameters was logically operated by multiple direct gradient analysis using Canoco 5.0. The following two standards must be met for bacterial data to reduce the possible influence of rare populations on multivariate analysis: 1) The same band appeared more than at least twice; and 2) The relative abundance in at least one lane was greater than 1%. Detrended correspondence analysis showed that the length of first gradient axis was 0.491, indicating linear model. Therefore, redundancy analysis was performed on physico-chemical parameters for determining the multiple relationships between parameters and bacterial populations [20]. The significant physico-chemical parameter that significantly affected bacterial population changes were identified by manual operation ($P < 0.05$). Moreover, the contribution of these significant parameters was calculated by using variation partitioning [21] after removing the influence on other significant ones on the bacterial community composition.

Results and Discussion

Change of physico-chemical parameters

The changes of physico-chemical parameters were shown in Figure 1. Pile temperature revealed a similar change trend for the two treatments, following the typical process of temperature changes (showed the stage of mesophilic, thermophilic, cooling and maturation) (Figure 1a). After the mesophilic stage of 6-7 days, the temperature of them both over 50°C. The TW rose over 50°C on day 6, which was earlier than the PM. However, the thermophilic stage of PM (7 to 26 day, 19 days) was obviously longer than TW (6 to 15 day, 9 days). Pile temperature is an important indicator of the microbial activity [22] and a universal index for composting process [12]. This quick rise in temperature indicated microbial activity established rapidly and was due to the biodegradation of simple organic compounds [12, 22]. Thermophilic stage of PM stayed longer than TW. It may indicate the quantity of easily degradable organic compounds in PM richer than in TW. TW entered the cooling stage first, because it contained more cellulosic substances that were difficult to be decomposed by microorganisms. While in PM,

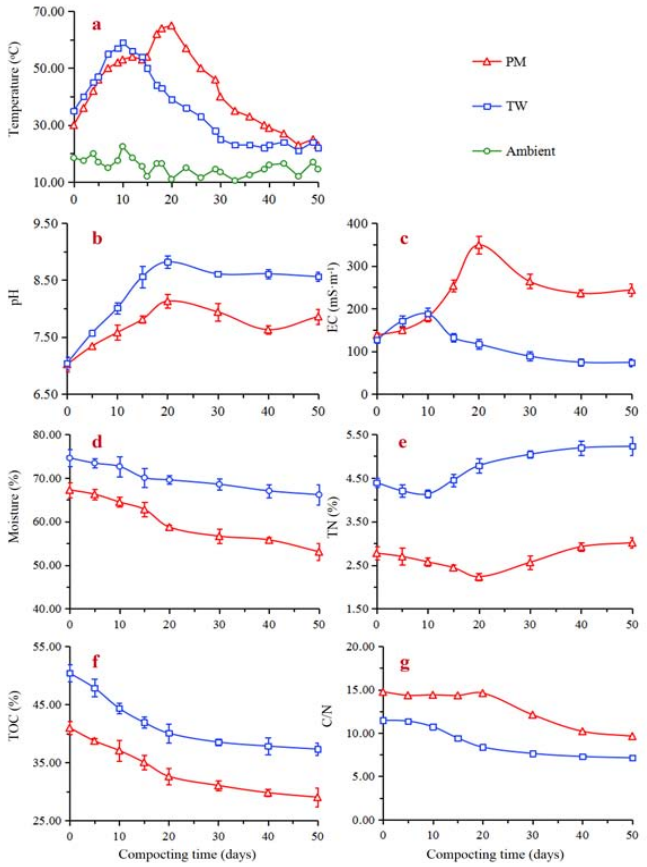

Figure 1: The changes of physico-chemical parameters (**a**) ambient and pile temperature; (**b**) pH; (**c**) EC; (**d**) moisture; (**e**) TN; (**f**) TOC; and (**g**) C/N ratio during composting process.

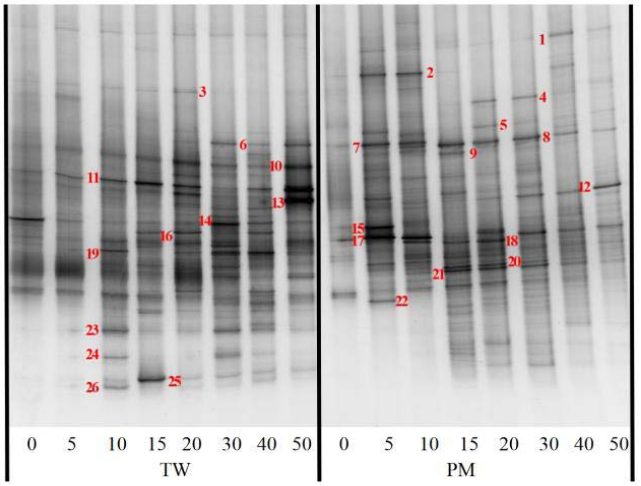

Figure 2: DGGE profiles of amplified 16S rDNA fragments from the compost samples.

thanks to it holds larger quantities of readily degradable material that benefits microorganism to maintain high activity. In addition, pile temperature exceeding 50°C was maintained for more than 9 days in all piles, which met the requirement condition for killing of most pathogens [23]. The pH of PM is lower than TW in whole composting process and the highest pH of PM was 8.13 while TW was 8.82 (Figure 1b). The pH not only affects the growth of microorganisms, but also has important effects on the biodegradation of organic matter. The pH of compost is an important parameter for evaluating the quality of composting products, because organisms will be influenced by changes in acid-base environment after compost application [12]. The pH surged is largely because the nitrogen nutrient sources in the composting material releases alkaline ammonia by ammonification and mineralization [24], thereby raising the pH. And, the pH of the compost only slightly rise even gradually fell during the mesophilic stage, likely because of the production of CO_2 and organic acids during the decomposition of organic matter [25, 26]. The different variations of pH originate in different raw materials (different initial pH and different components). Besides, after the maximum pH, the production of organic acids, NH3 stripping and the nitrification induced the slight decline and stabilize of pH [27, 28]. But both of them maintain the pH at 8 or so after the mesophilic stage. The EC (Figure 1c) both of them continuous rise during the thermophilic period and then gradually declined. The moisture (Figure 1d) maintains a reasonable range of 50-70% during almost whole composting process, meeting the needs of microbial activity and composting normal fermentation. Moisture content directly affected the rate of composting fermentation and maturity [29, 30]. The mesophilic stage was about 4-5 day in a good compost process. In this study, all pile satisfied this condition by our control. The changes of TN (Figure 1e), TOC (Figure 1f) and C/N (Figure 1g) in all piles are similar: 1) TN continued to decline during first fermentation and then it's proportion gradually increased by the decrease of total organic matter; 2) TOC is continuously consumed during fermentation, with the most consumed during the thermophilic period; 3) C/N gradually decreases and stabilizes during second fermentation and that indicates maturity.

Evolution of bacterial community structure

Bacterial community structure dynamics were showed in Figure 2. Each band of DGGE profile represented a class or a group of bacteria having similar 16S rDNA sequences [31]. Twenty-six bands were distinguished and detected in the DGGE profile. Neighbour-joining phylogenetic tree was constructed for the major taxonomic groups to investigate microbial phylogenetic diversity as well as to confirm the taxonomic affiliations of sequences obtained. *Firmicutes* (13 bands), *Proteobacteria* (6 bands), *Acidobacteria* (1 band), and *Bacteroidetes* (6 bands) were the five most dominant bacterial phyla for all composting samples (Figure 3). They are not interfered with by the pile material and the composting process, and are abundant. The relative abundance of *Proteobacteria* was very low during the first fermentation phase in all piles, indicating that *Proteobacteria* was inhibited by the high pile temperature. Higher relative abundance of Bacteroidetes (e.g., Flavobacterium sp., *Sphingobacterium gobiense, Leadbetteralla byssophila*) dominated during the thermophilic phase for all treatments composting. Some *Firmicutes* species (*Geobacillus lituanicus, Ureibacillus thermocloaceae, Tepidimicrobium xylanilyticm*) and *Proteobacteria* species (*Devosia geojensis, Ramlibacter tatouinensis, Pseudoxanthomonas suwonensis*) were abundant during the second fermentation phase. Most of major bands are different between different materials in the same periods.

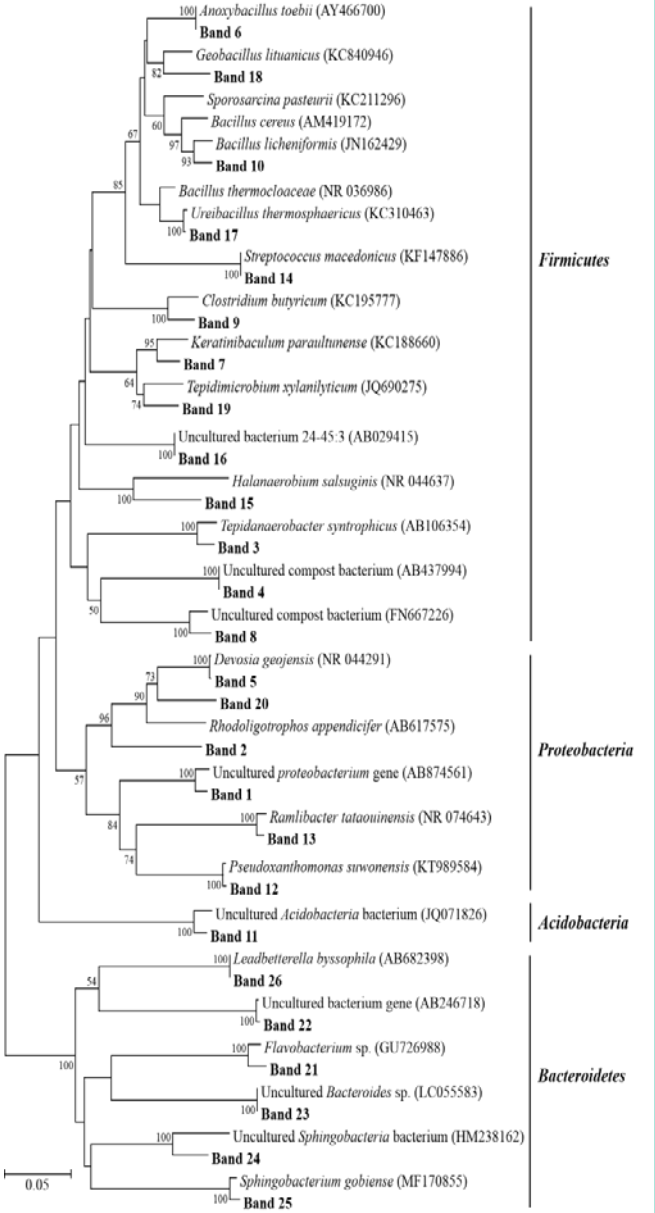

Figure 3: Bacterial phylogenetic tree.

Bacterial community structure was observed different in samples with different raw materials. For pig manure piles, *Anoxybacillus toebii* (Band 6), *Keratinibaculum paraultunense* (Band 7), *Peptoniphilus methioninivoras* (Band 18), et al. were remarkable observed at the stages of the cooling and maturation stages. While *Clostridium bytyricum* (Band 9), *Halanaerobium salsuginis* (Band 15) were widely distributed during the whole composting process for tea waste.

The evolution of bacterial communities is very diverse, as the various combinations of those bands were shown in different composting time. Precisely because the different raw materials in compost between PM and TW, and the ingredients of pig manure are more complex than tea waste. Combined with DGGE fingerprints and phylogenetic tree, it is not close relatives for most of dominant bacteria under different raw material during the same stage. Based on DGGE

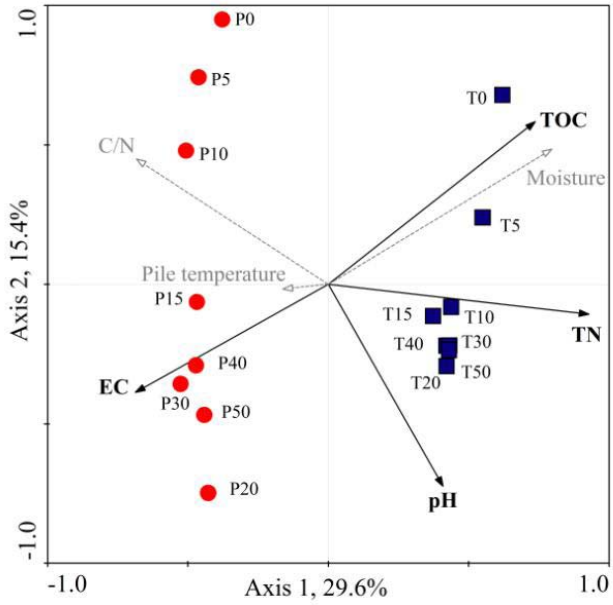

Figure 4: DGGE band data redundancy analysis for bacterial species. Significant composting parameters are indicated by solid lines with filled arrows while supplementary parameters are shown using grey dotted lines with unfilled arrows. Samples are represented by open circles and sample numbers refer to the sampling days.

fingerprints, phylogenetic tree and principal components analysis, different bacterial species existed in different stages of composting, and their dominant species were markedly different, which were changed by the change of composting conditions. Previous research showed that bacterial communities varied significantly between different initial materials (such as livestock manure, green waste, kitchen waste, municipal solid waste) [12]. Different initial material carried distinctive microbial communities and provided unique micro-environment for them [32]. Cellulolytic microorganisms in the compost piles were more numerous in the horticultural waste than municipal solid waste [8]. Moreover, the influences of the type of raw material (sludge from the fish processing industry, municipal sewage sludge, pig manure) on the microbial community succession during the maturity was analyzed by previous researcher [33]. The predominant microbial community for each compost treatment was dependent significantly on its corresponding waste type ($P < 0.001$) and still existed throughout the maturity stage. This suggests that which microorganisms can develop during the maturity stage was determined by the type of waste. In addition, microbial communities from different raw materials will show different community-level physiological characteristics during composting. Many studies shows that different additives of raw materials (leaf litter, wheat straw, sawdust) presented different microbial community functional diversity during kitchen waste composting [27,34].

Shaping factors for bacterial communities

In this research, in order to more accurately evaluating the influences of different raw material on the bacterial community structure, redundancy analysis was carried out base on inter-sample distances (Figure 4). The 16 samples were divided into 2 clusters classified by the difference of sample source and sampling time. It can

Table 1: The physico-chemical characteristics of raw materials and their mixing ratio.

	pH	Electrical conductance (EC) / mS·m^{-1}	Moisture / %	Total nitrogen (TN) / %	Total organic carbon (TOC)/ %	Carbon / nitrogen ratio (C/N)	Mixing ratio of treatment	
							PM	TW
Pig manure	6.91	144.5	19.87	2.46	35.27	14.31	10	0
Tea waste	7.02	137.7	22.5	4.95	50.16	10.14	0	10
Vegetable waste	5.46	112.7	69.06	2.38	48.6	20.41	3	3
Bran	7.42	34.2	14.06	2.77	54.13	11.36	2	2

Table 2: Redundancy analysis results of bacterial profile Monte Carlo significance tests for bacterial data: sum of all eigenvalues, 1.000; significance of first canonical axis, F value = 4.623, P = 0.002; significance of all canonical axes, F value = 4.237, P = 0.002. F and P values were estimated using Monte Carlo permutations.

Axis	Eigenvalue	Species-environment correlation	Cumulative % variation of species	Cumulative % variation of species-environment	Sum of all canonical eigenvalues
Axis 1	0.296	0.975	29.6	48.8	0.606
Axis 2	0.154	0.936	45	74.2	
Axis 3	0.11	0.836	56	92.3	
Axis 4	0.047	0.897	60.6	100	

Table 3: Eigenvalues, *F* values and *P* values obtained from the partial Redundancy analysis testing the influence of the significant parameters on the bacterial community structure Partial redundancy analysis based on Monte Carlo permutation (n = 499) kept only the significant parameters in the models. For each partial model, the other significant parameters were used as covariables. *F* and *P* values were estimated using Monte Carlo permutations. Sum of all eigenvalues for both partial redundancy analyses were 1.000.

Parameters included in the model	Eigenvalue	% variation explains solely	*F* value	*P* value
TN	0.193	19.3	2.888	0.002
EC	0.128	12.8	3.592	0.02
pH	0.077	7.7	2.144	0.032
TOC	0.071	7.1	1.997	0.048
All the above together	0.47	47	4.237	0.002

be found, as the raw material differences affect the physico-chemical parameters of the composting system, and thus affect the bacterial community structure. In order to more accurately evaluating the effects of different raw material (based on the six parameters) on evolution of bacterial community in the composting, redundancy analysis was used to analyze bacteria DGGE fingerprints. The result (Table 2) obtained from the highly significant (P = 0.002) in all axes through Monte Carlo tests, suggesting that these factors played critical roles in influence for bacterial community structure. As the bacterial species data showed 29.6% and 15.4% of the population succession were explained by the axes 1 and 2, respectively. Furthermore, the amount accumulating to 60.6 % of the community succession was explained by all canonical axes.

Each of significant parameters explained percentages of variation without the shared variation by variation partitioning analysis. The results of redundancy analysis (TN, EC, pH, and TOC) cumulatively explained 47.0% of the variation (P = 0.002) of bacterial population composition. Percentage of variation in bacterial community explained by each significant physical-chemical parameter was shown in Table 3. TN solely explained 19.3% (F = 2.888, P = 0.002) of the variation of bacterial community structure, EC 12.8% (F = 3.592, P = 0.020), pH 7.7% (F = 2.144, P = 0.032) and TOC 7.1% (F = 1.997, P = 0.048), respectively. Those parameters might have most influence on bacterial community structure during agricultural waste composting with different initial materials, respectively. The redundancy analysis biplot of bacterial community structure are shown in Figure 4. Sample points of bacterial population composition data from different treatment groups were obviously divided into two groups. It suggests that different raw materials are the main cause of bacterial population structure differences.

Mathematical tools, e.g., multivariate analysis, facilitate the analysis of important factors driving bacterial community evolution in different ecosystems. In this study, the aim was to explore which factor drives the variation of bacterial community composition. TN, EC, pH, and TOC were separated to analyze the variation in the bacterial community during composting. The variation of physico-chemical parameters confirmed to the change rule of typical organic agricultural material composting. Therefore, the collected samples will be more convincingly to reflect the response of bacterial community during composting [35]. Many studies have reported the important influence of C/N ratio on the evolution of microbial communities [6, 27, 36, 37]. Substrates with lower C/N ratio were more likely to result in higher bacterial/fungus ratios in samples during composting [37]. Higher microbial activity increased the pile temperature and duration of the mesophilic and thermophilic stages [23].

It may be the narrow range of the optimal growth pH of the bacterial community, resulting in the significant influence of pH on the bacterial community structure. Previous study showed that bacterial community structure was determined by soil pH [38]. Soil samples collected from North and South America indicate pH predominance in the construction of bacterial communities [38]. Bacterial community structure in soils across various sampling sites showed significant correlation with pH, regardless of the techniques used, e.g., pyrosequencing-based techniques [39], clone library-based techniques [40], and DNA fingerprinting-based techniques [41]. Moreover, many studies have been reported that the growth and activity of microbial population and even their community structure are strongly influenced by Water Soluble Carbon (WSC) [42], moisture [12] and pile temperature [43].

Conclusion

Differences in physico-chemical properties during composting

process were obtained with different raw materials (pig manure and tea waste). *Firmicutes, Proteobacteria, Acidobacteria*, and *Bacteroidetes* were the most dominant phyla in all samples. *Anoxybacillus toebii, Keratinibaculum paraultunense, Peptoniphilus methioninivoras*, were remarkable observed at the stages of the cooling and maturation stages for pig manure piles. While *Clostridium bytyricum, Halanaerobium salsuginis* were widely distributed during the whole composting process for tea waste. Redundancy analysis indicated that EC, pH, TN, and TOC were the significant factors influencing structure dynamics of bacterial community for pig manure and tea waste.

Acknowledgements

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