Characterization of bacterial community structure during in-vessel composting of agricultural waste by 16S rRNA sequencing

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Abstract

Bacterial diversity during in-vessel (rotary drum) composting of agricultural waste was characterized using NGS-based 16S rRNA sequencing for microbial identification. The activity of the bacteria was observed to vary with the composting materials and degradation pattern. Taxonomic hits distribution at domain level revealed that 89.5% sequences belonged to bacteria, 9% to eukaryota followed by 1.4% archaea during drum composting. The lowest common ancestor (LCA) classification plot showed the high abundance of the phylum proteobacteria followed by actinobacteria in compost sample. Taxonomic hit distribution at family level showed that compost sample was enriched with Thermomonosporaceae. *Thermomonospora curvata* is an aerobic, cellulolytic, thermophilic Gram-positive bacterium which produces a number of industrially important compounds, i.e., cellulase, alpha-amylase, and polygalacturonate lyase. *Thermomonospora* family of bacteria play a major role in organic matter degradation during composting. Hence, in the present study species such as *Actinomadura vinacea*, *Thermomonospora curvata*, *Actinoallomurus spadix*, *Actinomadura rubrobrunea*. *T. curvata* were identified from the compost mixture, which can utilize many organic compounds such as cellulose starch, xylose or pectin. The other biggest group in compost sample was Actinobacteria with *Thermoleophilum album* as the most abundant species followed by *Collinsella aerofaciens*. The compost was stabilized with higher volatile solids reduction, lower OUR (4.49 mg/g VS/day) and CO₂ (2.28 mg/g VS/day) values at the end of 20 days. The final compost was observed with 2.31% of TKN and 4.3% of phosphorus. Finally the results indicate that degradation of agricultural waste using drum composter was dominated by Bacilli, γ , β -proteobacteria, and actinobacteria.

Keywords 16S rRNA sequencing · Bacterial communities · Rotary drum composter · Agricultural waste · Thermophilic composting

Introduction

Composting is a self-heating, aerobic biodegradation process which is highly recommended for the treatment of organic solid waste, thereby reducing the overall biodegradable fraction in the municipal solid waste (Zuconi et al. 1987). The bio conversion of the organic fraction into stabilized end product is carried out by different composition and dynamics of microflora. The thermophilic temperature during the process reduces waste volume by 40–50% and eliminates pathogens, therefore, providing nutrient rich and sanitized end product (Bhatia et al 2013; Varma and Kalamdhad 2014b).

However, the dynamics of the microflora during composting is very important, as it reflects the overall quality of the product. The activities of these microbes are greatly influenced by the waste substrate, composting methodology, and other environmental conditions. Bacteria are considered to be the predominant group in overall composting and characterization of these microbes is important in understanding the overall treatment process (Trautmann and Olynciw 2012). They also have the capability to attack more complex material by releasing extracellular enzymes (Golueke 1992; Epstein 1997). They contribute to a major proportion (80%) of the total microbial count in the compost and are responsible for the degradation of variety of organic materials by releasing a wide range of enzymes.

Past few decades, culture-dependent and -independent approaches investigating microbial communities during composting have been extensively reported and provided

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great insight in the identification of bacterial diversity (Amann et al. 1995; Danon et al. 2008). Furthermore, the microbial community changes during different composting methods by pure culturing, 16S rRNA gene sequencing method, polymerase chain reaction denaturing gradient gel electrophoresis (DGGE), single strand conformational polymorphism (SSCP), terminal restriction fragment length polymorphism and phospholipid fatty acid (PLFA) profiling (t-PFLP) have also reported (Ryckeboer et al. 2003; Chandna et al. 2013; Egert et al. 2004; Varma and Kalamdhad 2014b; Wang et al. 2015; Villar et al. 2016; Meng et al. 2018; Mahon et al. 2018). This bacterial characterization has been reported on different waste materials such as cattle manure, chicken manure, kitchen waste, agricultural by products, lignocellulose biomass and municipal solid waste (MSW). The culture dependent bacterial characterization was widely used; however, the community characterization during the different stages of composting was still unclear (Bhatia et al. 2012; Ryckeboer 2003). The DNA extraction method allows the identification of unknown sequences and better understanding of their potential role in composting (Franke Whittle et al. 2014). The metagenomics molecular characterization has been increasingly used nowadays and allows the understanding of the microbial characterization during stages of the process with greater advantage (de Gannes et al. 2013). Most of the studies were reported during pile composting and lab scale reactors. Rapid molecular PCR-based techniques, such as amplified ribosomal DNA restriction analysis (ARDRA), allow the user to compare a large set of isolates at the phylogenetic level. Protocols have been advanced to extract the total DNA directly from the compost sample, and with combination to the PCR-mediated gene detection, it allow the user to detect the specific target of microbial interest. Nevertheless, there is still little information on the relationship between microbial species and environmental factors from different compostable materials during in-vessel composting methods.

Hence, the present study essentially focused on the bacterial characterization during in-vessel (rotary drum composter) composting of agricultural waste. Next-generation sequencing (NGS) based 16S rRNA sequencing of bacterial characterization was performed during the study. The specific objectives considered were: (i) to analyze mesophilic, thermophilic bacteria and actinobacteria community structure at different composting stages, (ii) to identify the bacterial and actinobacterial communities' potential role during the stabilization of waste and finally, (iii) to assess the overall community structure changes with respect to the varying environmental factors during composting.

Materials and methods

Feedstock materials and processing

Vegetable waste, cow dung, saw dust, and dry leaves mixture was used for preparing composting. The combinations of waste materials for preparing compost were in the order of 54 kg vegetable waste, 45 kg cow dung, 9 kg saw dust and 10 kg of dried leaves as suggested by Varma and Kalamdhad (2014a) for producing stabilized compost within shorter time period using rotary drum composter (20 days). Vegetable waste was collected from Vegetable Market, Fancy Bazaar, Guwahati, Assam, India and dry leaves from the Indian Institute of Technology Guwahati campus, Guwahati, India. Cattle manure (buffalo dung) was collected from dairy farm and saw dust from the nearby Amingaon village.

Drum specifications and analysis

A pilot-scale rotary drum composter of 550 l capacity was operated at batch mode. The drum is of 1.022 m in length and 0.76 m in diameter, fabricated by a 4-mm thick metal sheet. The composting period of 20 days was decided for both proper degradation and stabilization based on the performance of earlier studies on in-vessel composting reactors (Kalamdhad et al. 2008; Varma and Kalamdhad 2014a). Manual turning was done after every 24 h through one complete rotation of the rotary drum to ensure that the material on the top portion moved to the central portion, where it was subjected to higher temperature based on the performance of earlier studies regarding in-vessel composting reactors (Varma and Kalamdhad 2014a).

Physico-chemical and biological parameters analysis

Temperature was monitored using a digital thermometer throughout the composting period. pH of the compost (1:10 w/v waste:water extract) was analyzed as described by Kalamdhad et al. (2009). Total Kjeldahl nitrogen (TKN) was analyzed using Kjeldahl method. Volatile solids (VS) were determined by loss ignition method (on dry mass basis) at 550 °C for 2 h. The total organic carbon (TOC) was calculated from volatile solids. Total phosphorus (TP) (acid digest) was performed using stannous chloride method (APHA 1995). Analysis of stability parameters such as carbon dioxide (CO₂) evolution and oxygen uptake rate (OUR) were performed as described in Kalamdhad et al. (2008). Nutrient agar medium was used for the total count of prokaryotes. Cycloheximide (0.2 g/l) was added to inhibit fungal growth. The final pH of the medium was 7.3 ± 0.1 at 25 °C.

Finally, prepared plates were incubated in an inverted position for 24–48 h at 25 and 50 °C for spore forming bacteria.

Qualitative and quantitative analysis of gDNA

DNA was isolated using modified Xcelgen Soil gDNA kit (Xcelris Genomics, India). Quality of gDNA was checked on 1% agarose gel (loaded 5 µl) for the single intact band. The gel was run at 110 V for 30 min. 1 µl of each sample was loaded in Nanodrop 8000 for determining $A_{260/280}$ ratio. The DNA was quantified using Qubit dsDNA BR Assay kit (Thermo Fisher Scientific Inc., USA). 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Inc., USA).

Preparation of libraries for 2 × 300 bp run chemistry

The amplicon libraries were prepared using Nextera XT Index Kit (Illumina Inc., USA) as per the 16S Metagenomic Sequencing Library preparation protocol (Part # 15044223 Rev. B). Primers for the amplification of the V3–V4 hyper-variable region of 16S rDNA gene of Eubacteria and Archaea were designed in Xcelris NGS Bioinformatics Lab. These primers were synthesized in Xcelris Prime X facility and the details are provided in Table 1. The amplicons with the Illumina adaptors were amplified using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon libraries were purified by 1× AMPureXP beads and checked on Agilent DNA 1000 chip on Bioanalyzer 2100 (Agilent Technologies, USA) and quantified on fluorometer by Qubit dsDNA HS Assay kit (Thermo Fisher Scientific Inc., USA).

Cluster generation and sequencing

After obtaining the Qubit concentration for the library and the its mean peak size from Bioanalyser profile, the library was loaded onto MiSeq at appropriate concentration (10–20 pM) for cluster generation and sequencing. Paired-end sequencing allows the template fragments to be sequenced in both the forward and reverse directions on MiSeq. The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand

during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

Results and discussion

Physio-chemical characterization of the compost

Temperature rise within 24–48 h was observed in the present study due to higher biodegradable organic matter and higher bacterial activity. A maximum of 66.5 °C was observed in the present study with thermophilic phase observed for more than 7 days above 50 °C. The pH was observed to increase from neutral (6.7) to alkaline pH (7.7) towards the end of composting denoting the stabilization of waste (Tiquia and Tam 2000). A maximum of 11.4% of TOC was observed to degrade during the present due to higher microbial activity. Due to mass loss of the compost, the TKN increased from 1.4 to 2.2%, and phosphorous from 3.4 to 4.5% from day 0 to the end of composting (Table 2) (Kalamdhad et al. 2009).

Taxonomic hits distribution

Amplicon sequencing analysis has been carried out for compost sample on MiSeq platform. In total ~ 209 MB of data has been generated for compost sample. MG-RAST analysis showed that compost sample showed α -diversity = 143.7 species.

Total bacterial count and taxonomic hits distribution at domain level

The amount of mesophilic heterotrophic bacteria during the initial days of composting was observed in the range of 4.8×10^{11} CUF/g. The higher population of heterotrophic bacteria can be considered due to the initial substrate mixture and the environmental factors during composting (Hargerty et al. 1999). Domain level taxonomic hits distribution showed that compost had 89.5% bacteria, 9% eukaryota followed by 1.4% archaea as represented in Fig. 1. The higher abundance of bacteria can be considered due to the presence of higher biodegradable organic matter and lignocellulosic fraction. Bhatia et al. (2014) had also reported the higher abundance of bacteria during the composting of vegetable waste that were majorly involved in the degradation of organic matter. The presence of archaea during composting

Table 1 Primers used in the present study

Sr.no.	Oligo name	Oligo sequence (5' to 3')	Length of primer	Product size (approx)
1	V3-forward	CCTACGGGNGGCWGCAG	17	~ 460 bps
	V4-reverse	GACTACHVGGGTATCTAATCC	21	

Table 2 Physico-chemical and biological parameters during composting

Time of composting (days)	Physico-chemical and biological parameters								
	pH	Total organic carbon (%)	Total kjeldahl nitrogen (%)	C/N	Total phosphorous (%)	Oxygen uptake rate (mg/g VS/day)	CO ₂ evolution (mg/g VS/day)	Mesophilic phase	Thermophilic phase
0	6.76	40.99	1.68	24	3.41	21.26	14.97	4.80×10^{11}	5.10×10^7
4	7.41	40.29	2.03	20	3.62	16.15	12.91	5.40×10^8	7.35×10^9
8	7.41	40.11	2.08	18	3.79	13.91	9.59	5.80×10^7	3.75×10^9
12	7.68	39.15	2.16	18	4.21	10.16	7.77	3.60×10^7	6.80×10^8
16	7.69	37.61	2.17	17	4.24	7.44	4.57	4.80×10^6	4.30×10^6
20	7.76	36.3	2.31	15	4.3	4.49	2.28	6.10×10^5	5.30×10^5
(%) reduction	–	11% reduction	1.72 fold increase	37% reduction	1.79 fold increase	79% reduction	85% reduction	–	–

Fig. 1 Taxonomic hits distribution at domain level of compost

are majorly involved in the oxidation of ammonia and it can successfully correlated to the higher reduction of ammoniacal nitrogen during the process. The higher thermophilic temperature and prolonged thermophilic phase due to the higher microbial activity caused the stabilization of waste within shorter time period. The thermophilic bacteria were also observed in the range of 7.35×10^9 CUF/g during the thermophilic stage (day 4) and finally reduced to 5.30×10^5 CUF/g at the end of composting period. Finally the amount of mesophilic heterotrophic bacteria was observed in the range of 6.1×10^5 CUF/g at the end of composting period.

Taxonomic hits distribution at phylum level

Taxonomic hit distribution at phylum level showed that compost has 24.9% bacteroidetes, 21.6% firmicutes, as represented in Fig. 2. The bacteroidetes and firmicutes are

facultative anaerobic and obligate anaerobic pathogens that are common in rumen. The phylum bacteroidetes are composed of three large classes of Gram-negative, non-spore forming, anaerobic or aerobic and rod-shaped bacteria that are considered important during the initial and final stages of composting. The addition of cow dung as bulking agent during composting can be considered as the major source for these microorganisms to the compost (Neher et al. 2013). Furthermore, relative abundance of γ -proteobacteria, firmicutes and actinobacteria are reported as indicators of disease suppression during the process (Hadar and Papadopoulos 2012). It was also reported that these organisms are highly involved in the maturation of composting during the final stage of the process.

Taxonomic hits distribution at class level

Taxonomic hit distribution at class level shows that compost has 15.1% bacteroidia, 13.9% clostridia, 4.6% flavobacteria, as represented in Fig. 3. The presence of flavobacteria was reported during the higher temperatures of natural compost, while *Arthrobacter* sp. was reported at the high-temperature process in cellulose-decomposing strain compost (Liu et al. 2011). *Bacillus* sp. was considered to be dominant species at middle and later stages of composting. Flavobacteria are common in composts and include opportunistic pathogens. Its presence in composts and the importance of these flavobacteria in the degradation of phenolic and chlorinated compounds has also been reported (Danon et al. 2008).

Taxonomic hits distribution at order level and LCA

Taxonomic hit distribution at order level showed that compost had 15.1% bacteroidales, 13.8% clostridiales, 5.1% acholeplasmatales, as represented in Fig. 4. The identified lactic acid bacterium is known to produce enzymes and

Fig. 2 Taxonomic hits distribution at phylum level of compost sample

Fig. 3 Taxonomic hits distribution at class level of compost sample

natural antibiotics aiding effective digestion and has antibacterial properties, including control of salmonella and *E. coli*. This particular beneficial microorganism is often used in composting for stopping foul odors associated with anaerobic decomposition. Lactic acid bacteria thrive and feed on the ammonia released in the decomposition normally associated with foul odors. Rhizobiales is an order of alpha proteobacteria and are considered Gram-negative. These rhizobia can fix nitrogen and are symbiotic with plant roots. The four families bradyrhizobiaceae, hyphomicrobiaceae, phyllobacteriaceae, and rhizobiaceae contain

at least six genera of nitrogen-fixing, legume-nodulating, microsymbiotic bacteria. The role of these rhizobiales in the denitrification of manure compost pellets and their relationship to N_2O emissions has been successfully correlated by Yamane (2013).

The LCA classification plot showed the high abundance of the phylum proteobacteria followed by actinobacteria in compost sample (Fig. 5). Actinobacteria plays an important role in the later stages of composting and particularly for the degradation of relatively complex, recalcitrant compounds. The ability of actinobacteria to degrade lignocelluloses

Fig. 4 Taxonomic hits distribution at order level of compost sample

implies that this group of bacteria has potential to be useful indicators for compost maturity.

According to details listed in Fig. 5, the sequences related to proteobacteria made up the largest fraction in compost sample, which included alpha, beta, gamma, delta and epsilon subclasses. The alphaproteobacteria comprising 165 OTUs were the most dominant subclass of proteobacteria followed by 156 OTUs of Gammaproteobacteria. The species like *Chelatococcus asaccharovorans*, *Rhodobium orientis*, *Pseudomonas thermotolerans*, *Pseudoxanthomonas* sp. *ITRH31*, *Pseudoxanthomonas dokdonensis*, sulfur-oxidizing bacterium *OAI2*, *Chondromyces apiculatus*, *Sorangium celulosum*, etc., were present in compost sample.

Firmicutes group was the second most frequent group. *Geobacillus thermodenitrificans* followed by *Geobacillus stearothermophilus* were the most abundant species in compost sample. *Bacillus licheniformis*, *Moorella thermoacetica*, *Brevibacillus thermoruber*, *Thermoactinomyces vulgaris*, *Thermobacillus xylanilyticus*, *Ureibacillus thermosphaericus*, *Bacillus pumilus*, *Laceyella sacchari*, *Ureibacillus thermosphaericus*, *Aneurinibacillus thermoaerophilus*, were the other species found in compost sample.

The other biggest group in compost sample was actinobacteria with *Thermoleophilum album* as the most abundant species followed by *Collinsella aerofaciens*. *Thermomonospora* was one of the common genera including species i.e., *Actinomadura vinacea*, *Thermomonospora curvata*, *Actinoallomurus spadix*, *Actinomadura rubrobrunea*. The other species *Pseudonocardia thermophila*, *Streptomyces thermovulgaris*, *Streptomyces thermoviolaceus*, *Thermobispora bisporea*, were also found in the compost sample.

Strains belonging to the moderately thermophilic species have been detected during the study and are mainly involved

in the lignocellulose degradation. *Pseudoxanthomonas* spp. has been reported to enhance cellulose degradation by their acetate-consuming effect and consequent pH neutralization (Farris and Olson 2007). Moreover, the most common genera of actinobacteria in the compost were *Thermobifida* and *Thermomonospora*, which are well known for their cellulose- and hemicellulose-degrading ability (Steger et al. 2007). Since the temperature during composting had reached upto 66 °C, the compost explained the dominant presence of these bacteria that are extremely survival at higher temperature and involved in degrading lignocellulose. Therefore, it can be concluded that the different bacterial communities observed during study were influenced with the changing organic substrate degradation and environmental factors.

Conclusion

Taxonomic hits distribution at domain level showed that 89.5% sequences belonged to bacteria, 9% to eukaryota followed by 1.4% archaea in compost sample. *Bacillus* sp. and flava bacteria were observed predominant during the study that is substantially involved in the organic matter degradation. A number of industrially important microbes were observed from the drum compost i.e. *Thermomonospora curvata*, *Thermobifida*, *Pseudonocardia thermophila*, *Streptomyces thermovulgaris*, *Streptomyces thermoviolaceus* and *Thermobispora bisporea*, which can utilize many organic compounds present in the natural environment, such as cellulose starch, xylose or pectin. Taxonomic hit distribution at family level shows that compost sample has been enriched with *Thermomonosporaceae*. *Thermomonospora curvata* (strain ATCC 19995/DSM 43183/JCM

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Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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