Techniques to analyze microbial diversity during composting—A mini review

Akansha Bhatia1, Ankur Rajpal2, Sangeeta Madan3 and A A Kazmi1

1Department of Civil Engineering, Indian Institute of Technology Roorkee (IITR), Roorkee 247 667, India
2Department of Biology, Hong Kong Baptist University, Kowloon, Hong Kong
3Department of Zoology and Environmental Sciences, Gurukula Kangri University, Haridwar 294 404, India

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One of the most versatile and remunerative techniques for handling biodegradable solid wastes is composting. Composting refers to aerobic degradation of organic material and is one of the main waste treatment methods used for treating separated organic waste. Microbes play a key role as degraders during the composting-process and microbiology of composting has been studied for decades. Microorganisms participating in the self-heating phase of composting material could be characterized through cultural enumeration of microbe populations on one hand and, on the other hand, by a molecular investigation of the microbial community by restriction enzyme analyses of a clone library of bacterial 16S rRNA genes. It is well known that, with the traditional culture-based methods, only a small fraction (below 1%) of the species in a sample is normally detected. Furthermore, microbes can be mutualistic, symbiotic and parasitic and for these microbes growth conditions are hard to mimic. Use of polymerase chain reaction (PCR) and DGGE (denaturing gradient gel electrophoresis) have substantially enhanced the charm of the independent methods. The present review reveals the lack of sensitivity and specificity of culture dependent methods and preference of culture independent approach in microbial ecology to study the microbial diversity in a complex process of solid waste composting.

Keywords: Composting, culture dependent method, culture independent method, microbial diversity, PCR, 16S rRNA

Introduction

Composting is the process of recycling decomposed organic materials into a rich soil known as compost. The process involved complex interacting factors, in which biodegradable organic wastes are stabilized and converted by the action of some microorganisms under controlled conditions1. Microorganisms are the essential factors for the successful operation of composting. In order to effectively control the composting process, it is necessary to understand the microbial community structure and its change, especially its special role in decomposition of organic matters2. Variant kinds of compost systems have been evolved till date. The windrow system and the static piles are the most popular example of a nonreactor, agitated solids bed system. Vertical flow reactors allow the agitation of solids during their transit down the reactor and are termed moving agitated bed reactors. Horizontal flow reactors are divided into those that employ a rotary drum that used a bin structure of varying geometry and method of agitation to a wide variety of composting substrates including municipal solid waste (MSW), agricultural wastes and sewage sludges3. Vermicomposting is a process of bio-oxidation and stabilization of organics involving the joint action of earthworms and microorganisms that has been proved successful in processing paper wastes, food and garden residues, animal wastes and wastewater4.

The composting process can be divided into four different stages based on the temperature levels5 (Fig. 1). The latent phase corresponds to the time

![Fig. 1—Patterns of temperature and microbial growth during composting.](image_url)
necessary for the microorganisms to acclimatize and colonize in the new environment in the compost heap. The growth phase is characterized by the rise of biologically produced temperature to mesophilic level. In the mesophilic stages, the temperature is close to the ambient temperature and the pH is low. Mesophiles grow between 10 and 45°C\(^6\) and break down easily degradable components, such as, sugars and amino acids\(^7\). As the material starts to degrade due to microbial activity, the amount of mesophilic microbes, bacteria and yeasts, enhanced. The increase and activity of these acid-producing bacteria cause a drop in pH to acidic levels. As the temperature starts to increase as a result of the vigorous exothermic microbial activity, it reaches levels above 45°C and thermophilic microbes take over the degradation and the pH rise. A falling temperature indicates the start of the cooling phase, which is followed by the maturation phase. The maturation phase of compost begins when the compost temperature falls to that of the ambient air. During this phase, mesophilic microorganisms colonize the compost heap and slowly degrade complex organic compounds, such as, lignin. This last phase is important because humus-like substances are produced in this phase to form mature compost\(^6\). During composting, mineralization and humification occur simultaneously and are the main processes causing the degradation of the fresh organic matter.

**Microbial Diversity**

Microbes exist everywhere on earth. It has been estimated that the number of microbes on earth is as much as 4-6× \((10^{30})^8\) and the estimation for the number of different bacterial species in a gram of soil is \((10^9)^9\). They affect our health and agriculture, and have key roles in most of the planet’s geochemical cycles\(^10\). The high diversity of microbes has been revealed through the rapid development of sequencing techniques. The human gut has been found to be coated with microbes essential to human health\(^11\). Vast microbial diversity has been found in the seas where a huge number of novel genes have been detected\(^12\). Likewise, great diversity and many novel sequences have been discovered everywhere from farm soil to whale falls, *i.e.*, whale carcasses that have fallen to the ocean floor\(^13\), as well as in the air\(^14\). Recently, the basaltic lava in the oceanic lithosphere has been studied and the microbial diversity there\(^15\) was comparable to terrestrial soil. Terrestrial soil, itself, is considered to be extremely diverse because of its spatial heterogeneity, multiphase nature and complex chemical and biological properties\(^16\), which provide growth conditions for diverse groups of microbes.

In many studies, the number of analyzed clones is usually small, from tens to hundreds, while the number of individual microbes in the sample can be in billions\(^8\). It is now widely accepted that with cultivation based methods less than 1% of microbes are detected because the growth conditions do not represent the growth conditions of all the microbes present\(^17\). In other words, there is no growth condition or culture medium that is general enough to allow growth of all microbes in the studied samples. For example, their nutrient, pH and temperature requirements may be strict, while anaerobic microbes do not form colonies in aerobic conditions, and slow growing microbes can be out-grown by fast growers, which produce large colonies. In many cases it is thus possible to design the conditions so that a specific group of organisms is targeted, however in practice this is not a viable approach if the total microbial diversity of an environment needs to be revealed. Furthermore, microbes can be mutualistic, symbiotic and parasitic, and for these microbes growth conditions are hard to mimic. Since the development of polymerase chain reaction\(^18\) to amplify DNA, the molecular methods for studying microbes and the knowledge about microbial diversity have substantially increased.

**Diversity Identification and Enumeration**

Methods that rely on bacterial cultivation are currently thought to identify only a small fraction (0.01-10%) of the microorganisms in natural environments. A molecular alternative that involves DNA extraction, followed by PCR amplification and subsequent cloning of 16S rRNA genes was developed to alleviate the limitation associated with cultural approaches, although it is anticipated that this approach may also introduce bias. This technique has been used successfully for marine bacterio-plankton, soil environments hydrothermal vent systems and for a peat bog sample. Various methods, such as, DGGE\(^19,20\) (denaturing gradient gel electrophoresis), SSCP\(^21\) (single strand conformation polymorphisms), ARISA\(^22\) (automated ribosomal intergenic spacer analysis), cultivation\(^2,23\), restriction analysis and sequencing\(^24\), and microarrays\(^25\), have been used to
study compost microbial communities to find out which microbes are present in certain stages of the composting process. Compost microbiota have been studied from a wide spectrum of different composting scales, from 500 mL laboratory batch units, 15, 30 L synthetic compost made from dog food, 24, to actual composting drums, 26, 27, and composting piles. 20.

**Culture Dependent Approach**

A variety of methods have been used so far to investigate the microorganisms during composting, which includes the use of traditional plating (pour plate, spread plate & streaking methods) and identification of culturable microorganisms for determining microbial diversity during composting. 28, 29. The pour plate technique can be used to determine the number of microbes/mL or microbes/g in a specimen. It has the advantage of not requiring previously prepared plates. When using the pour plate method, a diluted sample is pipetted into a sterile Petri plate and then melted agar is poured in and mixed with the sample. Use of this method allows for a larger volume of the diluted sample, usually in the range of 0.1-1.0 mL. This method yields colonies that form throughout the agar, not just on the surface. Each colony represents a "colony forming unit" (CFU). For optimum accuracy of a count, the preferred range for total CFU/plate is between 30-300 colonies/plate. One disadvantage of pour plates is that embedded colonies will be much smaller than those which happen to be on the surface, and must be carefully scored so that none are overlooked. Also, obligate aerobes may grow poorly, if deeply imbedded in the agar. The spread plate method consists of evenly spreading the diluted sample over an agar plate. When using this method, a volume of 0.1 mL of the diluted sample should not be used since the agar will not be able to absorb the excess. Use of this method yields colonies that form on the surface of the agar. A streak plate technique is used to isolate a single species from a mixed species population.

**Molecular Techniques**

Several cultivation-independent or molecular techniques, including analyses of phospholipids fatty acids, 30 and quinolines, 31, oligonucleotide microarrays, 32, and the analyses of rDNA genes encoding for the small ribosomal subunit DNA (16S rRNA in bacteria) 31, have dramatically increased the knowledge about contribution of different microorganisms to various compost production phases. There is a general agreement among those in the field that the identification and classification of microorganisms should be performed according to phylogenetic analysis based on rDNA.

Molecular, nucleic acid-based characterization techniques are a promising approach to increase our knowledge about succession and function of microbial communities during composting processes. The use of PCR amplification of 16S rRNA genes of cultivated isolates, followed by restriction enzyme analysis of the amplified products (ARDRA) is an efficient way to differentiate bacterial isolates from compost and obtain information about their phylogenetic position. 33. Specific microorganisms from compost can also be identified by PCR detection of characteristic genes. 34, 35. The directly extracted DNA from composting material can be used as a template for PCR amplification and detection of specific microorganisms without the need of cultivation. Other target sequences can include 16S rRNA genes using universal primers for bacteria or 18S rRNA genes for fungi. From the mixture of PCR products of almost identical size, which will be generated from such a community DNA, patterns can be generated by electrophoretic techniques. Such genetic “fingerprints” should reflect the structure of the microbial community or a specific fraction of it.

More recently, DNA-DNA hybridization has been used together with DNA microarrays to detect and identify bacterial species or to assess microbial diversity. 36. This tool could be valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences with high specificity. The microarray can either contain specific target genes, such as, nitrate reductase, nitrogenase or naphthalene dioxygenase, to provide functional diversity information or can contain a sample of environmental ‘standards’ (DNA fragments with less than 70% hybridization) representing different species found in the environmental sample. 37. Reverse sample genome probing (RSGP) is a method used to analyze microbial community composition of the most dominant cultivable species and uses genome microarrays. RSGP has four steps: (1) Isolation of genomic DNA from pure cultures; (2) Cross-hybridization testing to obtain DNA fragments with less than 70% cross-hybridization; DNA fragments with greater than 70% cross-hybridization are considered the same species; (3) Preparation of genome arrays onto a solid support; and (4) Random labelling of a defined mixture of total community DNA and internal standard.
Like DNA-DNA hybridization, the use of RSGP and microarrays has the advantage that it is not confounded by PCR biases and microarrays can contain thousands of target gene sequences. However, it only detects the most abundant species. In general, the species need to be cultured but, in principle, cloned DNA fragments of unculturables could be used. The diversity has to be minimal, or enriched cultures used, otherwise cross-hybridization can become problematic.

Using genes or DNA fragments instead of genomes on the microarray offers the advantages of eliminating the need to keep cultures of organisms growing, as genes can be cloned into plasmids or PCR used to continually amplify the DNA fragments. In addition, fragments would increase the specificity of hybridization over the use of genomes and functional genes in the community could be assessed.

DGGE and TGGE (temperature gradient gel electrophoresis) are two similar methods for studying microbial diversity. These techniques were originally developed to detect point mutations in DNA sequences. Scientists have expanded the use of DGGE to study microbial genetic diversity. DNA is extracted from soil samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The 5V-end of the forward primer contains a 35-40 base pair GC clamp to ensure that at least part of the DNA remains double stranded. This is necessary so that separation on a polyacrylamide gel with a gradient of increasing denaturant will occur based on melting behaviour of the double-stranded DNA. If the GC-clamp is absent, the DNA would denature into single strand. On denaturation, DNA melts in domains, which are sequence specific, and will migrate differentially through the polyacrylamide gel. If the GC-clamp is present, the DNA would remain double stranded. Theoretically, DGGE can separate DNA with one base-pair difference. TGGE uses the same principle as DGGE except the gradient is temperature rather than chemical denaturant. DGGE/TGGE has the advantages of being reliable, reproducible, rapid and somewhat inexpensive.

Multiple samples can also be analyzed concurrently, making it possible to follow changes in microbial populations. Limitations of DGGE/TGGE include PCR biases and laborious sample handling, as this could potentially influence the microbial community and variable DNA extraction efficiency. It is estimated that DGGE can only detect 1-2% of the microbial population representing dominant species present in an environmental sample. In addition, DNA fragments of different sequences may have similar mobility characteristics in the polyacrylamide gel. Therefore, one band may not necessarily represent one species and one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slightly different sequences. While the rRNA genes have been the main target of microbial diversity studies using DGGE, some researchers have targeted catabolic genes, such as, methane monooxygenase for DGGE analysis. This would provide information on the diversity of specific groups of microorganisms in a defined function, such as, pollutant degradation.

Another technique that relies on electrophoretic separation based on differences in DNA sequences is SSCP. Like DGGE/TGGE, this technique was originally developed to detect known or novel polymorphisms or point mutations in DNA. Single-stranded DNA is separated on a polyacrylamide gel based on differences in mobility caused by their folded secondary structure. When DNA fragments are of equal size and no denaturant is present, folding and hence mobility will be dependent on the DNA sequences. SSCP has all the same limitations of DGGE. Also, some single-stranded DNA can form more than one stable conformation. Therefore, one sequence may be represented by more than one band on the gel. However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity.

Restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA), is another tool used to study microbial diversity that relies on DNA polymorphisms. PCR amplified rDNA is digested with a 4-base pair cutting restriction enzyme. Different fragment lengths are detected using agarose or non-denaturing polyacrylamide gel electrophoresis in the case of community analysis. RFLP banding patterns can be used to screen clones or used to measure bacterial community structure. This method is useful for detecting structural changes in microbial communities, but not as a measure of diversity.

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of the limitations of RFLP. This simplifies the banding pattern, thus allowing the analysis of complex communities as well as providing information on...
diversity as each visible band represents a single operational taxonomic unit or ribotype. The banding pattern can be used to measure species richness and evenness as well as similarities between samples. This procedure can be automated to allow sampling and analysis of a large number of soil samples. Reproducibility of the method has been tested and found that banding patterns within and between samples are highly reproducible. They have found that the use of different Taq polymerases increase the variability of same DNA sample. T-RFLP is limited not only by DNA extraction and PCR biases, but also by the choice of universal primers. None of the presently available universal primers can amplify all sequences from eukaryote, bacterial and archaeal domains. Additionally, these primers are based on existing 16S rRNA, 18S rRNA or ITS databases, which until recently have contained mainly sequences from culturable microorganisms, and therefore may not be representative of the true microbial diversity in a sample. In addition, different enzymes will produce different community fingerprints. It is, therefore, important to use at least two to four different restriction enzymes. T-RFLP, like any PCR-based method, may underestimate true diversity because only numerically dominant species are detected because of the large quantity of available template DNA. In addition, different species will have different gene copy numbers and this could bias the results. Incomplete digestion by restriction enzymes could also lead to an overestimation of diversity. Despite these limitations, some researchers are of the opinion that once it is standardized, T-RFLP can be a useful tool to study microbial diversity in the environment, while others feel that it is inadequate. T-RFLP has been used to measure spatial and temporal changes in bacterial communities, to study complex bacterial communities, and to detect and monitor populations.

The above review thus points out that each mentioned technique has its own benefits and short falls depending upon the needs of the study. Therefore, to make the best choice, the abilities of different methods should be compared in the same sample to analyze the phylogeny of microbial communities.

**Conclusion**

DNA-based direct community analysis is important since cultivation dependent methods intrinsically favour the growth of specific community members. Culture-independent methods have been proposed to provide a less biased picture of the richness of bacterial communities compared to the culture-dependent methods, because of the selective pressure imposed by the requirement of the latter for growth on a solid substrate, leading to the isolation of a plate-growth-adapted subpopulation from the communities. Quantification of the abundance in natural samples by DNA-DNA hybridization may, therefore, provide a good estimate of the actively growing and dominant species, as this method is not related to the abundance of the species in question on the plate surface. Dominant species on the plate surface may be minorities in natural samples and vice versa. At the same time, it is likely that many species fail to grow at all on the plate surface of a given medium or show so few CFU that they are overlooked in the isolation step. The cultivation-dependent strategy may, therefore, underestimate the richness in samples, which is better estimated by culture-independent methods.

**References**


