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Functional and Genetic Profiling Tools: Insight to the Black Box of Vermicomposting Process

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ABSTRACT

The process of vermicomposting is highly dynamic, as it involves a complex interaction of earthworms with diverse microbial species. Over the last two decades, the focus of research has been mainly on the conversion of various types of organic wastes and pollutants such as agricultural, municipal and industrial wastes into vermicompost and on the development of parameters to assess maturity. There is however a lack of knowledge on the process dynamics and the complex interactions that occur between the microbes and earthworms during vermicomposting. A plethora of techniques such as biomass measurement, respiration rate, and enzyme activities are in routine use for assessment of vermicomposting dynamics at process level. However, less attention has been paid to the techniques useful in revealing community-level changes, which can give a significant insight into the microbe-earthworm interactions that finally affects the quality of vermicompost. Methods to study community-level changes such as community-level physiological profiling (CLPP), phospholipid fatty acid analysis (PLFA) and genetic fingerprinting have been commonly used in research on microbial ecology of soil, sediments, gut, and aerobic and anaerobic reactors, but not applied widely in vermicomposting ecosystems. In this review, we discuss the inventory of techniques relevant to vermicomposting process and which can provide significant knowledge on organic matter transformations and community-level changes. Furthermore, this review also brings to light the role of multivariate statistical analysis in predicting the possible relationship between the process-level and the community-level changes. A polyphasic approach to vermicomposting research is thus suggested which will facilitate in opening the blackbox of this complex and dynamic process.

Keywords: Biolog™ method, DNA fingerprinting, multivariate analysis, process dynamics
Abbreviations: CLPP, community-level physiological profiling; **DGGE**, denaturing gradient gel electrophoresis; **DOC**, dissolved organic carbon; **FT-IR**, Fourier transform infrared spectroscopy; **HA**, humic acid; **NMR**, nuclear magnetic resonance; **PCA**, principal component analysis; **PCR**, polymerase chain reaction; **SSCP**, single-strand conformation polymorphism; **TGA**, thermogravimetric analysis

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INTRODUCTION

Vermicomposting has been commonly described as a natural decomposition process mediated by the action of earthworms and dominated by microbial activities. It is very similar to soil processes and is as essential as primary production besides detoxification of many environmental pollutants like industrial wastes and oil spills (Sen and Chandra 2007; Martín-Gil *et al*. 2008). In addition it provides a good model of microbial communities to study ecological issues such as diversity, succession and competition during biodegradation and bioconversion of organic matter. The complex interactions among the organic matter, microorganisms, earthworms, and other soil invertebrates makes this process highly dynamic, which ultimately results in the fragmentation, biooxidation and stabilization of the wastes.

It is well known that earthworms fragment and condition the substrate with a grinding gizzard, aided by grit and sand, and this increases the surface area of the organic matter and promotes very high microbial activity (Domínguez 2004). These in turn modify the biological, physical and chemical status of the raw material, gradually reducing its C/N ratio (Aira and Domínguez 2009). Earthworms prefer microorganisms over organic matter as their nutrient source (Brown 1995). The different requirement for optimal development, growth and productivity of the types of earthworm species, used especially for vermicomposting, has a strong influence on the efficacy of the process. Therefore, a number of aspects of the vermicomposting process need to be understood in spite of the extensive research done for over

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two decades, for e.g. the effect of earthworms on the microbial community at both the functional and genetic level.

Vermicompost, which is the end product of this process, is a stabilized, finely divided peat-like material with a low C/N ratio, high porosity, increased water-holding capacity and higher readily available nutrients for plants. Moreover the earthworm casts are rich in organic matter and have high rates of mineralization leading to formation of inorganic components like ammonium radicals and nitrates. Vermicomposting promotes organic matter humification and thus enhances the quality of the raw materials as soil organic amendments (Plaza *et al*. 2008). Therefore this process is considered the most efficient method for converting solid organic materials into eco-friendly and valuable product (Campitelli and Ceppi 2008a). The use of vermicompost as a soil amendment has increased tremendously in the recent years (Padmavathiamma *et al*. 2008). However, one of the important factors affecting their successful use for agricultural purposes is their stability (Campitelli and Ceppi 2008b), which depends on the amount of dissolved organic carbon (DOC) present in the end product. Thus, lesser the DOC the more stable is the end product; as a result DOC can be an important indicator of stability. The DOC of immature vermicompost contains both humic and non-humic substances, and thus any change in absorbance of water-extractable organic matter will reflect the degree of humification and maturity of vermicompost. Hence, the determination of both DOC concentration and its spectrophotometric properties can be a useful analysis to assess vermicompost maturity.

The raw materials used for vermicomposting process can be from various sources like municipal wastes, grass clippings, dry leaves, trash, hedge cuttings, fruit and vegetables waste from the food industry, kitchen wastes, solid and liquid manure from animal houses, wastes from the forest, wood and paper industries, sewage sludge from wastewater treatment plants etc. As a result, there is no homogeneity in the composition of the start-up material. In addition, there is also a seasonal variation of the raw materials. Due to the variation in the composition of the raw materials, the resident microbial community structure also varies. The composition of the microbial community is also determined by several other factors such as the feeding rate of the earthworms, type of earthworm species, extent of anaerobicity and the process conditions. Thus, it is difficult to generalize the microbial community composition in vermicomposts. Moreover, the biochemical composition of the various waste materials greatly influences their susceptibility to microbial degradation. Consequently, wastes containing carbohydrates, lipids and proteins would be easily degraded, whereas those with a high lignocellulose fraction and low nitrogenous compounds will be slowly degraded.

The main components of most of the raw materials used for vermicomposting are carbohydrates (cellulose and hemicellulose), proteins, lipids and lignin. The ability of the microorganisms to degrade and assimilate such raw materials depends on their production of hydrolytic enzymes such as total cellulases, β-glucosidase, phosphatases, proteases and urease (Trasar-Cepeda *et al*. 1998; Sinsabaugh *et al*. 2002; Tiquia 2002a). These enzymes catalyze the biochemical transformations of organic matter, thus characterizing and quantifying the enzymatic activities during vermicomposting can reflect the dynamics of the decomposition process and nitrogen transformations (Tiquia 2002b; Sen and Chandra 2009). In addition to enzyme activities, the Biolog^{TM} method, which is based on color development in a 96-microtitre plate containing 31 different carbon sources and a water blank, can also give significant information on the substrate utilization potential of the microbial community (Aira *et al*. 2007; Sen and Chandra 2009).

From a molecular perspective, vermicompost microbial diversity refers to different sequence types, and genetic analysis has shown a large diversity of vermicompost microorganisms (Sen and Chandra 2009; Vivas *et al*. 2009). Molecular techniques for the estimation of abundance and number of each species are essential for the understanding on the community composition and diversity at the genetic level. Moreover, information about the microbial community structure and their diversity is important for understanding the relationship between process function and community dynamics (Sen *et al*. 2008). The link between vermicompost stability and microbial diversity is not completely understood, though a medium to high diversity might indicate a good quality vermicompost. Thus, measurement of vermicompost microbial diversity gives valuable information about changes in the community structure and species richness in response to changes in the physico-chemical properties, and finally in the quality of the product. Therefore, vermicompost microbial diversity could be used as an indicator of quality and stability.

From the above, it is well understood that in order to get an insight into the blackbox of vermicomposting process it is essential to address the important factors involved in the process dynamics such as functional and genetic structure of the system. In this review, emphasis has been given to modern tools, which can be used to provide useful information in relation to the process dynamics at functional and genetic level (**Fig. 1**). An understanding of the factors involved in process dynamics using multivariate statistics technique will further help in the optimization of the process. This ultimately will lead to production of good quality vermicompost with optimum process time and low cost.

METHODS TO ASSESS ORGANIC MATTER TRANSFORMATIONS

The different analytical techniques used routinely, to study organic matter transformations are carried out either, on the whole vermicomposted materials, on the dissolved organic matter (DOM) or/and on the humic acids (HAs) fractions. The characterization of both, the organic colloidal fraction and the extractives, water-soluble products and lipids, is essential to follow changes in organic matter and to achieve a better understanding of the C and N cycles during the process. Typical chemical analysis carried out on the solid phase includes moisture, organic carbon, nitrogen, metal and ash content and lignocellulose fractionation (Suthar 2009; Khwairakpam and Bhargava 2009; Kwon 2009).

A few authors have also quoted the fractionation of humic substances and their analysis as a method for studying the vermicomposting process (Vinceslas-Akpa and Loquet 1997; Sen and Chandra 2007). The concentrations of the humic fractions are used to calculate humification parameters such as humification index, humification ratio and humic acid percentage, which can also assess the maturity and stability of vermicompost. The HA content in a vermicompost, along with its chemical and physico-chemical properties is an important indicator of the biological maturity and chemical stability of the product (Campitelli and Ceppi 2008a). Thus, advanced analytical techniques such as FT-IR (Fourier Transform Infrared) and solid-state ^{13}C-NMR (Nuclear Magnetic Resonance) spectroscopy are crucial in evaluating the changes in HAs structure in vermicompost. Castilhos *et al*. (2008) evaluated the quality and maturity of vermicomposts from different residues like cattle, sheep, pig and quail manure, coffee and mate-tee dregs by determining the humic substances content and their chemical composition. Based on HAs analysis, the maturity degree was greater in the vermicomposts of coffee and mate-tee dregs and also the proportion of oxygen containing functional groups and the aromatic degree were lowest in them. Thus, the determination of the amount and quality of humic substances in vermicompost is of primary importance for its agronomic efficacy, environmental safety and economic value. Thermal analysis and solid-state spectroscopy are highly reliable and valuable for assessment of changes in the humic substances and whole vermicomposts, which will help in defining the quality of end product.

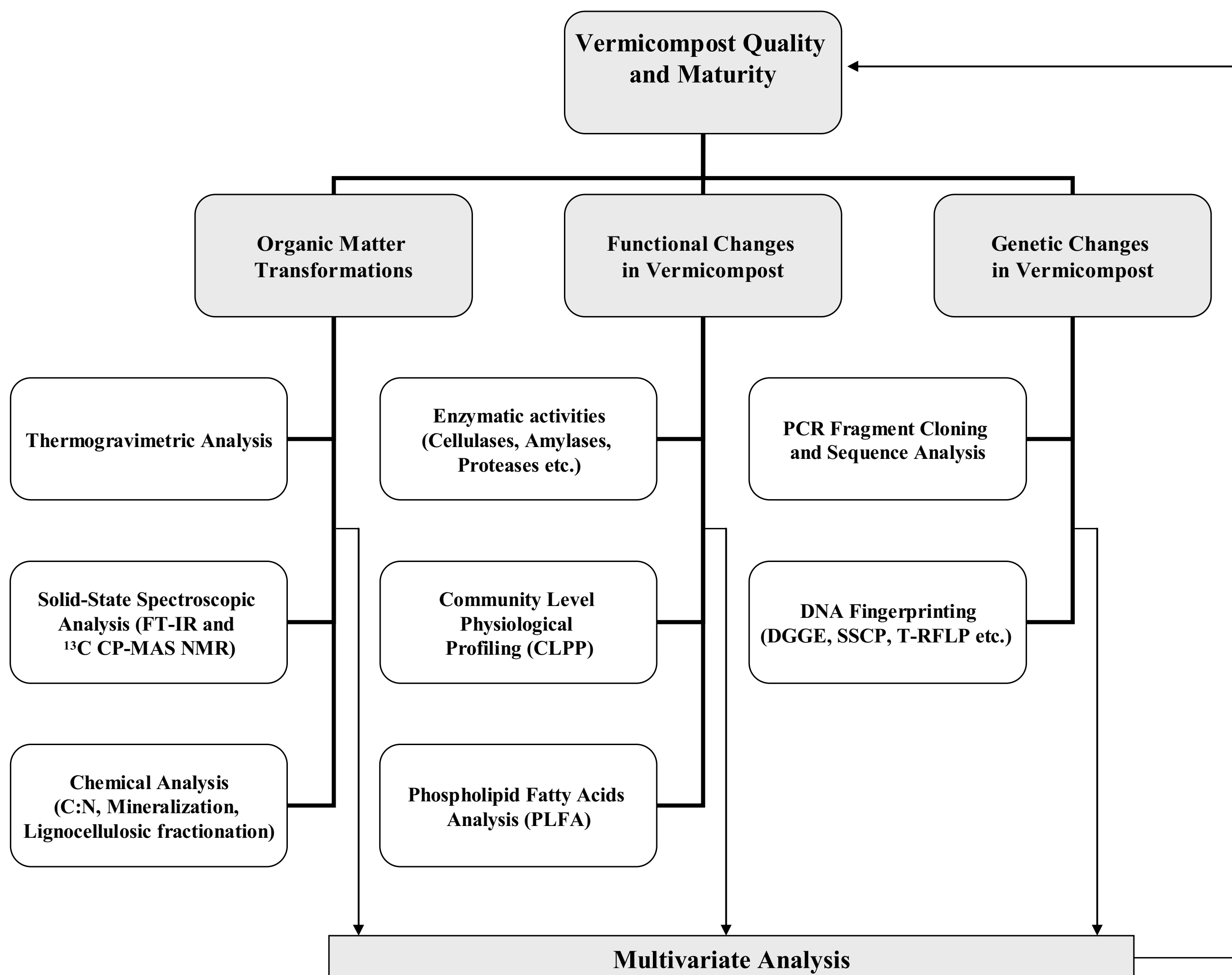

Fig. 1 Schematic illustration of the polyphasic approach to open the black box of vermicomposting process.

Thermal analysis

These methods include techniques based on thermal heating such as Thermogravimetry (TG), Differential Thermal Analysis (DTA) and Differential Scanning Calorimetry (DSC). They have been routinely carried out to characterize organic matter of composts (Dell'Abate *et al.* 1998) but not been applied to monitor changes in vermicompost samples. These methods are based on a programmed heating of the sample under a controlled atmosphere, which provides qualitative and quantitative information regarding the organic matter and humic substances content (Dell'Abate *et al.* 1998). The most important advantage of this method is its ability to analyze the whole samples without any pre-treatment (Melis and Castaldi 2004). These techniques have been successfully used to determine the stability of digestate and compost samples (Melis and Castaldi 2004; Gómez *et al.* 2007; Marhuenda-Egea *et al.* 2007). This suggests that thermogravimetric analysis can be a very rapid and powerful technique in the determination of vermicompost stability.

FT-IR Analysis

FT-IR spectroscopy is a modern, non-destructive analytical method which is used for the structural elucidation and quantification of a large variety of organic, inorganic and biological samples (Smidt *et al.* 2002). The two main advantages of using FT-IR spectroscopy are (1) no chemical treatment of the samples, thus avoiding any secondary reactions and (2) simultaneous measurement of all the compounds present in the sample, thereby minimizing sample analysis time. Major biochemical components such as carbohydrates, proteins, fats and lignocelluloses, and their composition can be easily determined using this technique. But FT-IR spectroscopy provides limited information about the functional groups in vermicompost samples. The changes in the pattern of the FT-IR spectra obtained at different time points during vermicomposting is very less except for the noticeable differences in the relative peak areas. However, there are several reports which has mentioned the use of FT-IR spectra as a qualitative tool for monitoring chemical groups and bands present in vermicompost sample (Sen and Chandra 2007; Campitelli and Ceppi 2008a; Gupta and Garg 2009).

The FT-IR spectra of humic acids extracted from vermicompost exhibited high levels of aliphatic (peaks at 2930-2850 cm^{-1}) and polysaccharide structures (peaks in the 1100-1000 cm^{-1} region) which decreased with process time (Sen and Chandra 2007; Gupta and Garg 2009). The main changes in the humic acid spectra with vermicomposting time are: (i) a reduction and transition to a small shoulder of the 1716 cm^{-1} peak (COOH groups) in the mature vermicompost; (ii) a sharp decrease in the aliphatic region (2930 cm^{-1} and 2850 cm^{-1}) and (iii) a relative increase in the aromatic peak (1650 cm^{-1}). Based on FT-IR spectral analysis, it can be concluded that the process ensues mainly via oxidation; the increase in aromatic structures is relative, which results from the decomposition of aliphatic components.

^{13}C NMR analysis

Solid-state ^{13}C NMR with CPMAS (Cross Polarization Magic Angle Spinning) technique provides useful information on the transformations of organic matter during vermi-

composting. Solid-state ^{13}C NMR spectroscopy offers the possibility of direct chemical characterization of organic materials in vermicompost. The initial application of solid-state ^{13}C NMR spectroscopy to soil organic matter studies produced spectra containing only broad featureless resonances due to strong ^{1}H-^{13}C dipolar interactions, low sensitivity and chemical shift anisotropy (Kögel-Knabner 1997). However, the use of high-power dipolar-decoupling and the development of cross-polarization (CP), and magic-angle spinning (MAS) techniques have allowed high resolution solid-state ^{13}C NMR spectra of vermicompost organic matter to be acquired (Vinceslas-Akpa and Loquet 1997; Sen and Chandra 2007).

The study of Vinceslas-Akpa and Loquet (1997) about decomposition of lignocellulosic wastes through composting and vermicomposting showed that NMR analysis could reveal more lignolysis in vermicompost, which was not apparent from chemical analysis. In addition, the authors reported a net production of new aromatic compounds and neosynthesis of polysaccharides especially in vermicompost. Sen and Chandra (2007) carried out ^{13}C NMR spectroscopy to predict changes in the HAs' structure during the vermicomposting process and found an increase in the alkyl C/O-alkyl C ratio.

METHODS TO STUDY COMMUNITY-LEVEL CHANGES DURING THE VERMICOMPOSTING PROCESS

Research on vermicompost microbial properties has been commonly carried out at the process-level, where biomass, respiration rate, and enzyme activities have been examined. These process-level measurements provide an important understanding of the gross microbial processes and their potential role in vermicompost quality. However, these methods explain little about the qualitative community-level changes, because any given microbial process may be carried out by diverse species. Moreover, these process-level measurements cannot characterize a particular microbial ecosystem.

The microbial interactions at the community-level are complex and depend on interactions of several different species. The quantitative and qualitative changes occurring in the composition of the vermicompost microbial communities may be a good indicator of changes in the quality of vermicompost. Recent advances in the methods to analyze microorganisms have significantly helped to gain insight into the resident microorganisms and their diversity. These approaches are based on the quantification and analysis of microbial physiology (CLPP and PLFA) and microbial DNA (fingerprinting).

Approaches to measure the functional changes in vermicompost

1. Community-Level Physiological Profiling (CLPP)

To understand the role of microbial communities in vermicompost, it is important to know their metabolic potential or functional diversity. Metabolic potential refers to the actual catabolic activity, while functional diversity indicates potential activity. The Biolog™ method developed by Garland and Mills (1991) is very useful for community-level physiological profiling, and can provide information on both, actual and potential activity of the microbial communities in vermicompost. This method provides a sensitive and ecologically meaningful measure of heterotrophic microbial community structure. Rather than relying on determination of changes in individual species abundances, which may not link to significant shifts in community function, this approach provides measures of the metabolic abilities of the community (Garland and Mills 1991). CLPP method requires the extraction of microbial cells from the vermicompost sample which are then inoculated directly onto commercially available sole-carbon substrate microtitre plates such as Biolog™ Ecoplates (31 different carbon substrates).

A data set of optical density (OD) values, one per substrate, is obtained from the colorimetric measure of the reduction of a tetrazolium dye. The pattern of oxidized substrates can be compared to indicate differences in the physiological functions of microbial communities in different samples. In addition temporal and spatial changes during the entire biological process can be assessed. Multivariate statistical techniques are necessary to analyze the data obtained on the substrate utilization profiles. This method is believed to be less biased than the traditional culturing techniques in spite of the response due to the non-culturable cells (Garland and Lehman 1999).

Several studies have shown that CLPP can be used to assess functional diversity (Zak *et al*. 1994; Garland 1997). However, there are some methodological considerations such as (i) the rate of color development in each well depends on the density of the initial inoculum which needs to be standardized (Garland and Mills 1991; Haack *et al*. 1995) (ii) visible color within a well is not achieved unless the total number of cells able to utilize that substrate reaches *ca*. 10^8 cells per ml (Haack *et al*. 1995). If there are less than 10^8 cells per ml, there can be a substantial lag phase leading to false negatives if wells are read too soon (iii) the color development in each well is assumed to be a function of the proportion of organisms present in the sample which are able to utilize a particular substrate (Garland 1997), which may not be valid since some strains may utilize more efficiently certain substrates than others (Smalla *et al*. 1998). In the presence of few generalists than specialists, the functional diversity is not equal to species diversity (Garland 1997; Konopka *et al*. 1998).

There are several studies, which report the use of CLPP method in composting process, while few authors have mentioned its use in vermicomposting. Using CLPP, differences in the functional response of microbial communities in compost and vermicompost was observed (Sen and Chandra 2009). Another study by Aira *et al*. (2007) reported that earthworms could modify the microbial biomass and activity and they found, using CLPP method, that the earthworms could even change the functional diversity of microbial community. The authors showed that earthworms promote changes in microbial communities towards more specialized community with potential use of diverse C pools. Yakushev and Byzov (2008) suggested a new method of multisubstrate testing (MST, Biolog) based on the kinetic parameters and showed that the method is very useful to differentiate three different lots of industrial vermicomposts. The authors concluded from their study that unification of substrates for composting is essential to produce standardized vermicomposts.

2. Phospholipid Fatty Acid Analysis (PLFA)

PLFA analysis has been used as a culture-independent method of assessing the structure of compost microbial communities and evaluating maturity of compost (Steger *et al*. 2003; Kato *et al*. 2005; Amir *et al*. 2008). PLFAs are useful signature molecules due to their presence specifically in cell membranes and also they are indicators of active microbial biomass because cell death leads to degradation of cell membranes. Moreover, using phospholipids, specific group of microbes can be tracked since they are unique to each group of microbes. Fatty acid methyl esters (FAMEs) analysis has been a very common biochemical test for bacterial taxonomy, and it makes the basis for using such signature molecules in revealing microbial community diversity. There are several important limitations that are associated with this method such as: (i) a general lack of knowledge and ambiguity in linking a particular fatty acid to a specific micro-organism or a group of micro-organisms, (ii) any error in the methodology can lead to variation in the signature molecules which may give false estimates of diversity, and (iii) the amount and type of PLFA produced by bacteria and fungi vary widely with respect to their growth condi-

tions and environmental factors.

There are several reports on the application of PLFA in determining microbial community changes during composting process. The PLFA profiles change over the composting process in a consistent and predictable manner (Lei and VanderGheynst 2000; Steger *et al*. 2003). Further to this Kato *et al*. (2005) have found that the proportion of branched FAMES and/or saturated PLFAs (SOH-FAMES) can be used as a tool for evaluating the maturity of poultry manure compost. While this method has found much importance in compost research, it has not been still implemented to track changes in microbial community over the vermicomposting process. However, Lores *et al*. (2006) investigated the FAME profiles of vermicomposts produced by different earthworm species, and found the fatty acid profiles were mainly determined by different earthworm species.

Approaches to measure genetic changes in vermicompost

Genetic changes in vermicompost means the succession of molecular sequences linked to microbial species surviving during the process. Nucleic acids have been the most useful sequences that helped in the understanding of the structure of microbial communities. Torsvik and colleagues (Torsvik *et al*. 1990, 1996) compared the re-association kinetics of DNA isolated from soil and sediment samples with that of pure cultures of microorganisms and found that the diversity of pristine soil and sediment may contain more than 10000 different bacterial types. They also reasoned that greater the sequence diversity of the DNA, greater is the DNA reannealing time and therefore a greater microbial diversity. Their study also estimated the genetic diversity of soil to be 200 times greater than the diversity among bacteria cultured from the same soil, which clearly indicated that soil microbial communities are much more complex than currently known. Thus, analysis of DNA sequences may provide a greater understanding of the microbial diversity that exists in vermicompost than could be gained from culture-dependent methods.

16S ribosomal RNA genes sequencing is the most commonly used nucleic acid technique for estimating microbial community composition and diversity whereas in eukaryotes 5S or 18S rRNA genes sequencing is used (Ward *et al.* 1992). The 16S sequence based methods are particularly suited for microbial diversity studies for a number of reasons: (i) 16S rDNA is found universally in all three forms of life: the domains Bacteria, Archaea, and Eucarya (Woese *et al.* 1990), (ii) it consist both highly conserved regions and hypervariable regions (Woese 1987). The phylogenetic relationships at several hierarchical levels can be calculated from comparative 16S gene sequence analyses, because of the differential rates of sequence evolution, (iii) the relatively large size (1.5 kb) and the presence of many secondary structural domains make this molecule suitable for obtaining greater phylogenetic information (Woese 1987). Moreover, the rate of change in one secondary domain does not affect the evolutionary changes in another domain and (iv) 16S gene can be easily amplified using PCR and can be further subjected to cloning and sequence analysis for species identification and diversity estimation (Huber *et al.* 2009).

Currently the most used nucleic acid based method is the partial community DNA analysis which consist the analysis of PCR-amplified 16S rDNA and intergenic spacer (16S-23S region) sequences. These methods include: (i) PCR fragment cloning and sequencing analysis, which gives more information on the number of different species than the relative abundance of these species and (ii) Genetic fingerprinting, which gives a global picture of the genetic structure of the bacterial community.

1. PCR fragment cloning and sequence analysis

This method involves generation of large clone libraries of 16S gene by PCR from isolated DNA. Each clone represents a single 16S gene sequence and thus a library of clones characterizes most of the sequences in a community. In order to separate and identify each clone, restriction fragment analysis and sequencing is carried out. The resulting sequences can be matched to sequences in databases to identify the closest phylogenetic relatives. Although this method of diversity estimation is considered the most acceptable nowadays, it still has inherent technical biases such as PCR, choice of primers and restriction enzymes, inhibition due to humic acids and formation of chimeras (von Wintzingerode *et al*. 1997). Moreover, due to high species richness found in soil or compost like communities, the number of clones generally analyzed (100 clones) gives an underestimate of the actual diversity. However, Dunbar *et al*. (1999) have shown that cultivation and 16S rDNA cloning provide similar assessment of the relationship among four arid soil communities based on several diversity indices. This implies that this approach will be useful in studying the relationship of microbial communities from vermicompost at different stages.

2. Genetic fingerprinting

These techniques are PCR based which do not involve the generation of a clone library. They involve the separation of amplified sequences based on different properties of the DNA by differential electrophoretic migration on agarose or polyacrylamide gels, such as size and sequence. The genetic fingerprints provide complex banding patterns representing the entire genetic structure of the community or a part of it, based on the selected primers. However, these profiles cannot give an accurate value of richness and evenness, since it is well known that one band may arise from different species and one species may be represented by several bands. Moreover, intraspecies operon heterogeneities can contribute significantly to complex genetic profiles in microbial community analysis, and studies based only on profile comparisons without sequencing may be misinterpreted as a high microbial diversity (Schmalenberger *et al*. 2001). The genetic profiles can further be subjected to multivariate analyses either in terms of similarities and relationships (Cluster analysis) or based on correlation and covariance matrix (PCA) to visualize the differences in the community structure of various samples (Fromin *et al*. 2002). Furthermore, the multivariate statistical analysis enables the correlation of community changes with various environmental factors (Ranjard *et al*. 2000).

3. Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)

This method was originally developed to detect specific mutations within the human genome, and is based on the different sequences of the same size of DNA. Muyzer *et al*. (1995) first reported its use in the analysis of bacterial diversity in complex communities. It involves the separation of PCR products on polyacrylamide gels containing a linear gradient of a DNA denaturing agent which can be either chemical such as urea or formamide (in DGGE) or physical factor such as the temperature (in TGGE). The extent of denaturation depends on the amount of differences between the sequences, thus a sample having different sequences will yield a banding pattern reflecting the genetic diversity of the sample (Muyzer *et al*. 1995). A severe limitation of this method is the laborious technical optimization including calibration of the linear gradient of DNA denaturants and the insertion of a GC base pairs clamp in the primers to obtain better electrophoretic separation of the fragments. Moreover, this method can only separate DNA of size smaller than 500 bps. Loisel *et al*. (2006) have further shown, using simulations, that this method does not reflect

the true diversity when there is a high background, and number of visible bands saturates around 35 for a complex community.

However, despite the technical difficulties, DGGE and TGGE are commonly used to compare the genetic structure of bacterial communities from different compost types and to study temporal successions. Using DGGE, Sen and Chandra (2007) have shown differences in the genetic structure of compost and vermicompost bacterial community. An advantage of this method is that the individual bands can be excised, reamplified and sequenced, or can be hybridized with probes for the taxonomic identification. Vivas *et al.* (2009) based on their study using DGGE concluded that in spite of the presence of several identical populations in the compost, vermicompost and non-processed materials, each process modified the original microbial communities of the waste in a diverse way indicative of the different roles of each process in the selection of bacterial community.

4. Single-Strand Conformation Polymorphism (SSCP)

This technique like DGGE was also originally developed to identify known or novel polymorphisms or point mutations in DNA (Orita *et al.* 1989). Single-stranded DNA is separated on a non-denaturing polyacrylamide gel based on differences in mobility due to their folded secondary structure (Liu *et al.* 2000), and this in turn depends on the DNA sequences. Some single-stranded DNA can form more than one stable conformation. Thus, one sequence may be represented by more than one band on the gel (Schmalenberger *et al.* 2001) as mentioned under DGGE technique. Unlike DGGE/TGGE, this method does not require a GC base pairs clamp or the construction of gradient gels, and has been successfully carried out to study bacterial or fungal community diversity in compost and soil samples (Peters *et al.* 2000; Stach *et al.* 2001). Peters *et al.* (2000) and Fracchia *et al.* (2006) used SSCP to measure diversity and succession of vermicompost bacterial communities. Recently, Sen *et al.* (2008) used this method based on capillary electrophoresis and showed structural divergence of bacterial communities in three similar vermicomposts produced from same start-up material.

5. Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Terminal Restriction Fragment Length Polymorphism (T-RFLP)

This method involves digestion of the PCR amplified 16S rDNA sequences using restriction enzymes, and further separating the restricted fragments on agarose or polyacrylamide gels. This technique has been used to study microbial diversity in different composts and to monitor changes in the genetic structure of contaminated soil bacterial communities following different amendments (Dees and Ghiorse 2001; Ntougias *et al.* 2004; Pérez-de-Mora *et al.* 2006). However, the main limitation of this method lies in the choice of restriction enzymes, which is crucial for obtaining optimum resolution and thus requires an initial screening for the selection of best enzyme. Moreover, this method will not provide valuable information, about the actual diversity, due to complex banding pattern and problems in band quantification.

An alternative method to ARDRA has been developed employing PCR, in which one of the two primers used is fluorescently labelled at the 5′ end (Liu *et al.* 1997). The PCR product is digested with restriction enzymes, and the fluorescently labelled terminal restriction fragment is accurately measured by using an automated DNA sequencer (Liu *et al.* 1997). Thus, only the terminal fragments are detected and is therefore known as T-RFLP or ‘terminal-RFLP’. The profiles obtained are much simpler than the previously described methods in terms of the numbers of bands. Based on our literature survey there are no reports on the use of this technique in studying the diversity of vermicompost samples.

INTERPRETATION OF DATA FROM POLYPHASIC APPROACH USING MULTIVARIATE ANALYSIS

A high variability of the parameters and the different sources of raw materials used during the vermicomposting process make it very difficult to assess the quality and the maturity of the final vermicompost. This difficulty, request for studies on quality assurance procedures, which can classify the vermicomposts produced from different sources and process time. Multivariate analysis tools such as PCA, cluster analysis, redundancy analysis (RDA), linear discriminant analysis (LDA) etc. are useful and reliable methods to predict the factors that influence the vermicomposting process. Such methods can also predict the relationship between the functional and genetic changes in the vermicompost.

PCA is useful for discerning patterns within the multivariate data whereas RDA can be used to test hypotheses regarding the importance of physico-chemical parameters in explaining variation in multivariate data. PCA describes the axes of maximum variability in the multivariate data set. RDA is a constrained ordination technique in which ordination axes are constrained to be linear combinations of process parameters (Legendre and Legendre 1998). This technique allows direct assessment of the relationship between known process parameters and variation in the functional and genetic data. The significance of the relationship can also be tested with Monte Carlo tests.

Sen *et al.* (2009) have shown in three similar vermicomposting beds, obtained with same raw material, divergent evolution of the bacterial genetic structure and a drift in the organic matter changes, using PCA and RDA analysis. Another study by Campitelli and Ceppi (2008b) suggested the use of statistical analysis to classify different organic amendments obtained from vermicomposting and composting from the point of view of their quality. Thus, multivariate statistical analysis methods provide significant information about the process dynamics and could discriminate the quality of the end products obtained from different vermicomposting processes.

CONCLUSION

In the last two decades, there has been a remarkable rise in vermicomposting research on several aspects, such as use of vermicompost as soil amendment, potential of various earthworm species in vermicomposting and recycling of different wastes. Although a good knowledge on the process technology and bioreactors development, using earthworms, currently exists, there are still lacunae in the basic information on the process dynamics and the role of different earthworm species participating in such a complex process. The advent of modern analytical tools in the recent past, has opened the doors to the investigation of such complex and dynamic microbe-earthworm mediated process. Currently, techniques like solid-state spectroscopy (FT-IR and NMR) are routinely carried out for composting research and warrant their use in vermicomposting process assessment. Besides, from following the process dynamics, these techniques can also aid in quality control of the start-up material being used for vermicomposting, like maintenance of uniformity, thus preventing the batch-to-batch variations. Designing and selection of new substrate combinations for vermicomposting and detection of process failure are few other areas where these techniques can find their use.

At the functional level of vermicomposting process, methods like Biolog™ and fatty acid analysis give an insight into the activity and abundance of microbial species. DNA based molecular tools (cloning and genetic fingerprinting) that are commonly applied to study the microbial ecology of terrestrial, aquatic and extreme environments have a great potential in revealing the community structure and bacterial selection during vermicomposting. Any change in the functional and genetic structure of vermicompost will have a strong influence on its quality and maturity. Thus,

studying these aspects using advanced techniques and multivariate statistical tools will not only give us an idea on the process dynamics but will also help to develop controlled vermicomposting process, which can overcome environmental stress and pollutant shock. It is a very common concept among the microbiologist that bioinoculants are helpful in the production of vermicomposts, which are effective in soil reclamation. These techniques would prove to be very effective as tracking tools in determining the role of bioinoculants, their fate during the process, and their usefulness in enhancing the vermicompost quality. Finally, vermicompost being a complex microbial process with species diversity similar to soil, can be a good model ecosystem proving ecological concepts, which are currently valid for plants and animal communities, through a polyphasic approach.

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