METAGENOMICS TO ASSESS BACTERIAL DIVERSITY IN THE SOIL AS INFLUENCED BY ORGANIC AND CHEMICAL INPUTS

By

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THESIS

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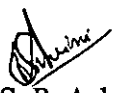
DECLARATION

I hereby declare that the thesis entitled "Metagenomics to assess bacterial diversity in the soil as influenced by organic and chemical inputs" is a bonafide record of research work done by me during the course of research and that the thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.



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ABBREVATIONS

CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxy ribonucleic acid
G	Grams
g^{-1}	Grams per litre
h	Hours
ha	Hectre
cm	Centi meter
m	Meter
kg	Kilo gram
kb	Kilo base
nm	Nano meter
min	Minutes
ml	Milliliter
mm	Millimeter
M	Molar
N	Normality
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
TAE	Tris acetate EDTA buffer
rpm	Revolutions per minute
µg	Micro gram
µl	Micro litre
µM	Micro molar
SDS	Sodium Dodecyl Sulphate
cfu	Colony forming unit
FYM	Farmyard manure

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF ANNEXURES

INTRODUCTION

1. Introduction

Soil is one of the most crucial factors for plant growth and also the best medium for the growth of the microorganisms. Soil microorganisms are very important, as almost every chemical transformation taking place in soil involves active contributions from soil microorganisms. They play an active role in soil fertility as a result of their involvement in the recycling of nutrients like carbon and nitrogen, which are required for plant growth. Soil microorganisms probably represents the world's greatest reservoir of biological diversity (Torsvik *et al.*, 1990). Moreover, microbial functions are active only in the healthy soil.

Non-judicious application of synthetic fertilizers decreases bacterial diversity in soil (Tan *et al.*, 2012). It may lead to soil acidification and neutralization and in turn, contribute to inactivation of enzymes present in microbes. Population of microorganisms is directly proportional to the organic contents present in the soil. Addition of organic inputs to soil enhances microbial growth and activities (Gelsomino *et al.*, 2004). Integrated nutrient management is proved to be better option as application of organic amendments along with synthetic fertilizers increases cereal crop yields (Saha *et al.*, 1998). Since the application of only synthetic fertilizer input has many negative impacts, there is tremendous enthusiasm to adopt organic farming. Mizoram left a mark on the world by turning into the principal state in India to changing its whole rural produce as organic. The act "Mizoram Organic Farming" was passed on July 12, 2004. The Government of Kerala has started efforts to advance organic cultivation in the state by 2016. Kasaragod is now proclaimed as an organic district.

Rice (*Oryza sativa* L.) is the most important staple food for a large part of the world's human population especially in east and south Asia (Sharif *et al.*, 2011). In Asia, more than two billion people obtain 60-70% of their energy requirements from rice. Rice is one of the most important food crops of India in terms of area, production and consumer preference. India is the second largest

producer and consumer of rice in the world. Microorganisms and their activities in rice rhizophere soil play important roles for rice production and soil fertility (Ishikawa *et al.*, 2010). Rice rhizopheric region is a hot spot of microbial interactions, due to exudates released by plant roots. Presence of microflora significantly increased the nutrients content of the plant (Miller and Chau, 1970). Thus, microflora and its diversity are very important for plant growth.

Despite the obvious importance of microbes, very little is known about their diversity, for example, how many species are present in the environment and what each individual species does or its ecological function (Singh *et al.*, 2008). There are no appropriate techniques available till date to answer these important questions due to the limitations encountered in the culturing of microbes. There is no single medium which permits growth of all microorganisms. It is widely accepted that up to 99 per cent of the microbes in the environment cannot be cultured readily (Sekiguchi, 2006).

To overcome these limitations, a DNA-based technique, metagenomic approach, has been developed. It is a recent branch of biology, which is a tool to study the microbes in an environment as a whole. It helps us to study the microorganisms in a particular environment as a community, at the molecular level. To assess the bacterial diversity, metagenomic approach is more preferential than conventional microbial techniques; traditional methods of culturing microorganisms limit analysis to those that grow under laboratory conditions (Rondon *et al.*, 2002). In general, methods based on 16S rRNA gene analysis provide extensive information about the taxa and species present in an environmental sample. High throughput sequencing has opened a new era for environmental microbial studies as large amounts of genetic information can be obtained. The ability to recover and analyse 16S rRNA gene directly from environmental DNA provides a means to investigate microbial populations and diversity without the need to culture them (Dojka *et al.*, 2000).

Researchers use 16S rRNA gene amplicon and next generation sequencing to characterize soil microbial communities. Next-generation sequencing (NGS); "culture-free method" enables analysis of the entire microbial community within a sample. The NGS-based 16S rRNA gene sequencing is a cost-effective technique to identify strains that may not be found using other methods. One of the NGS technologies include Illumina Miseq^{TM} sequencing. It is based on sequencing by synthesis technique; this approach generates several billion reads of nucleotide sequence (Bentley *et al.*, 2008), allows sequencing of up to 500 bp through paired-end sequencing, read length of 2X 150bp can be sequenced within 24 hours.

With this background, an attempt was made to assess the bacterial diversity of soil, from the Permanent Manurial Trial (PMT) rice plots at Regional Agricultural Research Station, Pattambi, Kerala. These plots have been receiving organic inputs (Cattle manure + green manure @ 9 t ha^{-1} each), integrated inputs (Cattle manure + green manure @ 4.5 t ha^{-1} each + inorganic fertilizers to supply 45:45:45: kg ha^{-1} N, P_2O_5 and K_2O) and inorganic inputs (Inorganic fertilizers to supply 90:45:45 kg ha^{-1} N, P_2O_5 and K_2O each) treatments since 1973. The soils of these plots were considered for analysis of bacterial diversity. The present study entitled "Metagenomics to assess bacterial diversity in the soil as influenced by organic and chemical inputs" was carried out with the objective to assess the diversity of bacterial community in the soil, as affected by the organic, integrated and inorganic inputs, using metagenomic approach.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The term "Metagenomics" was coined by Handelsman, Department of Plant Pathology at the University of Wisconsin, in 1982 and it is a novel culture-independent approach that permits direct access to total gene pool present in a specimen such as soil, sea water and sediment (Handelsman, 2004).

Metagenomics can be defined as "the application of modern genomics to the analysis of groups of microbial organisms directly in their natural environments, bypassing the need for isolation and laboratory cultivation of individual species"(Chen and Pachter, 2005). Only one per cent of the total microorganisms present in the soil is culturable and remaining 99 per cent cannot be cultured by standard techniques. New and effective techniques in DNA sequencing can circumvent these obstacles, as DNA can be isolated directly from the environment, living cells, old samples, and dead cells. Such methods have opened up a new field of study, referred to as Metagenomics (Susannah and Edward, 2005).

Soil is the most important component of earth's biosphere, as it supports production of food and also maintains environmental quality. A healthy soil, in turn, produces a healthy crop and maintains animal health. Soil health can be defined in terms of physical, chemical and biological indicators. The abundance and diversity of microflora serve as biological indicators of soil health. Functional diversity of microflora is also important, because microbes play a key role in nutrient cycling and hence soil fertility.

There is a general belief that soil health is degraded if synthetic fertilizers, herbicides and plant protection chemicals are extensively and continuously used over a long period of time. Intensive cultivation with fertilizer-responsive varieties of crops may also lead to soil pollution.

The present study is an attempt to assess the diversity of bacteria present in the soil samples so as to understand the effect of organic, integrated and inorganic inputs on diversity of microorganisms. This chapter is a review of research work carried out on soil health (physical, chemical and biological parameters) as influenced by management practices, and application of metagenomics in assessing microbial diversity in soil, water, environment and gut of animals.

2.1 Effect of soil microorganisms on soil health

Microorganisms are an essential component of a living soil. These microorganisms play an important role in soil processes that determine plant productivity. It is broadly being perceived that abundance and diversity of microbes make the soil healthy. Microbes also enhance plant growth and offer protection against pests and diseases. The significance of rhizosphere microbial populations for the improvement of the root health by promoting nutrient uptake and tolerance of environmental stress have been the major research areas (Bowen and Rovira, 1991) in the past.

Microorganisms in organic soil are more active. They play an important role in the development of stable soil organic matter like humus and other natural carbon complexes. Microbes use the applied inputs quickly and soil structure was improved (Singh and Singh, 2010).

2.2 Effect of organic and inorganic inputs on microbial population

Microbial communities are key drivers of soil fertility and agriculture productivity. Based upon the different inputs received by the soil, its health and microbial communities change, which is an important aspect in the development of sustainable agriculture.

2.2.1 Effect of organic inputs on microbial population

Soil microbes are the living part of organic matter and the soil biological activity was found more in the soil receiving organic inputs under long-term management (Fauci and Dick, 1992). Incorporation of organic amendments in soil is reported to increase soil microbial activity (Elliott and Lynch, 1994), microbial diversity (Girvan *et al.,* 2004; Grayston *et al.,* 2004) and population of bacteria (Bruggen-Van and Semenov, 2000). Gelsomino *et al.* (2004) found that addition of organic amendments increased microbial biomass and microbial activity compared to the conventional agricultural system. Combined use of organic manures improved the microbial load of the soil rather than single organic manure application (Krishnakumar *et al.*, 2005). According to Zhong and Cai (2007), the microbial parameters were correlated mainly with soil organic carbon content rather than phosphorous and nitrogen, indicating that the application of phosporous and nitrogen did not directly affect microbial parameters in soil, but indirectly increases the crop yields and accumulation of soil organic matter.

The highest population of bacteria was observed in vermicompost incorporated soil (55.19 $x10^5$ CFU g^{-1}dry soil) followed by farmyard manure (54.26 $x10^5$ CFU g^{-1} dry soil) and the least was observed in control (30.89 $x10^5$ CFU g^{-1} dry soil) as investigated by Das and Dkhar (2011). The soil bacterial, fungal, actinomycetes and N fixing bacteria were more in organic fields than inorganic field (Padmavathy and Poyyamoli, 2011)

The application of farm yard manure improves soil struture which leads to a better environment for root development (Dejene and Lemlem, 2012). Wiseman *et al.* (2012) assessed the potential of organic amendments *viz* leaf based and bio-solid compost in alteration of soil biological characteristics. The results showed an increase in soil microbial biomass (12%) in leaf based compost compared to other organic amendments.

The integrated use of organic manures and inorganic fertilizers improved the enzymatic activities as well as the microbial population of bacteria, fungi and Actinomycetes (Meena *et al.*, 2014). Application of Panchagavya and Beejamruth to soil recorded highest rhizosphere microbial population (Shubha *et al.*, 2014).

The organic nutrient management is important for increasing the number and diversity of soil organisms and inputs such as compost was effective as the commercial synthetic fertilizer on crop growth and yield (Chhogyel *et al.*, 2015). Bajgai *et al.* (2015) reported that organic fertilizer application could reduce nutrient loss to the environment due to its slower nutrient releasing mechanism compared with that of synthetic fertilizers.

The organic inputs like farm yard manure increased the soil beneficial mycoflora population, species diversity and nutrient availability to the crops. This ultimately increased the growth and yield of crop plants compared to inorganic inputs applied to the field which adversely affect mycoflora diversity (Singh and Kaur, 2016). Velmourougane (2016) reported that organic inputs treated soil was found to have the higher microbial population (34%) and microbial diversity indices compared to conventional systems.

2.2.2 Effect of inorganic inputs on microbial population

Mineral fertilisation of soil could bring about a reduction in population and activity of soil organisms, due to the toxicity of metal contaminants contained in mineral fertilisers. In general, N and K fertilisers contain very low levels of contaminants, whereas P fertilisers often contain significant amounts of cadmium, mercury and lead (McLaughlin *et al.*, 2000). Sarathchandran *et al.* (2001) reported that application of inorganic fertilizers such as nitrogen and phosphate fertilizers had no significant effects on soil microbial populations but nitrogen application reduced the functional microbial diversity in pasture soils. Similarly, Barabasz *et al.* (2002) concluded that

application of high doses of synthetic nitrogen fertilizers resulted in decline of some beneficial microorganisms.

Inorganic fertilizers were reported to lower rhizosphere microbial population and diversity (Nelson and Mele, 2006). Tan *et al.* (2012) predicted that high inputs of agrochemicals leads to an increase of phosphorus level in the soil and a concomitant reduction of the bacterial diversity. Meena *et al.* (2014) conducted studies on soil enzyme activity as influenced by concentrate manures and synthetic fertilizers in alluvial soils of Varanasi and concluded that judicious application of 100% NPK + 300 kg organic manure concentrate ha^{-1} was the best treatment for soil enzymatic activity as well as microbial population.

2.3 Effect of organic and inorganic inputs on soil properties

Mineral fertilizers and organic manures induce different changes in the chemical, physical and biological properties of soil. The numerical presence of microorganism influences the chemical properties. The changes in chemical properties, in the long-term, are believed to have significant influences on the quality and productive capacity of the soil (Acton and Gregorich, 1995; Belay *et al.*, 2002; Zhong and Cai, 2007).

Long-term use of organic inputs in soil improves the soil properties and physical condition of the soil. Synthetic fertilizers offer nutrients which are readily soluble in soil solution and thereby are instantly available to plants (Sarker *et al.*, 2004).

Microbial activities act like a function of soil properties such as nutrition, texture, pH, temperature, soil water content and these parameters are sensitive indicators of changes in soil properties (Mele and Crowley, 2008). Soil organisms decompose organic residues and mobilize plant nutrients. The interaction of soil organisms and organic matter in the soil, helps to improve the

ecosystem of rhizosphere by improving the physico-chemical and biological properties of soil (Perez *et al.*, 2006).

The soil under integrated nutrient management noted greater organic C and total N compared to soils receiving synthetic fertilizers (Goyal *et al.*, (1999). Nakhro and Dkhar (2010) concluded that soil from organic plot showed an increase in organic carbon compared to the inorganic plot. Research related to the organic amendment application by Diacono and Montemurro (2010) also reported that long-term application of organic amendments increased organic carbon per cent.

The application of farmyard manure increased the availability of Mn and Zn, and also an increase in Fe was found in vermicompost applied soil. An increase in total soil microbial biomass carbon was also found in combined application of FYM, vermicompost and mineral fertilizers. (Rathod *et al.*, 2013)

Shaikh and Gachande (2013) conducted an experiment to compare the influence of organic and inorganic inputs on soil physico-chemical properties of jowar field. The results of this experiment from first year to next year showed that pH in organic cropping systems (organic manures used were farm yard manure, Jeevamruth and Beejamruth) showed the highest decrease in 2012-13 (1.23), followed by 2010-11 (0.81) and 2011-12 (0.79) over inorganic farming. Increase in available phosphorous of the organic field showed highest increase in 2012-13 (15.16 kg/h), followed by 2011-12 (10.36 kg/h) and 2010-11 (6.62 kg/h) over inorganic farming. Soil potassium in the organic field during 2010-13 showed highest in 2010-11 (20.6 kg/h), followed by 2011-12 (11.4kg/h) and lower in 2012-13 (34.2 kg/h) over inorganic farming.

The proper management of crop residues and organic material incorporation ensures improved soil properties and sustainability in crop productivity (Bajgai *et al.*, 2015). The physical properties of soil such as electrical conductivity and bulk density were found to increase by 34 and 21 per cent respectively in conventional farming and organic farming systems and a

significant increase was observed in organic carbon, nitrogen, potassium and phosphorous in soil receiving long term organic inputs (Velmourougane, 2016).

2.4 INTRODUCTION TO METAGENOMIC APPROACH

It is a culture-independent technology in genomic analysis of all the microorganisms in a particular environmental niche (Handelsman *et al.,* 1998). Studies have revealed that only 0.001- 0.1 per cent of the total microbes in seawater; 0.25 per cent in freshwater; 0.25 per cent in sediments and 0.3 per cent of soil microorganisms were culturable under *in vitro* condition (Amann *et al.,* 1995).

Metagenomics provides an effective tool for the discovery of new, valuable natural products and functions of microbes (He *et al.*, 2007). Next Generation Sequencing (NGS) technology enables us to get a snapshot of the blueprints of DNA of 'unculturable' microbes straight out of the environment. High-throughput sequencing of metagenomic DNA using second generation sequencing technology led to the accelerated collection of environmental metagenomic sequences, which provide data on the prevalence of species, antibiotic resistant genes (ARGs) and genetic elements in various environments (Monier *et al.,* 2011).

The technology is applied in the study of an array of microbial diversities. The Sargasso Sea near Bermuda is the first metagenomic sequencing project which provides one of the comprehensive studies of marine microbial diversity (Venter *et al.*, 2004; Tringe *et al.,* 2005). Several other ecosystems of various environmental niches have been explored, such as the analysis of ruminal bacterial diversity (Kocherginskaya *et al.,* 2001; Edwards *et al.,* 2004), drainage of acid mine (Ram *et al.*, 2005; Tringe *et al.,* 2005), in the termite hindgut microflora (Warmecke *et al.,* 2007) and permafrost-influenced soils in the Arctic (Ganzert *et al.*, 2007).

These studies provided information on the ecology of microbes and evolution of species and directions for harnessing novel genetic and biochemical data. Initially, uncultured microflora and ancient DNA analysis were the prime targets of metagenomics studies (Gabor *et al.*, 2007; Singh *et al.*, 2008). However, the technology is now being applied in the study of microbial diversities like deep sea aquatic microflora, soil microbes and gastrointestinal ecosystems of living beings (Lu *et al.*, 2007).

2.4.1 Environmental Metagenomics

The first extensive large-scale environmental sequencing project was carried out by J. Craig Venter Institute in 2004 in which they sequenced fragments of DNA derived from the entire microbial population of the nutrient-limited Sargasso Sea, an intensively studied region of the Atlantic Ocean close to Bermuda (Venter *et al.*, 2004).

Random shotgun sequencing of DNA from a natural acidophilic biofilm, was employed for reconstruction of near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II from a biofilm. Single-nucleotide polymorphisms were the predominant form of heterogeneity at the strain level. The uniformity of the community structure allowed to sequence almost the whole microflora with a high degree of accuracy (Tyson *et al.*, 2004). A shotgun sequencing approach yielded over 1.6 billion base pairs of DNA and reveled the 1.2 million new genes. An aggregate of 7, 94,061 of these genes were assigned out to a conserved hypothetical protein group, the functions of which are unknown. The acid mine drainage microbiota was found to contain three bacterial and three archaeal species (Schoss and Handelsman, 2005). The bacterial genera included *Leptospirillum, Sulfobacillus, Acidomicrobium* and the dominant archaeal species was *Ferroplasma acidomicrobium.*

2.4.2 Soil metagenomics

Microorganisms which are soil borne are one of the world's tremendous sources of biodiversity (Curtis *et al.*, 2002) with evaluations running somewhere around 3,000 and 11,000 microbial genomes per gram of soil (Schmeisser *et al.*, 2007). Additionally, nearly 140 mega bases of a sequence taken from Minnesota farmland soil contained less than one per cent of sequences with any overlaps and formed no contigs (Kowalchuk *et al.*, 2007).

However, soil has the vast diversity and its history as a source of commercially important molecules in agriculture, chemical, industrial and pharmaceutical industries remains the most common intention for studies of functional metagenomics (MacNeil *et al.*, 2001; Courtois *et al.*, 2003; Daniel, 2005).

Souza *et al.* (2013) conducted analysis of microbial diversity in soil under different soil and crop management, using pyrrosequencing. The study concluded that within the domain Bacteria, Proteobacteria represented the dominant phylum in all treatments- 41.5%. The major classes were Alphaproteobacteria (51.1%), Betaproteobacteria (20.8%), Deltaproteobacteria (19.6%) and Gammaproteobacteria (8.55%). Actinobacteria was the second most abundant phylum of bacteria (24.0%), and consisted of Actinomycetales, Solirubrobacterales and Rubrobacterales. Bacterial composition was more influenced by tillage practices than crop management practices. Sah (2014) reported that soil samples collected from '*pokkali*' rice fields of Kerala comprosed the phyla Proteobacteria, Chloroflexi and Acidobacteria.

2.5 Soil sampling and nucleic acids extraction

In the metagenomics process, the samples could be analysed from any environment, soil or habitat including the Geographical Indication ecosystem (Ghazanfar and Azim, 2009). Specifically, soil microbial communities are composed of a mixture of archaea, bacteria and protists, exhibiting the diversity of

cell wall characteristics and fluctuating in their resistance to lysis (Kauffmann *et al.,* 2004). Some unique methods are required for their extraction. Although, different kits are available for DNA isolation from environmental specimens, numerous research institutes have built up their own particular techniques with the aim of enhancing extraction and reduction of predisposition brought by unequal lysis of various individuals from the soil microbial community group (Frostegard *et al.,* 1999; Krsek and Wellington, 1999; Miller *et al.,* 1999).

There are two different types of extraction techniques: (1) direct, *in situ,* extraction where the cells are lysed in the soil sample followed by DNA is isolation; and (2) indirect extraction techniques, where the cells are expelled from the soil and further lysed for DNA isolation (Schmeisser *et al.*, 2007).

Soil is a complex framework containing numerous substances, like humic acids, which can be co-separated during DNA extraction. Expulsion of humic acids is a key step before the DNA can be prepared for downstream processes. For this reason, scope of DNA purification procedures has been researched. One of the purification protocol given by Miller *et al.* (1999) was using the Sephadex G-200 spin columns. This is an ideal approach to expel contaminants from soil DNA. Pulsed field electrophoresis strategy utilizing two-phase agarose gel, with one phase containing poly vinyl pyrrolidone (PVP) was used for removal of humic acid (Quaiser *et al.*, 2002).

Siddhapura *et al.* (2010) examined DNA extraction and its quality evaluation for PCR applications from saline soils of coastal Gujarat and Sambhar Soda Lake, Rajasthan in India. The mechanical and soft lysis techniques were straight forward and effective for fast isolation of PCR amplifiable aggregate genomic DNA. The same method was also followed by Girija *et al.* (2013) for extracting metagenomic DNA from cowdung and Sah *et al.* (2014) *Pokkali* soils of Ernakulam district, Kerala.

2.6 16S rRNA gene amplification

Winker and Woese (1991) used the 16S rRNA gene sequences to understand bacterial phylogeny and taxonomy. It was the most widely recognized housekeeping genetic marker utilized because of various reasons including (i) its presence in all microorganisms regularly existing as a multigene family or operons; (ii) the function of the 16S rRNA gene after some time was not changed suggesting that random sequence changes were more precise in measure of time (iii) the 16S rRNA gene (1,500 bp) is sufficiently extensive for informatics purposes.

Schmalenberger *et al.* (2001) concentrated on parallel examination of 3 distinctive hypervariable regions of 16S rDNA sequence (V2–V3, V4–V5 and V6–V8 locales). This was used as a powerful tool finding in deciding the organization of bacterial consortia in maize rhizospheres. Baker *et al.* (2003) demonstrated the methodology to recognize organisms in the complex community by exploiting universal and conserved targets, such as rRNA genes. By amplifying selected target region inside 16S rRNA genes, microorganisms (particularly bacteria and archaea) can be identified by the effective combination of conserved primer-binding sites. Further intervening variable sequences encouraged genus and species identification. The 16S rRNA gene in bacteria comprises of conserved sequences scattered within variable sequences that include 9 hypervariable *i.e.* V1 to V9 areas. The lengths of these hypervariable regions range from around 50 to 100 bases, and the sequences differ with respect to variation and in their corresponding utility for universal microbial identification. Among 9 hypervariable regions the V2 and V3 regions were most effective for universal genus identification (Chakravorty *et al.*, 2007). Fadrosh *et al.* (2014) used sequencing strategy to evaluate the composition of microbial communities from clinical samples utilizing V3-V4 area of the 16S rRNA gene on the Illumina MiSeq platform and this approach yielded high-quality sequence information from 16S rRNA quality amplicons utilizing both 250 bp and 300 bp paired-end end MiSeq protocols.

2.7 TOOLS FOR METAGENOMICS

Metagenomics often defined as the analysis of DNA from microbial communities in environmental samples without prior need for culturing. Many metagenomics computational tools and databases have been developed in order to allow the exploitation of the huge influx of data such as Next Generation sequencing (NGS); is also known as high-throughput sequencing. The biochemistry of platforms is different, but in all these technologies, massively high throughput is achieved. These technologies do not require cloning of DNA fragments in any vector, instead these depend on preparation of NGS libraries in a cell-free system. Several millions of sequencing reaction run parallel. No electrophoretic separation is required to read the bases. The enormous amount of reads generated makes it simpler to analyse whole genomes of microorganisms. A library is prepared by random fragmentation of DNA, followed by *in vitro* ligation of common adaptor sequences. PCR amplicons derived from any given single library molecule end up spatially clustered, either to a single location on a planar substrate or to the surface of micron-scale beads, which can be recovered and arrayed (emulsion PCR). The sequencing process consists of alternating cycles of enzyme-driven biochemistry and imaging-based data acquisition. These technologies have helped in bringing down the cost of sequencing considerably. The disadvantage of NGS lies in the short read length and lower accuracy of sequence reads. This is the term used to illustrate various distinctive, present day sequencing advances in sequencing technologies including Illumina (Solexa) sequencing, Roche 454 pyrosequencing, Ion torrent: Proton/ PGM sequencing, Applied Biosystems SOLiD^{TM}Sequencer.

NGS has become most important to a complex genomic research and this technology is the answer to all the questions of the researcher. Innovative NGS sample preparation and data analysis options enable a broad range of applications. Through next generation sequencing, one can know the depth of information which is beyond the capacity of traditional DNA sequencing technologies.

NGS technology includes some features, which are not present in the technology of traditional DNA sequencing. Some of them are 1) Whole genome sequencing (the most comprehensive method for analysing the genome), 2) Deeply resequence the target gene. 3) Utilize RNA sequences to discover novel RNA variants and splice sites, or precisely quantify mRNAs for gene expression analysis, 4) Enhances epigenetic studies with high coverage density and flexibility, 5) Sequence the entire sample with no loss of information, 6) Survey the genome of the entire community.

2.8 Sequencing Platforms

454 pyrosequencing, Solexa (Illumina) and Ion Torrent are the NGS platforms available. In 2006, Solexa discharged the genome analyzer (GA) and was acquired by the Illumina. The Illumina system utilizes a sequencing-by-synthesis approach in which all four nucleotides are added at the same time to the stream cell channels, along with DNA polymerase, for fusion into the oligo-primed cluster fragments specifically.

Zepeda *et al.* (2015) described that the nucleotides carry a base-specific fluorescent label and the 3'-OH group is synthetically blocked such that every fuse is an extraordinary event. An imaging step follows each base incorporation step, during which each stream cell lane is figured in three 100-tile segments by the instrument optics at a cluster density per tile of 30,000. After each imaging step, the 3'blocking group is synthetically removed to prepare each strand for the next incorporation by DNA polymerase.

This array of steps proceeds for a particular number of cycles, as determined by user-defined instrument settings, which allows discrete, read lengths of 25–35 bases. A base-calling algorithm assigns sequences and associated quality values to each read and a quality checking pipeline assesses the Illumina information from each run, removing poor-quality sequences.

2.9 APPLICATION OF SOIL METAGENOMICS

The advancement and use of metagenomics has empowered access to the uncultivated soil microbial community, benefiting a rich source of novel and useful biomolecules.

2.9.1 Bioprospecting for novel genes

Metagenomics is one of the best tools for locating novel genes encoding important traits, from a complex environment. Several genes have been identified and some of them are amidases, lipolytic genes, esterases, oxidoreductases *etc.* Voget *et al.* (2003) identified 12 agarase genes, while screening of a soil metagenomic library. Gabor and Janssen (2004) cloned a novel amidase gene from a study employing soil metagenomics, using enrichment method. Li and Qin (2005) cloned a novel lipase gene from a soil metagenomic library, in vector pEpiFOS-5.

Esterase (*EstCE1*) was derived from a soil metagenome (Elend *et al.*, 2006). This enzyme displayed remarkable characteristics like high level of stability and unique substrate specificities. Functional screening of a soil metagenomic library for cellulases yielded eight cellulolytic clones, one of which was purified and characterized (Voget *et al.*, 2006).

Metagenomics offers a comprehensive tool in prospecting for genes which can be used for bioremediation, synthesis of natural products, and in pharmaceutical industry.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Metagenomics to assess bacterial diversity in the soil as influenced by organic and chemical inputs" was carried out at the Department of Agricultural Microbiology and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2014-2016. The materials utilized and the methodologies adopted in the present study have been outlined in this chapter.

3.1 MATERIALS

3.1.1 Chemicals, Glassware and Micropipette

The chemicals used for the study were of good quality from various agencies. Molecular Biology Grade enzymes and buffers were supplied by MERCK, SRL and HIMEDIA. Micropipettes and tips for molecular work were supplied by Eppendorf. Equipment items used in the present study are given in Annexure I.

3.2. METHODOLOGY

3.2.1 Experimental block details

Soil samples were collected from the permanent manurial trial plots at the Regional Agricultural Research Station, Pattambi (Plate 1), started in 1973. The station is situated at 10° 48" N latitude and 76° 12" E longitude and at an altitude of 25 m above mean sea level.

The details of the experiment are:

Design	: RBD
Replication	: 4
Variety	: Jaya
Spacing	: 15 X 15 cm
Plot size	: 7.8 x 5.25 cm (gross) 7.5 x 4.95 m (net).
Treatments	: 8

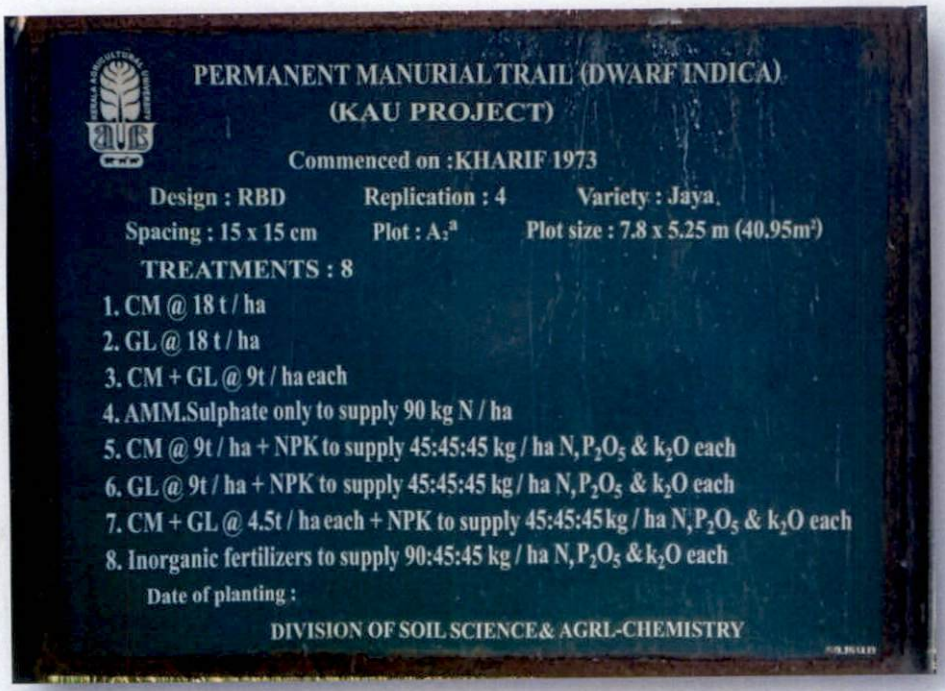


PLATE 1. Experimental plot

Treatments details are listed below:

Treatments

T_1 : Cattle manure @ 18 t/ ha

T_2 : Green manure @ 18 t/ ha

T_3 : Cattle manure + green manure @ 9 t/ha each

T_4 : Ammonium sulphate only to supply 90 Kg/ ha

T_5 : Cattle manure @ 9 t / ha + NPK to supply 45:45:45 kg / ha N, P_2O_5 and K_2O each

T_6 : Green leaves@ 9t / ha + NPK to supply 45:45:45 kg / ha N, P_2O_5 and K_2O each

T_7 : Cattle manure + Green manure @4.5t /ha each+ NPK to Supply 45:45:45: kg/ha N, P_2O_5 and K_2O each

T_8 : Inorganic fertilizers to supply 90:45:45 kg/ha N, P_2O_5 &K_2O each

Among these only three treatments were considered for the present study. The treatments receiving organic inputs (T_3), integrated inputs (T_7) and inorganic inputs (T_8) were considered for the present study.

3.2.2 Collection of rhizosphere soil samples

The soil samples were collected from T_3, T_7 and T_8 of paddy plots receiving organic inputs, inorganic inputs, and integrated inputs respectively. These were designated as ONM, INM and INF, in the present investigation. Rice rhizosphere soil samples were collected at panicle initiation stage during the month of August 2015. From each plot, soil samples were collected from four different rice plants in polythene bags, and brought to the laboratory. Soil samples from each plot were pooled together to get a representative composite sample. The samples were then air dried, stones and other debris were removed and sieved using 2.00 mm sieve. It was then stored under refrigerated condition. The soil samples were further processed for physical, chemical, biological characteristics and DNA extraction for metagenomic studies.

3.3 Analysis of physico-chemical properties of soil

Physical and chemical properties of the three composite soil samples were analysed, as mentioned in Table 1.

Table 1. Methods used for analysis of physico-chemical properties of soil

Parameters	Method	Reference
Bulk density	Core sampler	(Piper, 1966)
Soil reaction (pH)	Soil water suspension of 1:25 and read pH meter	(Jackson, 1958)
Electrical conductivity	Soil water suspension of 1:25 and read electrical conductivity meter	(Jackson, 1958)
Organic carbon	Walkley and Black method	(Walkley and Black, 1934)
Total Nitrogen	Micro-Kjeldahl method	(Jackson, 1973)
Available calcium and magnesium	Using atomic absorption spectrophotometer	(Hesse, 1971)
Available sulphur	Extraction by using 0.15 per cent $CaCl_2$ turbidimetry method	(Massoumi and Cornfield, 1963)
Available phosphorus	Ascorbic acid reduced molybdo phosphoric blue color method	(Watanabe and Olsen, 1965)
Available potassium	Neutral normal ammonium acetate extract using photometry	(Jackson, 1958).
Available Fe, Mn, Zn and Cu	Extraction using 0.1 M HCl by atomic absorption spectrophotometer	(Sims and Johnson, 1991)
Available boron	Azomethine–H using spectrophotometer	(Berger and Truog, 1939, Gupta, 1972)

3.3.3 Enumeration of rhizosphere microflora

Isolation and enumeration of microorganisms in rhizosphere soil samples was carried out by serial dilution and plate count method (Johnson and Curl, 1972). The dilutions and media used for enumeration of different functional groups of microorganisms are given in Table 2.

Table 2. Media and dilutions used for isolation of microorganism

Media	Target organisms	Dilutions used
Nutrient agar	Bacteria	10^{-5},10^{-6}
Martin's rose Bengal agar	Fungi	10^{-2},10^{-3}
Kenknight agar	Actinomycetes	10^{-3},10^{-4}
Pikovskaya's agar	Phosphate solubilisers	10^{-3},10^{-4}
King's B agar	Fluorescent pseudomonads	10^{-5},10^{-6}
Jensen's agar	Nitrogen fixers	10^{-3},10^{-4}
Trichoderma selective media	*Trichoderma*	10^{-2}.10^{-3}

Ten gram of soil from each sample was weighed, transferred aseptically in to 250 ml conical flask containing 90 ml sterile distilled water and was shaken for 5 min at 150 rpm. After shaking, a series of tenfold dilutions of suspension was made for each sample by pipetting 1 ml of aliquots in to 9 ml sterile water blank until the final dilution was 10^{-6} fold. The dilutions used for plating are indicated in Table 2. One ml of suspension from respective dilutions was transferred aseptically in to Petri dishes. 20 ml of molten and cooled agar media was then poured in the Petri dishes. The plates were then rotated clockwise and anticlockwise manually for uniform distribution of the suspension in medium. After solidification, Plates were incubated at 32 ± 2°C in an incubator. Observations were taken as and when colonies appeared. The number of colonies on each media was counted and expressed in (cfu g^{-1}). Observations were also taken on the different morphotypes of microorganisms.

3.3.4 Determination of microbial biomass carbon

Microbial biomass carbon in soil was estimated by chloroform fumigation and extraction method (Jenkinson and Powlson, 1976). For this, five sets of 10 g of soil samples were taken, one set kept in an oven for the determination of moisture gravimetrically at 105°C. Two sets of samples were kept in vacuum desiccators containing vacuum created using a vacuum pump. Then from the fumigated and non-fumigated samples, organic carbon was extracted using 0.5 M potassium sulphate. To the 10 ml extract 0.2 M potassium dichromate, concentrated sulphuric acid and orthophosphoric acid were added and kept on a hot plate at 100°C for half an hour under refluxing condition. After that 250 ml water was added and titrated against standard ferrous ammonium sulphate to determine microbial biomass carbon.

3.4.1 Metagenomic DNA extraction

3.4.1.1 Direct method of DNA extraction by soft lysis (Siddhapura *et al.*, 2010)

One gram of soil sample was taken in a 30 ml centrifuge tube and 10 ml of extraction buffer was added and incubated at 37°C for 10-12 h under constant shaking at 150 rpm. The sample was re-extracted in 1 ml of extraction buffer and the supernatant was collected by low speed centrifugation (5000 rpm) for 10 minutes. Then 4 ml of lysis buffer was added and incubated at 65°C for 2 h with vigorous shaking at every 15 min. The sample was centrifuged at 10,000 rpm for 10 min at 4°C. After centrifugation, the upper aqueous layer was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) at 10,000 rpm for 20 minutes at 4°C. After spin, the upper aqueous phase was again extracted with equal volume of chloroform: isoamyl alcohol (24:1) at 10,000 rpm for 10 minutes at 4°C. DNA ~~thus~~ was prepared further ... treated by adding 1/10 volume of 7.5 M potassium acetate and subsequently precipitated by adding two volume chilled ethanol. DNA precipitate was collected by centrifugation at 10,000 rpm for 10 min, air dried and suspended in 50 µl sterile distilled water.

Chemicals used in this protocol are given in Annexure II

3.4.1.2 Direct method-short procedure (Siddhapura *et al.*, 2010)

About 20 mg of soil sample was suspended in 400 µl of extraction buffer in 1.5 ml micro centrifuge tube and vortexed for 10-15 min. The mixture of soil and extraction buffer was then incubated at room temperature for 1 h. After incubation it was centrifuged at 12,000 rpm for 5 minutes and supernatant was collected. The upper aqueous layer was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:10) at 10,000 rpm for 20 minutes at 4°C. The aqueous phase was transferred to a fresh tube and the DNA was precipitated with equal volume of ice-cold isopropanol and incubated at room temperature for 15 minutes. It was then centrifuged at 13,000 rpm, at room temperature for 5

minutes. The supernatant was discarded and 600µl of ethanol was added. DNA precipitate was further collected by centrifugation at 10,000 rpm for 10 minutes, air dried and suspended in 25µl of sterile distilled water. Chemicals used in this protocol are given in Annexure III.

3.5 Agarose Gel Electrophoresis

3.5.1 Preparation of agarose gel

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of DNA. Materials used for preparation is given in Annexure IV

3.5.2 Electrophoresis

The quality of isolated DNA was evaluated though agarose gel electrophoresis (Sambrook *et al.*, 1989). About 100 ml of 1X TAE buffer was prepared from the 50X TAE (pH 8.0) stock solutions and 0.8 g of agarose was added to 1X TAE buffer in conical flask. Agarose was dissolved in buffer by heating and cooled to 42-45°C. Ethidium bromide prepared from a stock of 10 mg ml^{-1} was added to it at a concentration of 0.5 µg ml^{-1} and mixed well without the formation of bubbles. After wiping the gel casting tray and comb with alcohol, the comb was placed properly in the casting apparatus. The prepared agarose was poured into the tray and left for solidification for 30-45 minutes. To make the well, the comb was pulled out and gel was placed in the buffer tank containing 1X TAE buffer with well side directed towards the cathode. Then 5 µl of the DNA mixed with 2 µl of gel loading dye was carefully loaded into the wells using a micro pipette. The ladder λDNA/*Eco*R1+ *Hind* III double digest (Sisco research laboratory; Biolit, Mumbai) was used as the molecular weight marker. The cathode and anode of the electrophoresis unit were connected to the power pack and the gel was run at constant voltage of 80 V. The power was turned off when the tracking dye reached to about 3 cm from the anode end.

3.5.3 Gel documentation

Gel documentation was done with BioRad Gel Documentation system using 'Quantity one^{R}' software. Quantity one^{R} is software package for imaging, analyzing and datasing the electrophoresed gels. The gel containing DNA was viewed under UV light due to ethidium bromide dye. The image of the gel was captured using the Quantity one^{R} controls in the imaging device window and band size was confirmed.

3.5.4 Assessing the quantity of DNA

The purity of DNA was further analyzed by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample reading, the instrument was set to zero by taking 1µl autoclaved water as blank. The absorbance of the nucleic acid sample was measured at a wavelength of 260 nm and 280 nm. 260/280 ratio was used to assess the purity of nucleic acids. A ratio of 1.8 to 2 indicated that the DNA preparation was pure and free from protein.

1 OD at 260nm = 50 µg DNA/ µl

Therefore, OD_{260} X 50 gives the quality of DNA in µg/ µl.

3.5.5 Metagenomic DNA sequencing

Metagenomic DNA of three samples were checked for quality and quantity and after obtaining values of quantity and quality of DNA as per as standard values described for metagenomic soil DNA sequencing, samples were sequenced at the NGS Facility, M/S. SciGenom Lab, Cochin.

3.6. 16S RNA gene amplicon library sequencing using Next Generation Illumina Miseq^{TM}

Amplicon library was prepared with specific primers spanning hypervariable region V3 region of 16S rRNA gene and used for sequencing and subsequent classification.

3.6.1. 16s rRNA gene library preparation

3.6.1.2 Amplicon PCR

The extracted soil metagenomic DNA was pooled and normalized to 5 ng/µl (purified DNA, 10 mM Tris pH-8.5) and amplicon PCR was earned out using V3 primers (341F 5'CCTACGGGAGGCAGCAG 3', 518**R** 5'ATTACCGCGGCTGCTGG 3') (Muyzer *et al.,* 1993). The PCR master mix consisted of 2 µl each 10 pmol/ul forward and reverse primers, 0.5 µl of 40mM dNTP, 5 µl of 5X Phusion HF reaction buffer, 0.2 µl of 2 U/ul F-540 Special Phusion HS DNA polymerase, 5 ng input DNA and water to make up the total volume to 25 µL. PCR reaction was programmed, initial denaturation of 98°C for 30 sec, 30 cycles of denaturation 98°C for 10 sec, annealing temperature of 55°C for 30 sec, primer extension of 72°C for 30 sec and a final extension at 72°C for 5 min followed by 4°C hold. The PCR product was quantified using the fluorescence quantitative (Qubit 2.0®) fluorometer with the Qubit dsDNA HS assay kit (Invitrozen, USA).

3.6.1.3 PCR clean-up

PCR clean up was carried out using AMPure XP beads to purify the 16S r RNA gene V3 amplicon away from free primers and primer dimers species. The reagents consisted of 10 mM Tris pH 8.5 (52.5 µl per sample), AMPure XP beads (20 µl per sample), freshly prepared ethanol (EtOH) (80%) (400 µl per sample). Standard protocol was followed and the cleaned up PCR product was stored at -20°C.

3.6.1.4 Index PCR

Illumina™ Truseq adapters and indices were added to the cleaned up PCR products. PCR master mix consisted of 2 µl each 10 pmol/ul forward and reverse primers, 1 µl of 40 mM dNTP, 10 µl of 5X Phusion HF reaction buffers, 0.4 µl of 2 U/ul F-540 special Phusion HS DNA polymerase, 10 µl (minimum 5 ng) of PCR I amplicon and water to make up the total volume to 50 µL.

PCR reaction was programmed as follows: initial denaturation of 98°C for 30 sec, 15 cycles or denaturation 98°C for 10 sec, annealing temperature of 55°C for 30 sec, primer extension of 72°C for 30 sec and final extension at 72°C for 5 minutes followed by 4°C hold.

3.6.1.5 PCR clean-up 2

AMPPure XP beads were used to clean up the final library before quantification. The reagents consist of 10 mM Tris pH 8.5 (27, 5 µl per sample). AMPure XP beads (56 µl per sample), freshly prepared 80 per cent ethanol (EtOH) (400 µl per sample). Standard protocol was followed and PCR product was stored at - 20°C.

3.7 Library, quantification, normalization and pooling

Library was quantified using a fluorometric quantification method and concentrated final library was diluted using distilled water. Diluted DNA (5 µl) from each library pooled with unique indices.

3.7.1 Library, denaturing and MiSeq sample loading

In preparation for cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq® sequencing. Each run included a minimum of PhiX (5%) to serve an internal control for these low diversity libraries. The denatured library was loaded into the reagent cartridge of Illumina Mi Seq™ sequencer for sequencing, the output files (Fastq) generated from sequencer was used for analysis.

3.7.2 Analysis of NGS data

Total raw sequencing reads obtained from sequencer were checked for quality parameters *viz.*, base quality parameters, base composition, distribution and GC distribution. After trimming the unwanted sequences from originally paired-end data, a consensus V3 region sequence was constructed using Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Then multiple filters

were applied *viz.*, conserved region filter, spacer filter and mismatch filter and the highest quality V3 region sequences were taken for various downstream analyses.

As a part of pre-processing of sequence reads, singletons that were likely due to the sequencing errors and could result in spurious operational taxonomic units (OTUs) were removed. This step was achieved by removing the reads that did not cluster with other sequences (abundances <2). Chimeras were also removed using the de-novo chimera removal method UCHIME implemented in the tool USEARCH.

Pre-processed reads from all samples were pooled and clustered into OTU-based on their sequence similarity using Uclust program (similarity cutoff = 0.97). QIIME (Caporaso *et at.*, 2010) used for downstream analysis.

Representative sequences were identified for each OTU and aligned against Greengenes core set of sequences using PyNAST program (DeSantis *et al.*, 2006). Further these representative sequences were aligned against reference chimeric datasets. Then taxonomic classification was performed using RDP classifier and Greengenes OTU database and the sequence data were uploaded to MG-RAST (http://metagenomics.anl.gov/) (Meyer *et al.*, 2008) server to obtain the quantitative insights into microbial population.

The Illumina sequencing data have been submitted to Sequence Read Archive (SRA) of GenBank database as a file under accession number.

3.7.3 Statistical analysis

Analysis of variance was done on the data collected using the statistical package WASP 2.0. Multiple comparisons among the treatment means done using DMRT.

RESULTS

4. Results

The results of the investigation entitled "Metagenomics to assess bacterial diversity in the soil as influenced by organic and chemical inputs" carried out during the period of 2014-2016 at the Department of Agricultural Microbiology and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, are presented below.

4.1. PHYSICO-CHEMICAL PROPERTIES OF RHIZOPHERE SOIL COLLECTED FROM RARS, PATTAMBI

The rhizosphere soil samples collected were analyzed for various physico-chemical properties. The data on bulk density (mg m^{-3}), electrical conductivity (dS m^{-1}), pH, total nitrogen (%), available potassium (kg ha^{-1}), phosphorous (kg ha^{-1}), calcium (mg kg^{-1}), manganese (mg kg^{-1}), sulphur (mg kg^{-1}), iron (mg kg^{-1}), magnesium (mg kg^{-1}), copper (mg kg^{-1}), zinc (mg kg^{-1}) and boron (mg kg^{-1}) are given in Tables 3.

4.1.1 Bulk density

Bulk density was found to be highest in INF (1.28 mg m^{-3}) followed by INM (1.26 mg m^{-3}) and ONM (1.2 mg m^{-3}).

4.1.2 pH and electrical conductivity

All the three soil samples included in the study were acidic in nature with pH ranging from 4.6 to 4.7. Electrical conductivity was in the normal range for all the samples and highest electrical value of 0.15 dS m^{-1} was recorded by INM. This was followed by ONM (0.10 dS m^{-1}) and INF (0.10 dS m^{-1}).

Table 3. Physico-chemical properties of the soil samples

Parameters	ONM		INM		INF	
	Quantity	**Remarks**	**Quantity**	**Remarks**	**Quantity**	**Remarks**
pH	4.7	Very Strongly Acidic	4.6	Very Strongly Acidic	4.7	Very Strongly Acidic
Bulk density (mg m^{-3})	1.20	Normal	1.26	Normal	1.28	Normal
Electrical Conductivity (dS m^{-1})	0.10	Normal	0.15	Normal	0.10	Normal
Macronutrients						
Total Nitrogen (%)	0.19	Normal	0.18	Normal	0.16	Normal
Available Phosphorus (kg ha^{-1})	68.76	High	74.43	High	48.53	High
Available Potassium (kg ha^{-1})	34.72	Low	35.84	Low	47.04	Low
Available Calcium (mg kg^{-1})	466.25	Sufficient	277.75	Deficient	342.25	Sufficient
Available Magnesium (mg kg^{-1})	79.75	Deficient	73.00	Deficient	79.50	Deficient
Available Sulphur (mg kg^{-1})	13.54	Sufficient	11.20	Sufficient	6.77	Sufficient
Micronutrients (mg kg^{-1})						
Available Copper	10.52	Sufficient	9.12	Sufficient	8.50	Sufficient
Available Iron	214.80	Sufficient	196.30	Sufficient	315.40	Sufficient
Available Zinc	7.05	Sufficient	5.56	Sufficient	4.79	Sufficient
Available Manganese	14.42	Sufficient	8.47	Sufficient	10.73	Sufficient
Available Boron	0.42	Deficient	0.35	Deficient	0.23	Deficient

4.1.3 Total nitrogen

Total nitrogen content was in the normal range for all the samples. The maximum value of 0.19 % was recorded in ONM, followed by INM (0.18 %) and INF (0.16 %).

4.1.4. Available phosphorous

Available phosphorus was high in all the three samples tested. Maximum P was observed in INM (74.43kg ha^{-1}) followed by ONM (68.76 kg ha^{-1}) and INF (48.53 kg ha^{-1}).

4.1.5. Available potassium

In case of available potassium, the levels were low in all the three samples. However, maximum value was recorded in INF (47.04 kg ha^{-1}) and the lowest in ONM (35.72 kg ha^{-1}).

4.1.6. Available calcium

Calcium content was sufficient in ONM (466.25 mg kg^{-1}) and INF (342.25 mg kg^{-1}), but deficient (277.75 mg kg^{-1}) in INM .

4.1.7. Available magnesium

All the three samples analysed were deficient with respect to magnesium content and the values ranged from 79.75 mg kg^{-1} (ONM) to 73.00 mg kg^{-1} (INM).

4.1.8. Available sulphur

The available sulphur content ranged from 13.54 mg kg^{-1} (ONM) to 6.77 mg kg^{-1} (INF). These values corresponded to sufficient levels.

4.1.9. Available copper

All the three samples were sufficient with respect to Cu levels. ONM recorded highest level (10.52 mg kg^{-1}) and INF, the lowest level (8.5 mg kg^{-1}).

4.1.10. Available iron

The results of available iron showed highest in INF (315.40 mg kg^{-1}) followed by ONM (214.80 mg kg^{-1}) and INM (196.30 mg kg^{-1}).

4.1.11. Available zinc

The available zinc ranged from 7.05 to 4.79 mg kg^{-1}. The lowest available zinc was recorded in INF (4.79 mg kg^{-1}) and highest in ONM sample (7.05 mg kg^{-1}).

4.1.12. Available manganese

In case of manganese ONM recorded highest (14.42 mg kg^{-1}) followed by INF (10.73 mg kg^{-1}) and INM (8.47 mg kg^{-1}).

4.1.13. Available boron

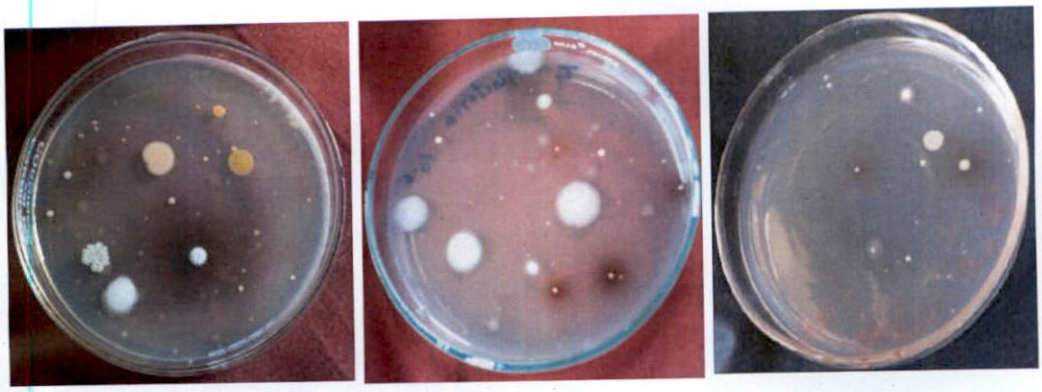
The lowest value of available boron of 0.23 mg kg^{-1} recorded in INF and highest in ONM (0.42 mg kg^{-1}).

4.2. ENUMERATION OF RHIZOSPHERE MICROFLORA FROM SOIL

The population of bacteria, fungi, actinomycetes, nitrogen fixers, phosphate solubilizers, fluorescent pseudomonads and *Trichoderma* was estimated in rhizosphere soils using different media and dilutions. Population of microorganisms in three soil samples are represented in Table 4 and Plates 2-3.

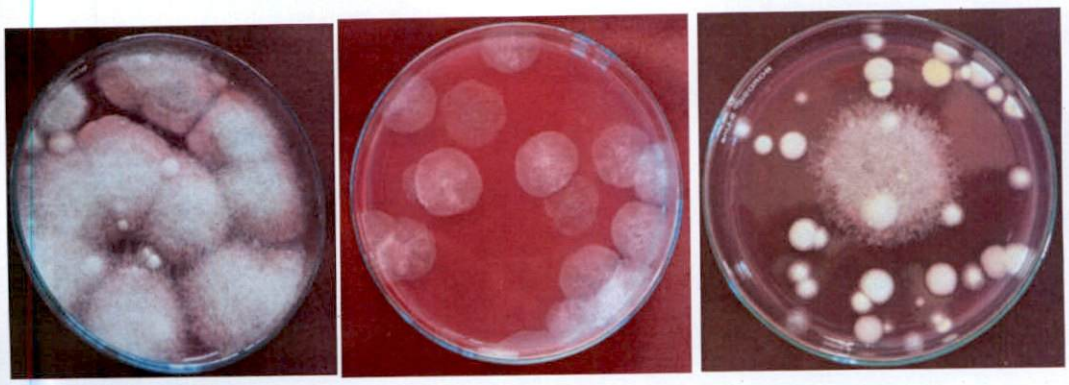
The results of bacterial population were not significantly different among the treatments, However, rhizosphere sample INM harboured more number of bacteria ($33.22x10^6$ cfu g^{-1}) compared to ONM sample ($29.13 x10^6$cfu g^{-1}) and the least was recorded in INF sample ($13.00x10^6$cfu g^{-1}).

Among the different samples, fungal population was significant and was highest in INF ($93x10^2$ cfu g^{-1}) followed by INM (54x102 cfu g^{-1}) and ONM ($38.50x10^2$ cfu g^{-1}). Highest population of actinomycetes ($57.50x10^4$ cfu g^{-1}) was recorded in INM followed by ONM (50.85x102 cfu g^{-1}) and least population of



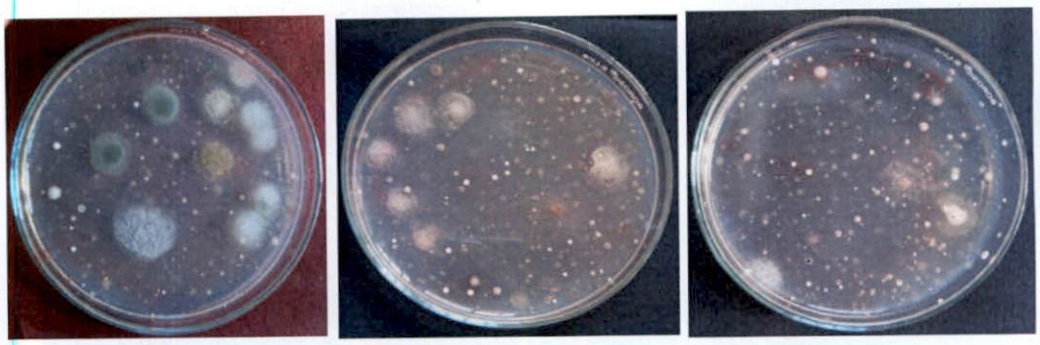
ONM INM INF

A. Bacteria on Nutrient agar



ONM INM INF

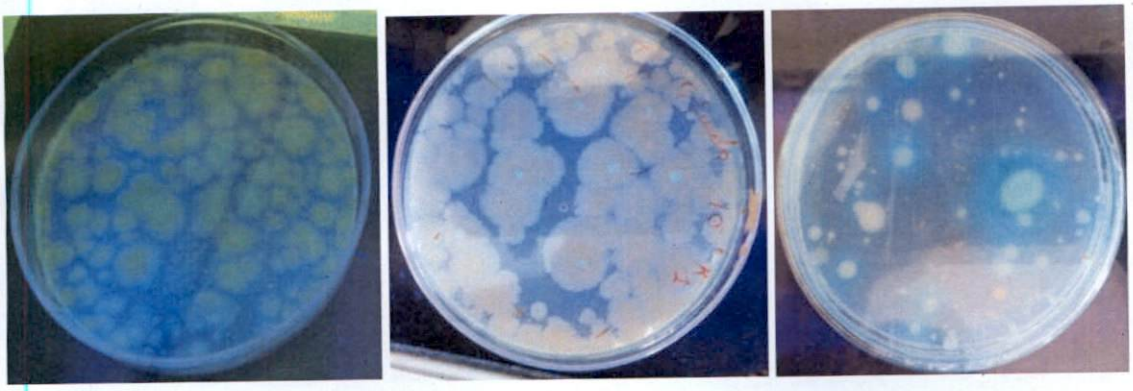
B. Fungi on Martin's Rose Bengal agar



ONM INM INF

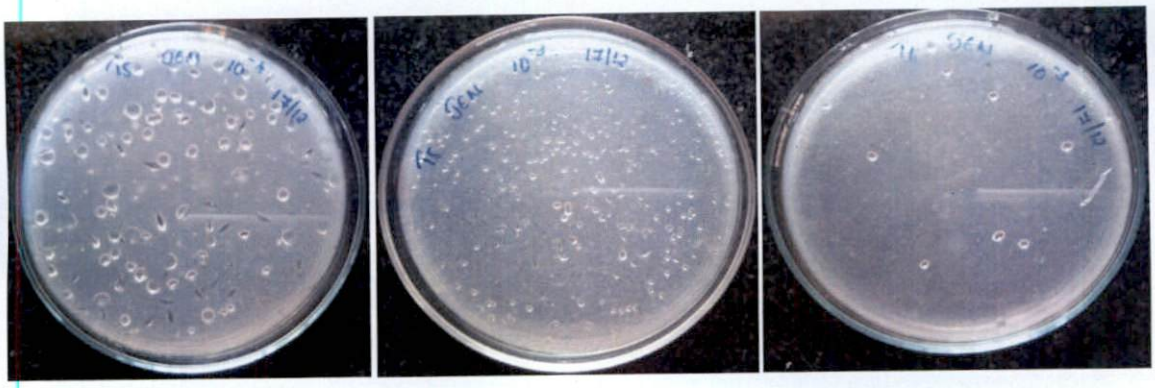
C. Actinomyetes on Kenknights's agar

PLATE 2. Population and colony morphology of microflora



ONM INM INF

D. Fluorescent pseudomonads on King's B agar



ONM INM INF

E. Nitrogen fixer's on Jensen's agar

PLATE 3. Population and colony morphology of microflora

Sample	Population of microorganisms* (cfu per g of soil)				
	Bacteria x 10^6	Fungi x 10^2	Actinomycetes x 10^4	Fluorescent Pseudomonads x 10^5	Nitrogen fixers x 10^3
ONM	29.13 (1.46)	38.50 (1.58)^c	50.85 (1.70)	36.00 (1.55)	22.25 (1.34)
INM	33.22 (1.52)	54.00 (1.73)^b	57.50 (1.75)	10.00 (0.98)	9.25 (0.91)
INF	13.00 (1.10)	93.00 (1.96)^a	23.00 (1.35)	05.00 (0.60)	7.00 (0.82)
C.D (0.05)	NS	0.07	NS	NS	NS

Figures in parenthesis indicate log transformed values

NS- Non-significant

*Each value is an average of three replications

Table 4. Population of rice rhizosphere microflora under different nutrient management systems

actinomycetes was noticed in INF ($23x10^2$ cfu g^{-1}) and found non-significant. No phosphate solubilizers could be obtained from any of the three soil samples at 10^{-3} dilution.

Population of fluorescent pseudomonads was non-significant but found to be highest ($36x10^5$ cfu g^{-1}) in ONM and lowest in INF ($5x10^5$ cfu g^{-1}). Nitrogen fixers were found to be non-significant however, sample ONM recorded maximum population ($22.25 x10^3$cfu g^{-1}) of nitrogen fixers and this was followed by INM ($9.25x10^4$cfu g^{-1}) and INF ($7x10^4$cfu g^{-1}). No *Trichoderma* could be obtained in any of the three soil samples. Details of morphotype of microflora from three soil samples are given in Tables 5-7 and Plate 4-5.

4.3. Organic carbon

The organic carbon status of the soils varied from 1.69 to 2.41 per cent and the status was rated as high in all the samples (Table 8). Soil sample INF recorded lowest (1.69%) and highest was found in ONM (2.41%) and intermediate in INM (1.90%).

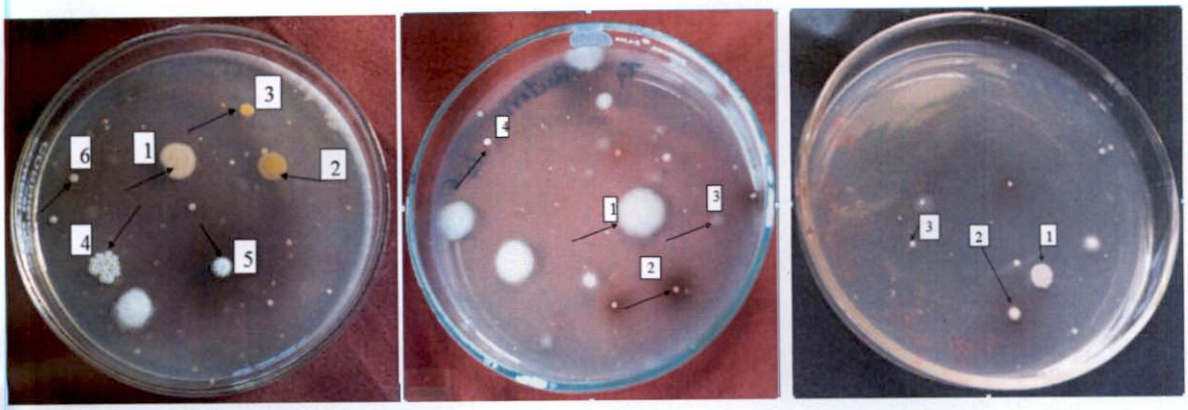
4.4. Microbial biomass carbon

Biomass carbon was found to be normal in all the samples (Table 8) and was highest INM (266 µg C g^{-1}) followed by in ONM (244 µg C g^{-1}) and lowest in INF (177 µg C g^{-1}).

4.5. Checking the quality and quantity of metagenomic DNA

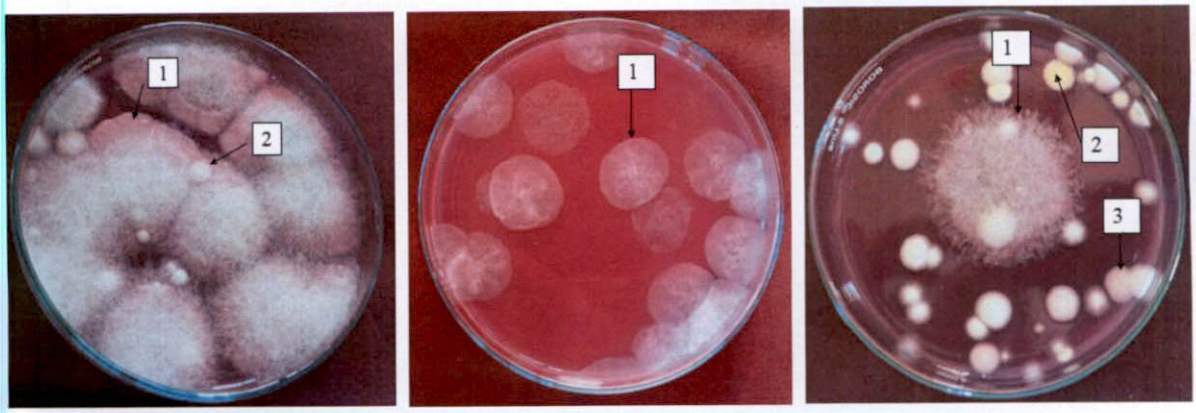
The quality of metagenomic DNA extracted from ONM, INM and INF soil samples was ensured by electrophoresis on 0.8 per cent agarose gel. A single intact band of DNA with high intensity withoutshearing was obtained by direct lysis method (Plate 6), whereas DNA obtained through soft lysis showed the absence of intact band (data not shown).

The quantity of DNA from three samples (isolated by direct lysis method) was estimated by spectrophotometry using NanoDrop. The ratio of



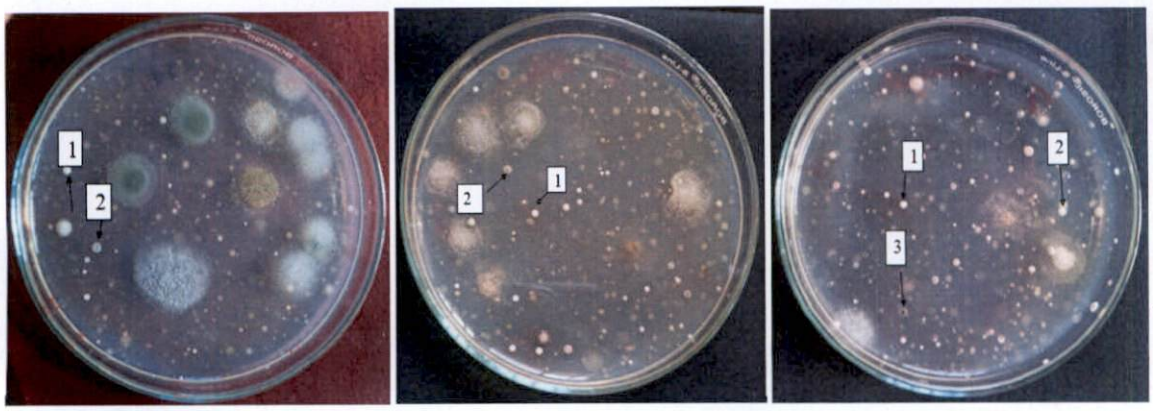
ONM INM INF

A. Bacteria on Nutrient agar



ONM INM INF

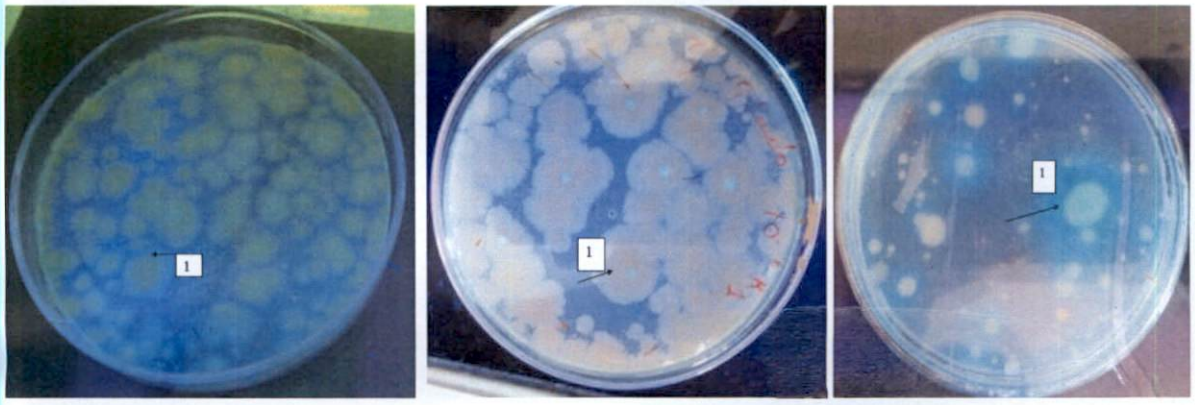
B. **Fungi on Martin's Rose Bengal agar**



ONM INM INF

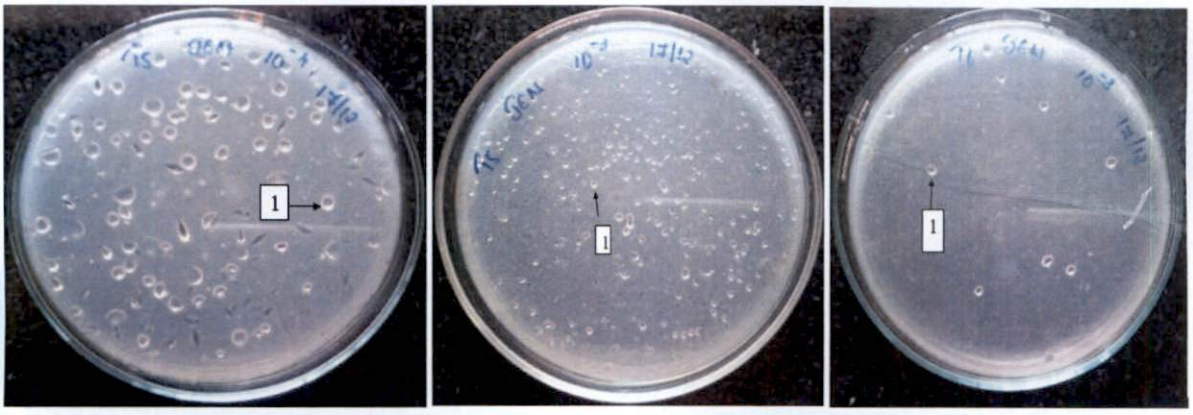
C. Actinomyetes on Kenknights's agar

PlATE 4. Colony morphology of microflora



ORG INT CHE

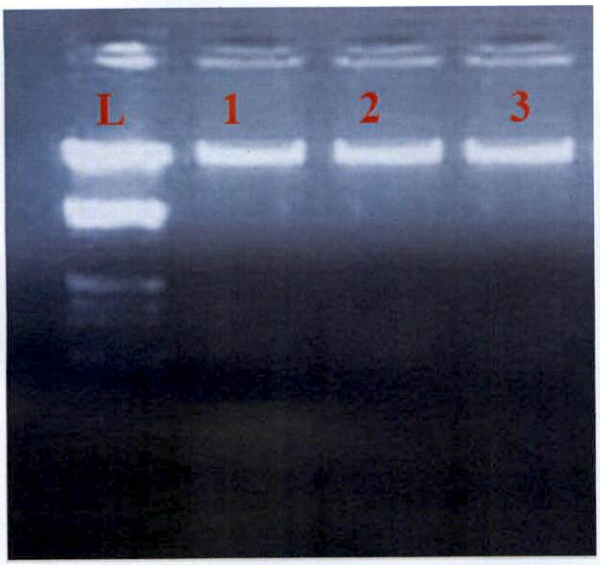
D. Fluorescent pseudomonads on King's B agar



ORG INT CHE

E. Nitrogen fixer's on Jensen's agar

PLATE 5. Colony morphology of microflora



L- Marker (λ DNA/*Eco*R1+*Hind*III)

1- ONM
2- INM
3- INF

PLATE 6. Metagenomic DNA extracted from three samples on 0.8% agarose gel

Table 5. Abundance of morphotypes of microorganisms from sample ONM

Microrganism	Media used	Morphotypes	Characteristics					
			Size	Margin	Elevation	Colour	Form	Surface
Bacteria	Nutrient agar	Morphotype I	Large	Entire	Flat	Cream	Circular	Smooth
		Morphotype II	Large	Entire	Flat	Light yellow	Circular	Smooth
		Morphotype III	Medium	Entire	Raised	Circular yellow	Circular	Smooth
		Morphotype IV	Medium	Filamentous	Raised	Cream	Irregular	Smooth
		Morphotype V	Medium	Undulate	Raised	White	Circular	Rough
		Morphotype VI	Small	Entire	Flat	Grey	Circular	Rough
Fungi	Martin's Rose Bengal agar	Morphotype I	Large	Filiform	Raised	White	Filamentous	Rough
		Morphotype II	Small	Entire	Raised	White	Circular	Smooth
Actinomycetes	Kenknight and Munaier's agar	Morphotype I	Medium	Entire	Umbonate	White	Circular	Powdery
		Morphotype II	Medium	Entire	Flat	Grey	Circular	Powdery
Fluorescent pseudomonads	King's B	Morphotype I	Large	Undulate	Flat	Green	Irregular	Smooth
Nitrogen Fixer	Jensen's agar	Morphotype I	Medium	Entire	Raised	White	Circular	watery

Table 6. Abundance of morphotypes of microorganisms from sample INM

Microrganism	Media used	Morphotypes	Characteristics					
			Size	Margin	Elevation	Colour	Form	Surface
Bacteria	Nutrient agar	Morphotype I	Large	Entire	Raised	White	Circular	Smooth
		Morphotype II	Small	Entire	Flat	Yellowish -cream	Circular	Smooth
		Morphotype III	Medium	Entire	Flat	Off- white	Circular	Watery
		Morphotype IV	Small	Entire	Flat	White	Circular	Smooth
Fungi	Martin's Rose Bengal agar	Morphotype I	Medium	Entire	Flat	White	Circular	Rough
Actinomycetes	Kenknight and Munaier's agar	Morphotype I	Medium	Entire	Umbonate	White	Circular	Powdery
		Morphotype II	Medium	Entire	Flat	Grey	Circular	Powdery
Fluorescent pseudomonads	King's B	Morphotype I	Large	Lobate	Flat	Green	Circular	Smooth
Nitrogen Fixer	Jensen's agar	Morphotype I	Small	Entire	Raised	White	Circular	watery

Table 7. Abundance of morphotypes of microorganisms from sample INF

Microrganism	Media used	Morphotypes	Characteristics					
			Size	Margin	Elevation	Colour	Form	Surface
Bacteria	Nutrient agar	Morphotype I	Large	Undulated	Flat	White	Circular	Rough
		Morphotype II	Medium	Entire	Raised	Creamy-white	Circular	Smooth
		Morphotype III	Small	Entire	Flat	White	Circular	Smooth
Fungi	Martin's Rose Bengal agar	Morphotype I	Large	Filiform	Raised	White	Filamentous	Rough
		Morphotype II	Medium	Entire	Flat	Light-yellow	Circular	Smooth
		Morphotype III	Medium	Entire	Flat	White	Circular	Smooth
Actinomycetes	Kenknight and Munaier's agar	Morphotype I	Medium	Entire	Flat	White	Circular	Powdery
		Morphotype II	Medium	Entire	Umbonate	Grey	Circular	Powdery
		Morphotype III	Medium	Entire	Flat	Black with white margin	Circular	Powdery
Fluorescent pseudomonads	King's B	Morphotype I	Large	Entire	Flat	Bluish-green	Circular	Smooth
Nitrogen Fixer	Jensen's agar	Morphotype I	Medium	Entire	Raised	White	Circular	watery

Table 8. Biological properties of soil

Sample	Organic carbon (%)	Remarks	Biomass carbon ($\mu g\ C\ g^{-1}$)	Remarks
ONM	2.41	High	244	Low
INM	1.90	High	266	Low
INF	1.69	High	177	Low

absorbance at 260/280 nm and concentration of DNA present in the samples are given in Table 9. Sample ONM recorded maximum yield of 80.4ng/µl.

The DNA samples were then used for Next Generation Sequencing (NGS) using Illumina MiSeq^{TM}sequencer at SciGenom, Cochin.

Table 9. Quality and quantity of DNA in samples

Sample name	OD 260/280	Concentration (ng/µl)
ONM	1.54	80.40
INM	1.60	36.01
INF	1.75	65.11

4.6. Illumina sequencing data

The raw and paired end sequences were obtained from SciGenom.

4.6.1 Raw fastaq sequences

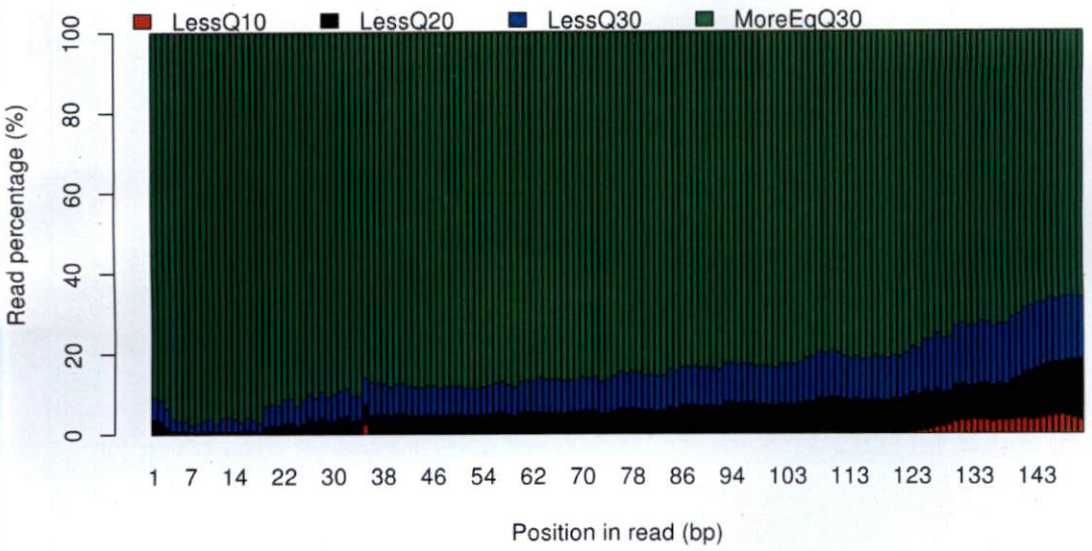
Total raw sequencing reads (paired-end) of ONM, INM and INF DNA samples were 3,74,632 , 3,35,666 and 3,81,295 respectively with average sequence length of 150 bp each was obtained from Illumina MiSeq^{TM} sequencer.

4.6.2. Sequence quality checking

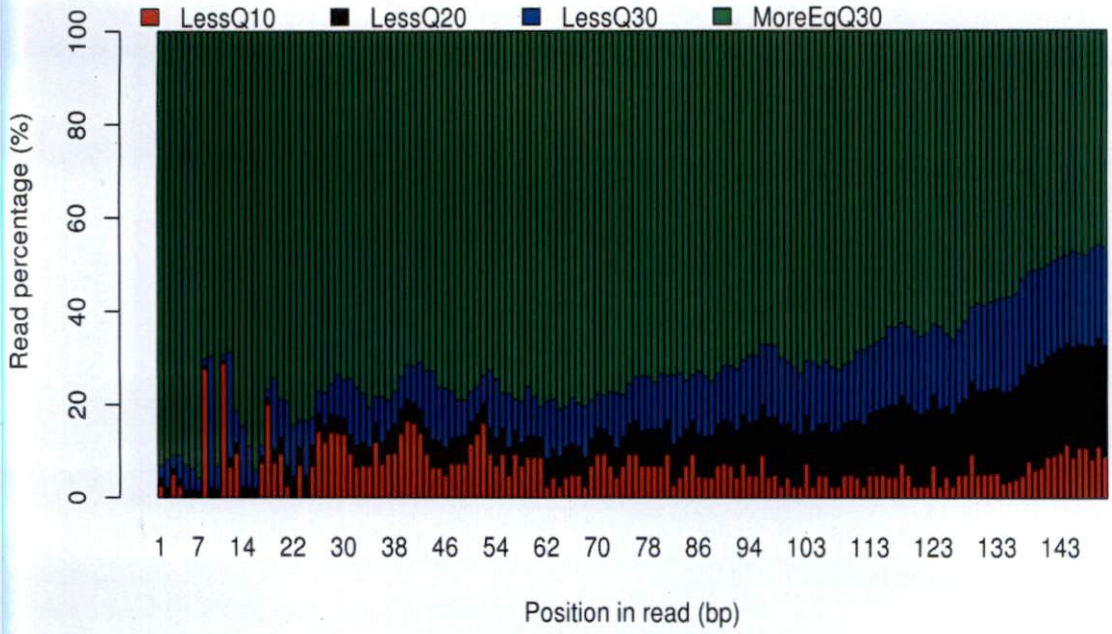
Quality parameters such as base quality score distribution, base composition distribution and GC distribution for sequencing reads were checked.

4.6.2.1 Base quality score distribution

The Phred score distribution of the paired-end reads samples is provided in Table 10. Base quality of each cycle for all samples is shown in Plates 7-9. The X-axis represents sequencing cycle and Y-axis represents the per cent total reads. The quality of left and right end of the paired-end read sequences of the sample

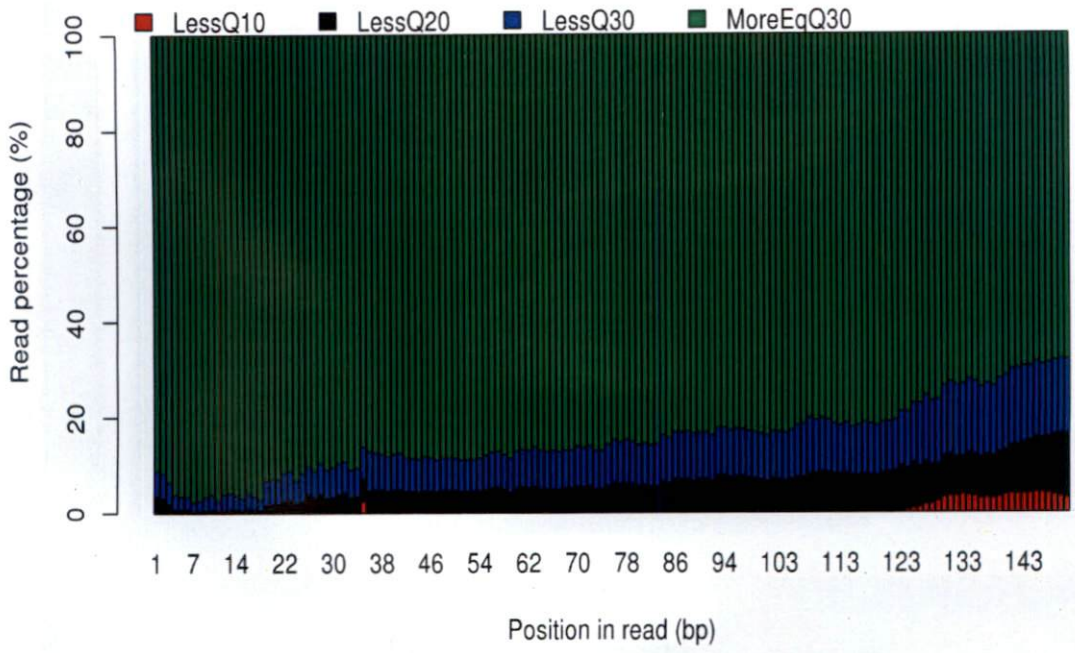


A. Base quality distribution of Read 1 (R1)

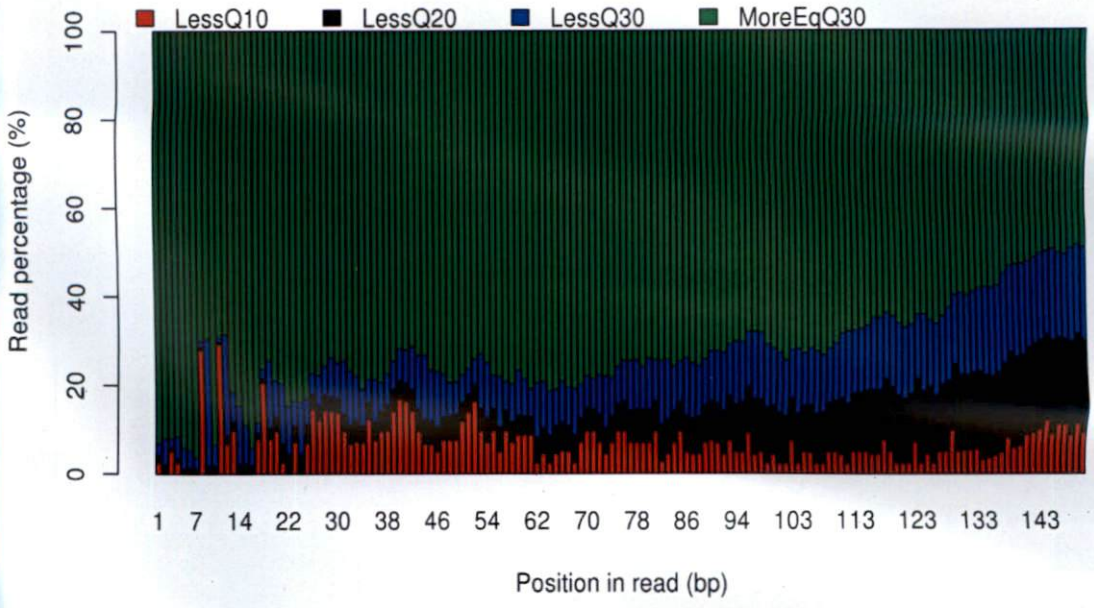


B. Base quality distribution of Read 2 (R2)

PLATE 7. Base quality distribution of sample ONM

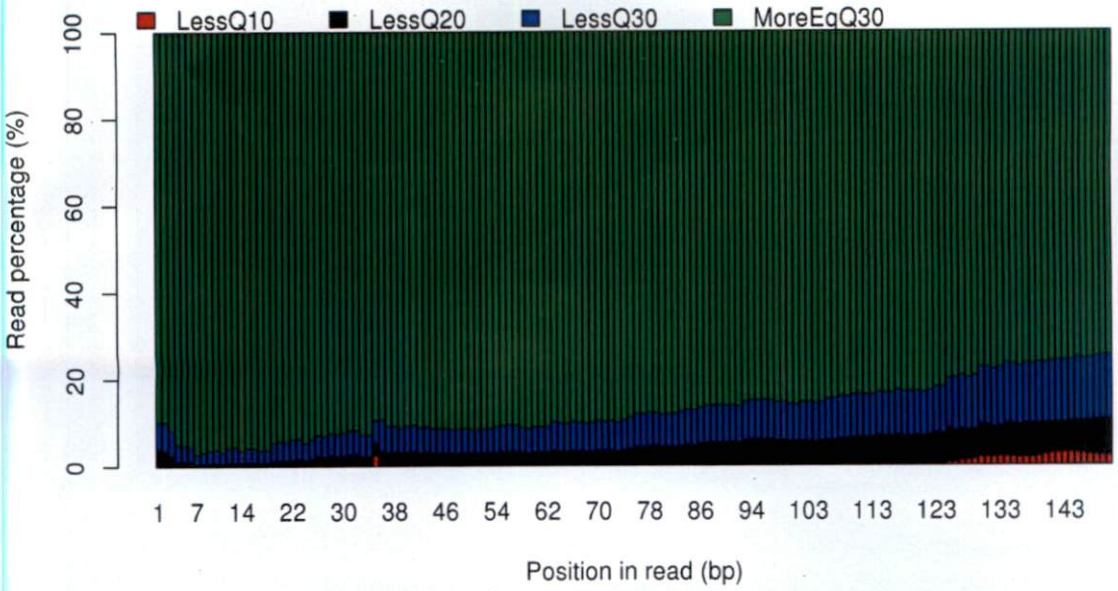


A. Base quality distribution of Read 1 (R1)

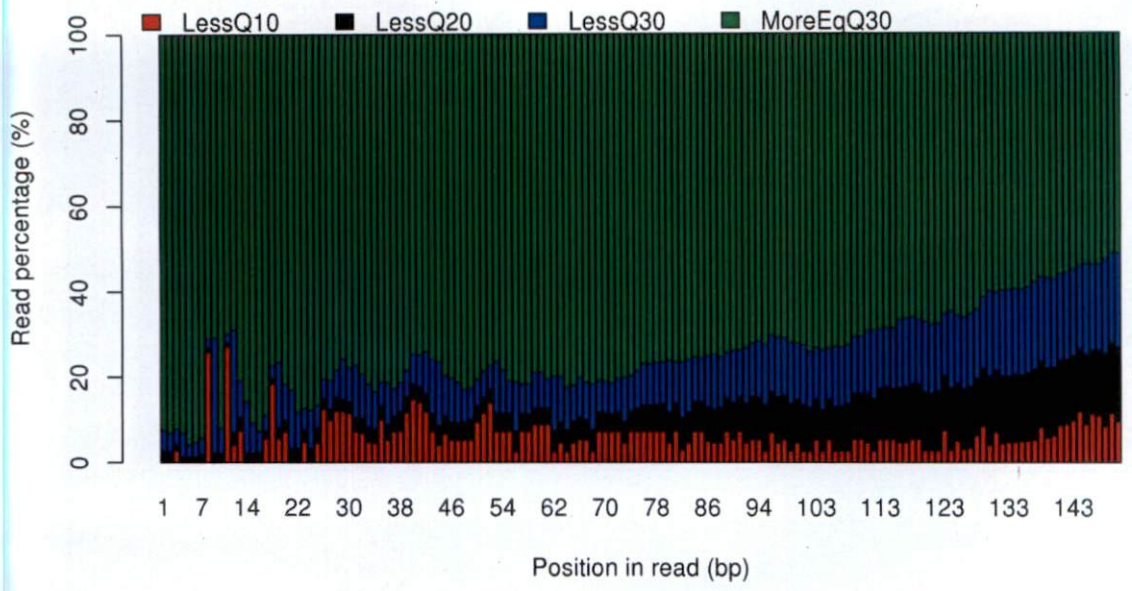


B. Base quality distribution of Read 2 (R2)

PLATE 8. Base quality distribution of sample INM



A. Base quality distribution of INF (R1)



B. Base quality distribution of INF (R2)

PLATE 9. Base quality distribution of sample INF

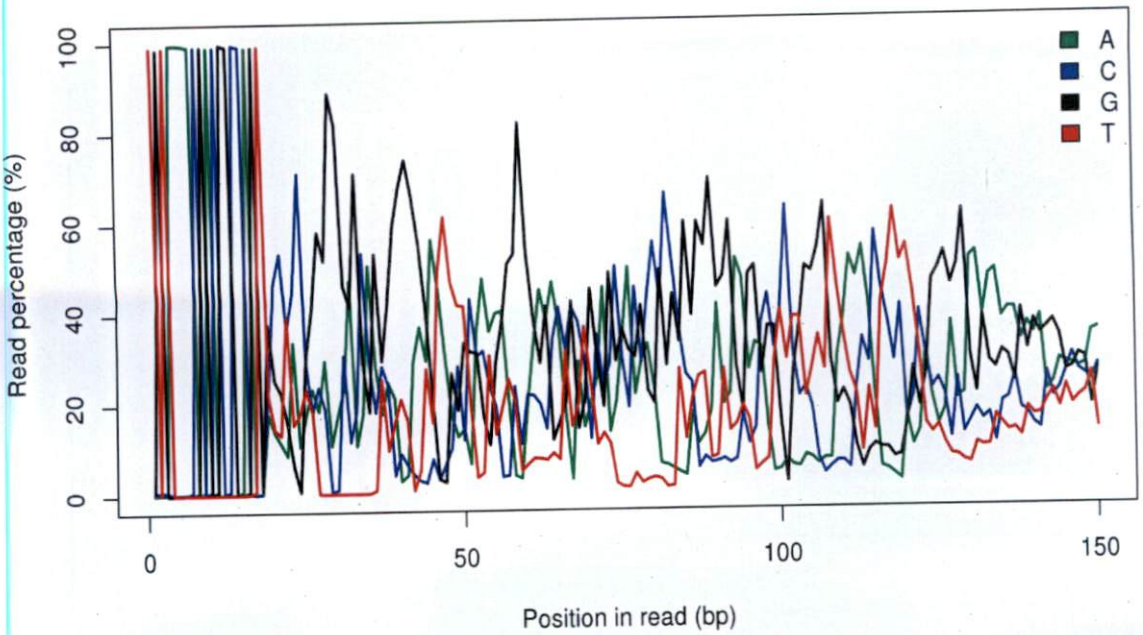
clearly indicates that nearly 90 per cent of the total reads had Phred score greater than 30 (>Q30; error-probability >=. 0.001).

Table 10. Phred score distribution of the paired-end reads for the samples

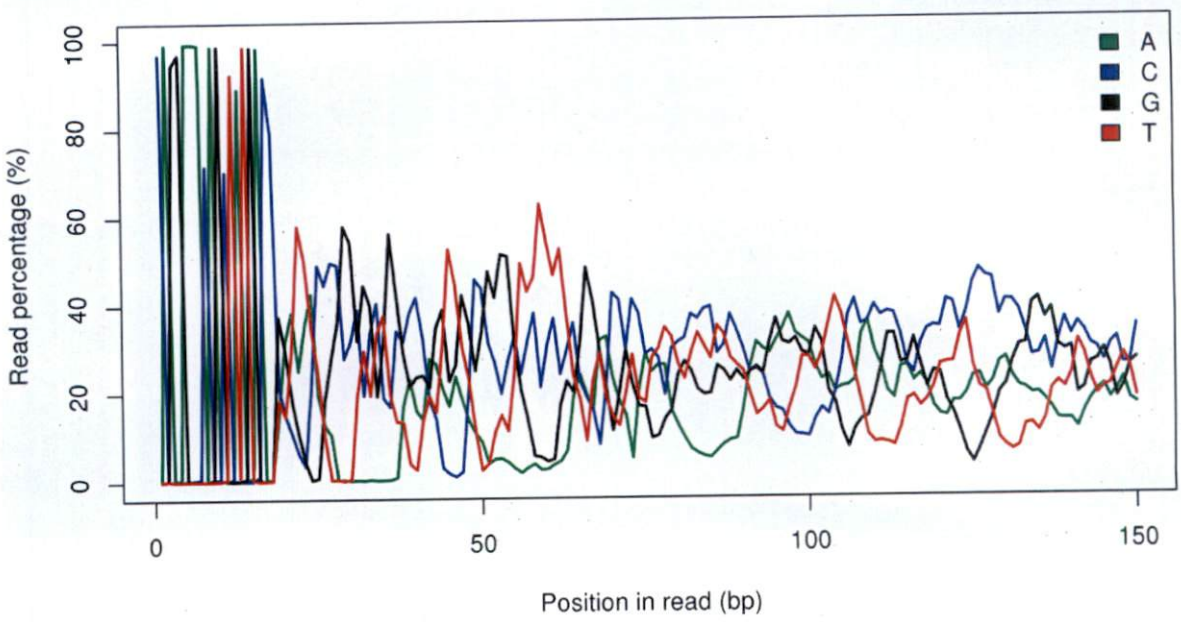
Sample	Read Phred quality score distribution (%)			
	Q0-Q10	Q10-Q20	Q20-Q30	>= Q30
ONM	3.72	7.68	10.50	78.10
INM	3.75	7.27	10.39	78.59
INF	3.23	6.01	9.91	80.85

4.6.2.2. Base composition distribution

The base composition distribution of nucleotides in the sequence read for each sample was graphically represented (Plates 10-12). The X-axis represents sequencing cycle and the Y-axis represents nucleotide per cent. The base composition of left and right end of the paired-end read sequences was calculated. Since the target sequence was that of V3 region, sequence composition bias was observed in the sample. Overall base composition of the samples is provided in Table 11.

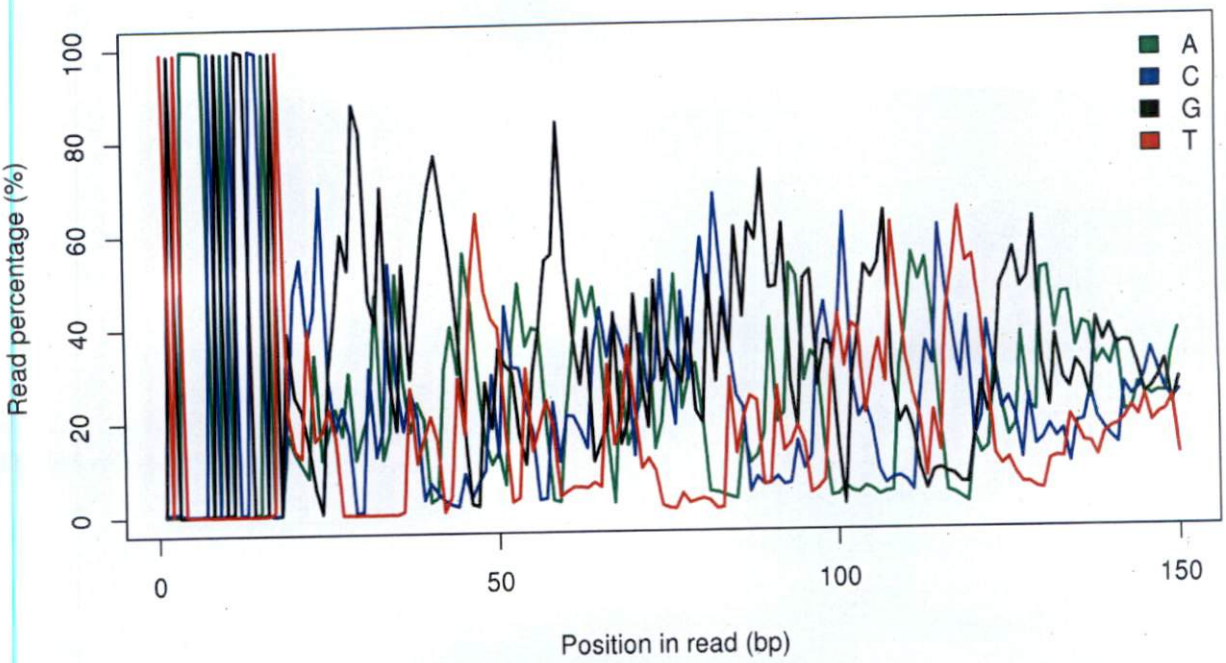


A. Base composition distribution of sample ONM (R1)

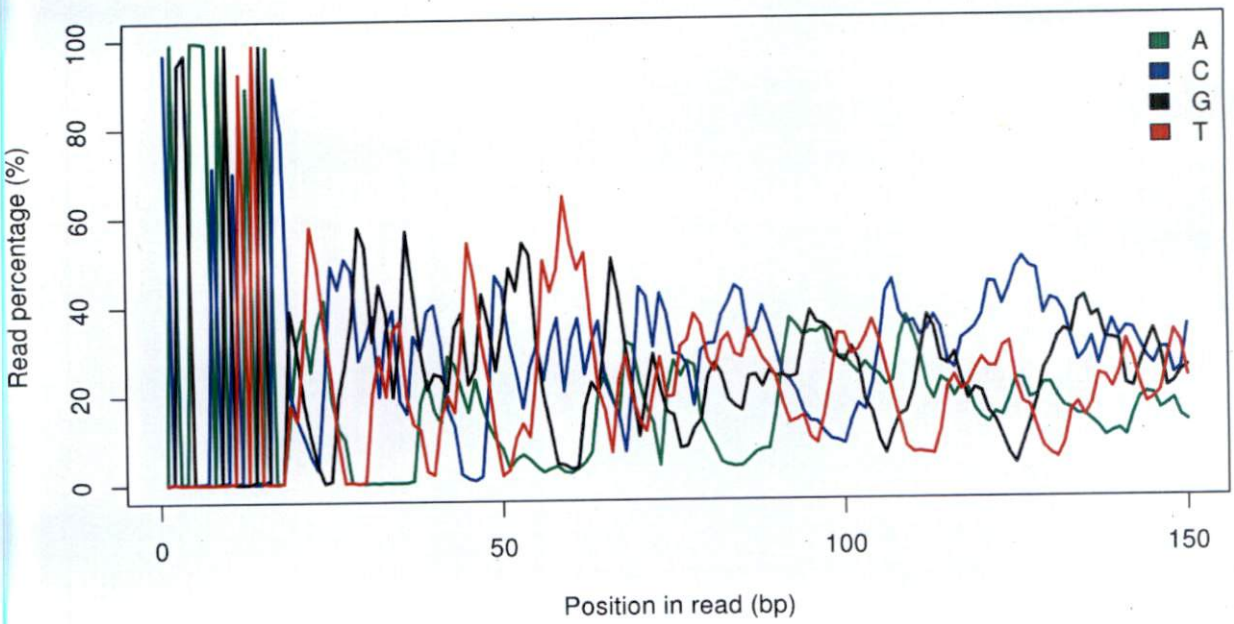


B. Base composition distribution of sample Read 2 (R2)

PLATE 10. Base composition distribution of sample ONM

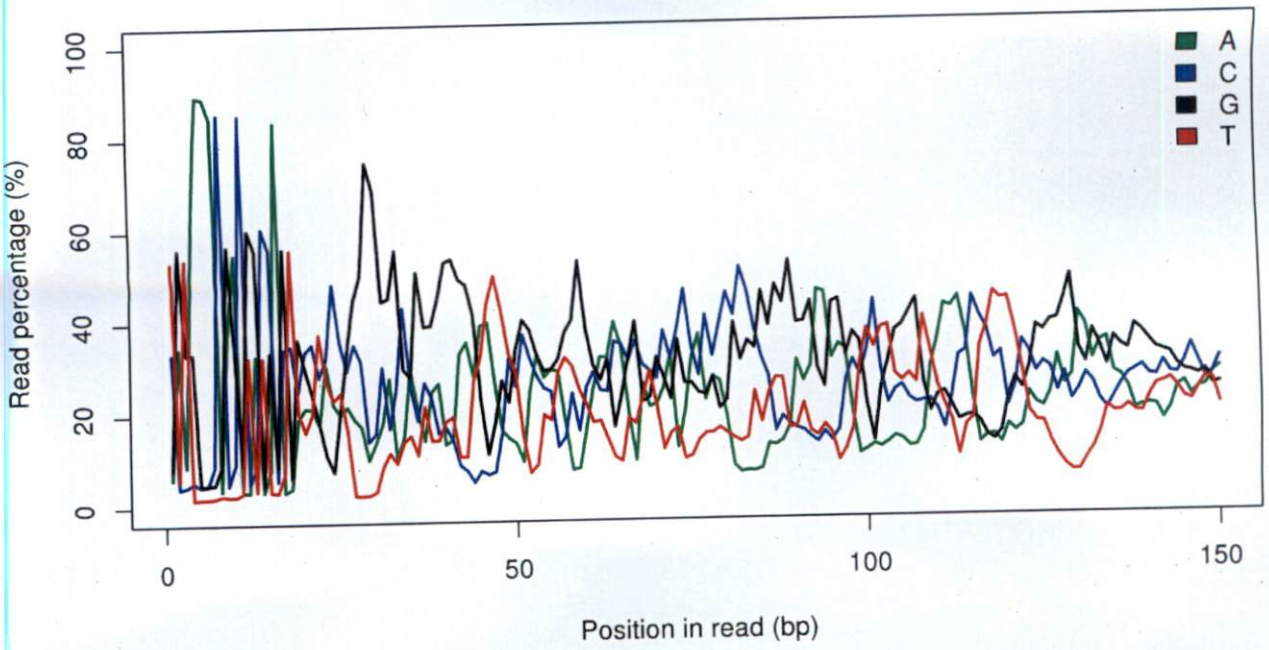


A. Base composition distribution of Read 1 (R1)

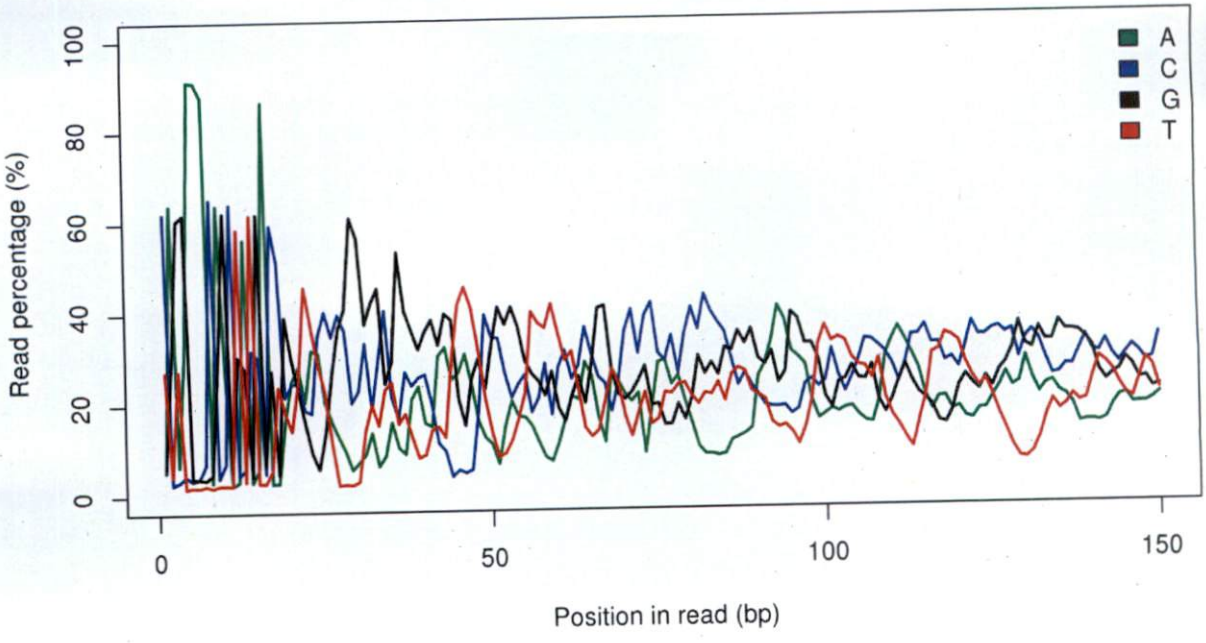


B. Base composition distribution of Read 2 (R2)

PLATE 11. Base composition distribution of sample INM



A. Base composition distribution of Read 1 (R1)



B. Base composition distribution of Read 2 (R2)

PLATE 12. Base composition distribution of sample INF

Table 11. Base composition of the samples

Sample Name	Base Composition (%)			
	A	C	G	T
ONM	22.61	25.78	28.93	19.48
INM	22.17	25.99	29.19	19.41
INF	22.15	26.35	29.13	19.59

4.6.2.3 GC distribution

The average distribution of GC content for the samples ranged from 55.18 to 54.71 per cent. Per cent of GC estimated were 55.48 per cent in INF DNA sample, 55.18 per cent in INM DNA sample and 54.71 per cent in ONM DNA sample.

4.7 Identification of V3 region from paired-end reads

Sequences of the samples after checking for quality parameters were subjected to identification of V3 region from paired-end reads. Paired-end reads were processed and multiple filters were applied to remove the conserved region, spacer region, mismatch sequences these regions were trimmed and with the good paired-end reads consensus V3 region was constructed using ClustalO program and results are given in Table 12. While making consensus V3 sequence, the passed reads were aligned to each other with 0 mismatches with an average contig length of ~130 to ~160bp.

Table 12. Summary of reads that passed each filter

Sample Name	Total Reads	Passed Conserved Region Filter	Passed Spacer	Passed Read Quality Filter	Passed Mismatch Filter
ONM	374,632	227,938	227,928	227,868	113,229
INM	335,666	213,772	213,762	213,728	108,609
INF	381,295	172,006	171,224	171,198	81,963

4.8. Pre-processing of reads and selection of OTUs

To obtain consensus reads, chimeras were removed using the UCHIME algorithm. A table of filtered consensus reads based on individual sample is given in Table 13. Pre-processed reads from all samples were pooled and clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity using Uclust program (similarity cutoff = 0.97). A total of 32,207 OTUs were identified from 297,859 reads and the graphical representation of reads and OTUs proportion is given in Plate 13. The blue bar represents the percentage of total OTUs in the read-count groups. The red bar represents the percentage of total read contributed by the OTUs in the read-count group. From 32,207 OTUs identified 19,347 singletons were removed and 12,860 OTUs were selected for further analysis.

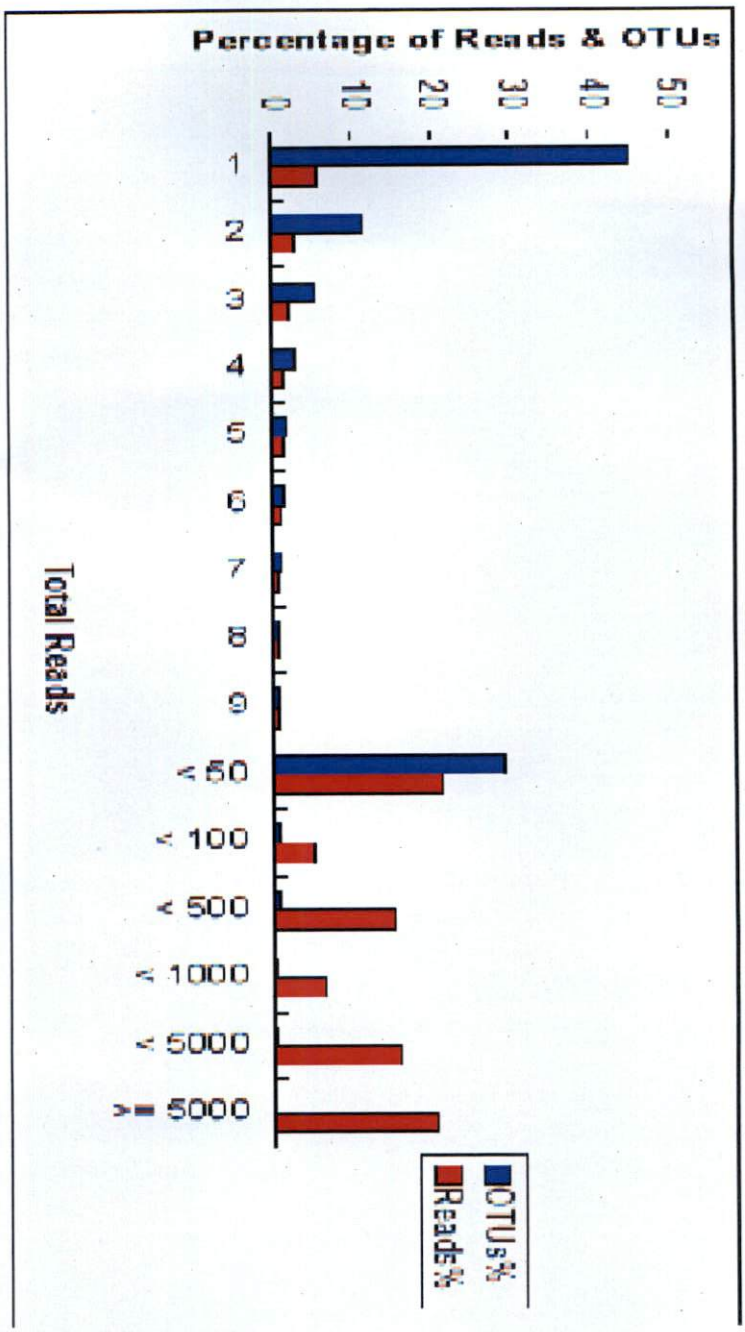


PLATE 13: The percentage of total OTUs and percentage of total read contributed by OTUs.

Table 13. Pre-processing reads statistics

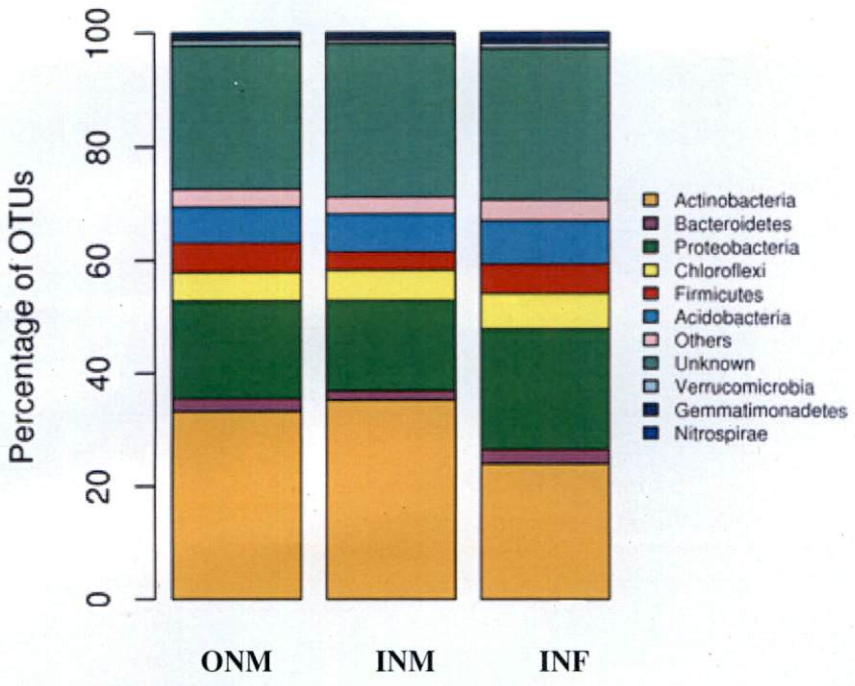
Sample Name	Consensus Reads	Chimeric Sequences	Pre-processed Reads
ONM	113,229	1,802 (1.59%)	111,427 (98.41 %)
INM	108,609	1,917 (1.77%)	106,692 (98.23 %)
INF	81,963	2,223 (2.71%)	79,740 (97.29 %)

4.9 Taxonomic classification and relative abundance

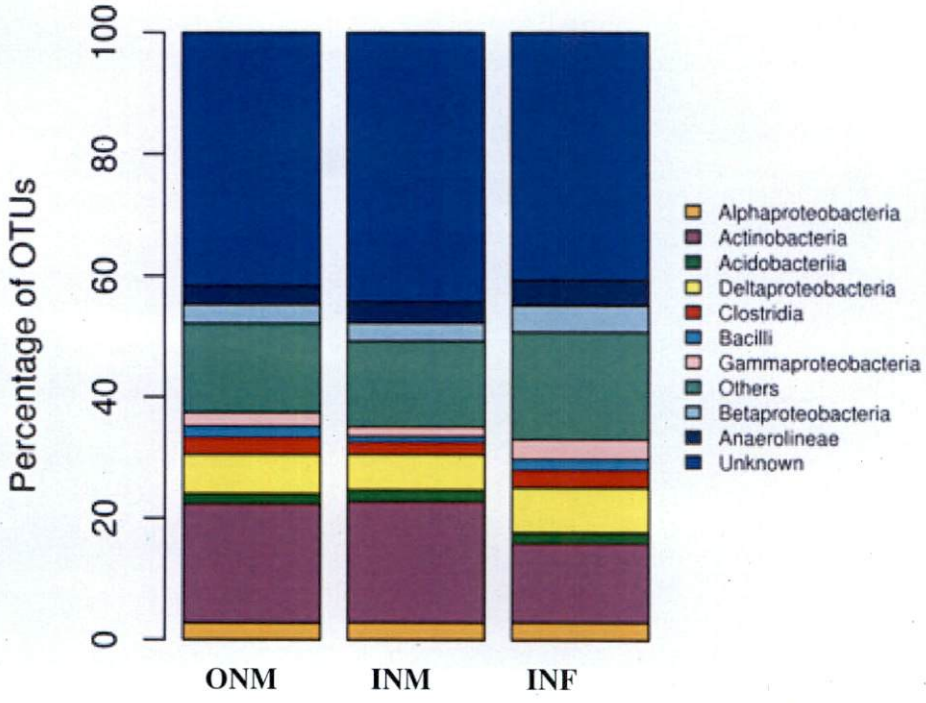
QIIME program was used for the microbial community analysis. The representative sequence was identified for each OTU and aligned against Greengenes core set of sequences using PyNAST program. Further sequence were aligned the representative sequences against reference chimeric data sets. Then, taxonomy classification was performed using RDP classifier against Greengenes 16S rRNA genes database. The phylum, class, order, family, genus and species distribution for each sample based on OTU and reads are shown in Plates 14-16. The sequences do not have any alignment against taxonomic database was categorized as "Unknown". Category "Others" belongs to the taxa other than top 10.

4.10. Quantitative insights into the microbial population

The sequence data were uploaded to the MG-RAST analysis tool and the taxonomic annotation was obtained. The quantitative insights into microbial population present in the samples were calculated for three samples automatically by the MG-RAST analysis tool. The sequence data was compared to RDP using maximum e-value of $1e^{-5}$, a minimum identity of 90 per cent, and a minimum alignment length of 15 measured in base pair for RNA databases and

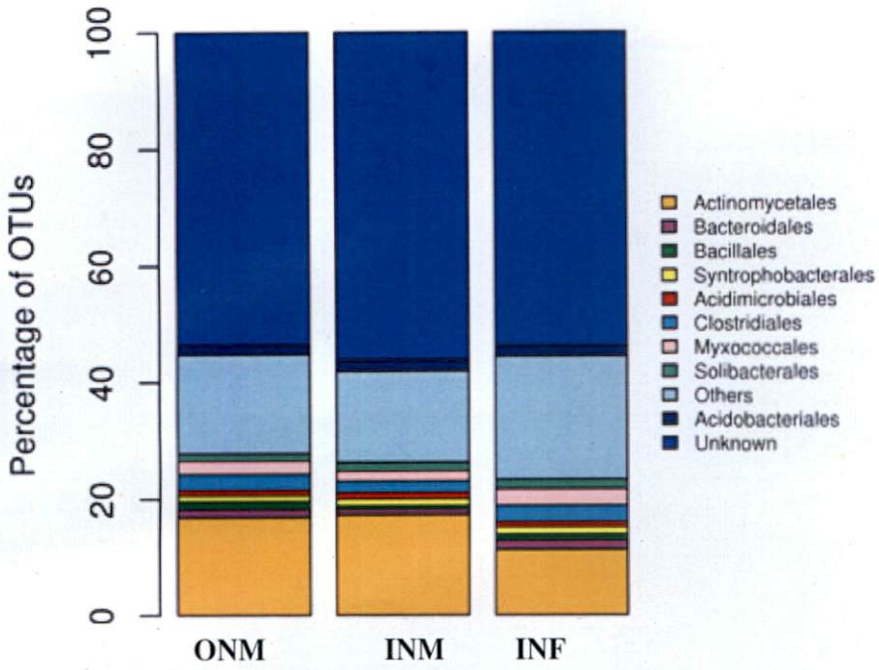


A. Taxonomy classification of OTUs at phylum level

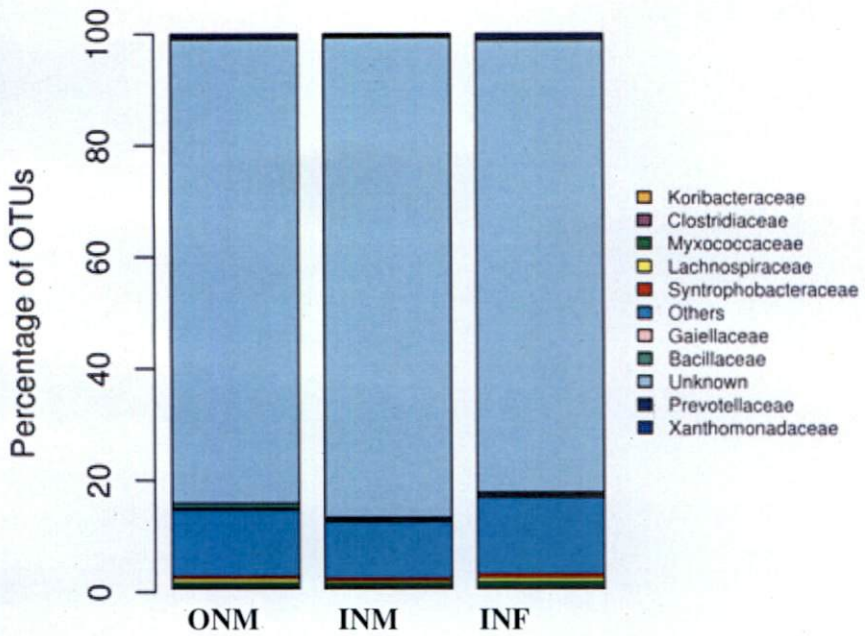


B.Taxonomy classification OTUs at class level

PLATE 14. Taxonomy classification



C. Taxonomy classification of OTUs at order level



D. Taxonomy classification of OTUs at family level

PLATE 15. Taxonomy classification

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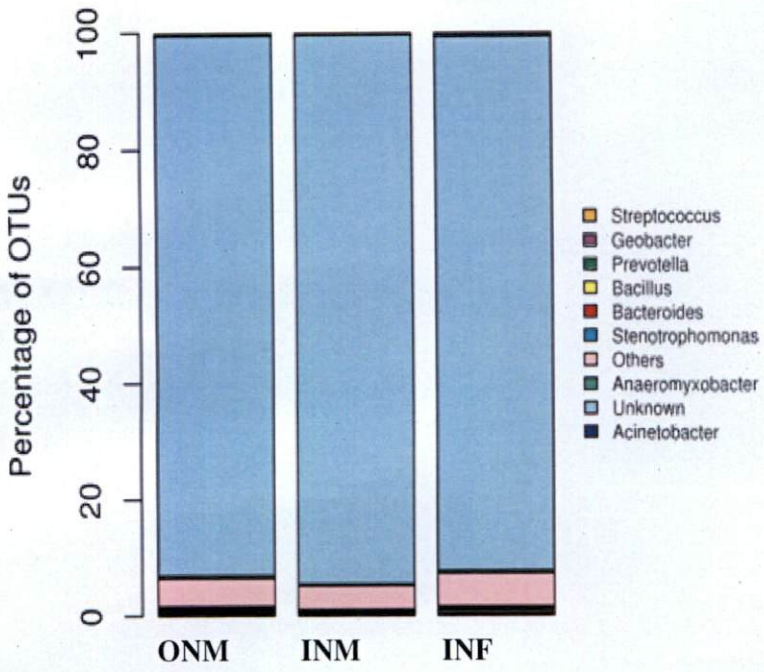
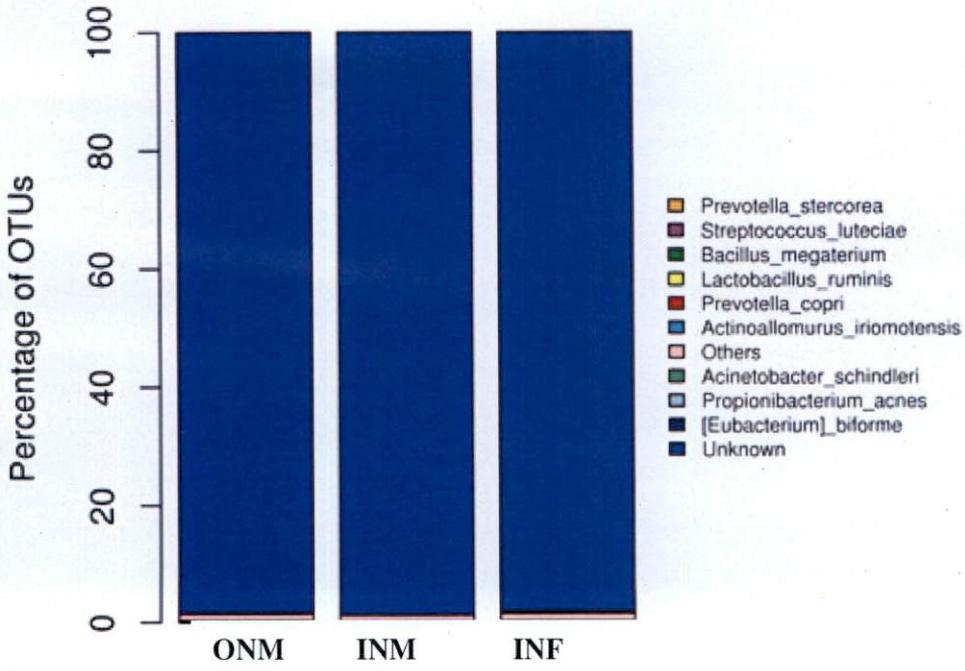


E. Taxonomy classification of OTUs at genus level



F. Taxonomy classification of OUTs at species level

PLATE 16. Taxonomy classification

data was visualized by using different tools for data visualization such as table, tree, barchart, heatmap and rarefraction. Analysed sequence data revealed that two domains were detected viz.: Bacteria and Eukaryota (Tables 14-16). Unassigned are category which doesnot shown any similarity with the present database and unclassified sequences were also reported in the samples and given in Tables 17-19. The barchart depiciting the sequence belonging to bacteria, eukaryota, unassigned and unclassified sequences is given in Plate 17. A total number of phylum, class, order, family, genus and species under domain bacteria obtained by using "table" data visualization tool are given in Table 20.

Table 20. Total number of taxonomic category obtained in the samples

Sample Name	Phylum	Class	Order	Family	Genus	Species
ONM	21	40	82	169	352	853
INM	18	35	66	134	272	670
INF	21	40	85	173	365	867

4.11. Bacterial diversity as analysed by MG-RAST.

4.11.1 Organic inputs treated soil sample

First twenty most abundant taxonomic categories from phylum to species are given in Table 21 and phylogenetic tree in Plate 18. Altogether, 21 phyla were detected in the organic treated soil, and phylum Actinobacteria (54.39%) dominated over other phyla. Other phyla included unclassified (derived from bacteria, 17.78%) followed by Acidobacteria (10.31%), Proteobacteria (7.05%), Firmicutes (6.76%) and Bacteroidetes (2.81%). Some of the phyla with less than

Table 14. Eukaryotic taxonomic category obtained in sample ONM

Domian	Phylum	Class	Order	Family	Genus	Species	Abundance
Eukaryota	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*	*Verticillium dahliae*	81
Eukaryota	Bacillariophyta	Bacillariophyceae	Naviculales	Phaeodactylaceae	*Phaeodactylum*	*Phaeodactylum tricornutum*	16
Eukaryota	Bacillariophyta	Bacillariophyceae	unclassified (derived from Bacillariophyceae)	Bacillariaceae	*Nitzschia*	*Nitzschia frustulum*	2
Eukaryota	Chlorophyta	Prasinophyceae	Chlorodendrales	Chlorodendraceae	*Scherffelia*	*Scherffelia dubia*	1
Eukaryota	Chlorophyta	Ulvophyceae	Bryopsidales	Bryopsidaceae	*Bryopsis*	*Bryopsis hypnoides*	1
Eukaryota	Chlorophyta	Ulvophyceae	Ulvales	unclassified (derived from Ulvales)	*Pseudendoclonium*	*Pseudendoclonium akinetum*	3
Eukaryota	Streptophyta	Coleochaetophyceae	Coleochaetales	Chaetosphaeridiaceae	*Chaetosphaeridium*	*Chaetosphaeridium globosum*	3
Eukaryota	Streptophyta	Coniferopsida	Coniferales	Pinaceae	*Pinus*	*Pinus taeda*	63
Eukaryota	Streptophyta	Liliopsida	Alismatales	Araceae	*Lemna*	*Lemna minor*	353
Eukaryota	Streptophyta	Liliopsida	Poales	Poaceae	*Festuca*	*Festuca ovina*	17
Eukaryota	Streptophyta	Polypodiopsida	Schizaeales	Lygodiaceae	*Lygodium*	*Lygodium japonicum*	1
Eukaryota	Streptophyta	Sphagnopsida	Sphagnales	Sphagnaceae	*Sphagnum*	*Sphagnum palustre*	1
Eukaryota	Streptophyta	Zygnemophyceae	Desmidiales	Desmidiaceae	*Staurastrum*	*Staurastrum punctulatum*	4
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Ericales	Ericaceae	*Vaccinium*	*Vaccinium corymbosum*	568

Table 15. Eukaryotic taxonomic category obtained in sample INM

Domian	Phylum	Class	Order	Family	Genus	Speices	Abundance
Eukaryota	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	Verticillium	*Verticillium dahliae*	43
Eukaryota	Bacillariophyta	Bacillariophyceae	Naviculales	Phaeodactylaceae	Phaeodactylum	*Phaeodactylum tricornutum*	8
Eukaryota	Streptophyta	Coleochaetophyceae	Coleochaetales	Chaetosphaeridiaceae	Chaetosphaeridium	*Chaetosphaeridium globosum*	1
Eukaryota	Streptophyta	Coniferopsida	Coniferales	Pinaceae	Pinus	*Pinus taeda*	10
Eukaryota	Streptophyta	Liliopsida	Alismatales	Araceae	Lemna	*Lemna minor*	138
Eukaryota	Streptophyta	Liliopsida	Poales	Poaceae	Festuca	*Festuca ovina*	5
Eukaryota	Streptophyta	Sphagnopsida	Sphagnales	Sphagnaceae	Sphagnum	*Sphagnum palustre*	2
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Arabidopsis	*Arabidopsis thaliana*	1
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Capsella	*Capsella bursa-pastoris*	1
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Crucihimalaya	*Crucihimalaya wallichii*	1

Domian	Phylum	Class	Order	Family	Genus	Species	Abundance
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Fabales	Fabaceae	*Phaseolus*	*Phaseolus vulgaris*	2
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Magnoliales	Magnoliaceae	*Liriodendron*	*Liriodendron tulipifera*	4
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Malpighiales	Euphorbiaceae	*Ricinus*	*Ricinus communis*	3
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Solanales	Solanaceae	*Solanum*	*Solanum bulbocastanum*	8
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Solanales	Solanaceae	*Solanum*	*Solanum lycopersicum*	8
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Solanales	Solanaceae	*Solanum*	*Solanum tuberosum*	8
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Vitales	Vitaceae	*Vitis*	*Vitis hybrid cultivar*	231
Eukaryota	unclassified (derived from Eukaryota)	Cryptophyta	Pyrenomonadales	Pyrenomonadaceae	*Rhodomonas*	*Rhodomonas salina*	14
Eukaryota	unclassified (derived from Eukaryota)	unclassified (derived from Eukaryota)	unclassified (derived from Eukaryota)	unclassified (derived from Eukaryota)	*Bigelowiella*	*Bigelowiella natans*	1

Table 16. Eukaryotic taxonomic category obtained in sample INF

Domain	Phylum	Class	Order	Family	Genus	Species	Abundance
Eukaryota	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	Verticillium	*Verticillium dahliae*	375
Eukaryota	Bacillariophyta	Bacillariophyceae	Naviculales	Phaeodactylaceae	Phaeodactylum	*Phaeodactylum tricornutum*	4
Eukaryota	Bacillariophyta	Bacillariophyceae	unclassified (derived from Bacillariophyceae)	Bacillariaceae	Nitzschia	*Nitzschia frustulum*	1
Eukaryota	Bacillariophyta	Coscinodiscophyceae	unclassified (derived from Coscinodiscophyceae)	Skeletonemataceae	Skeletonema	*Skeletonema costatum*	1
Eukaryota	Chlorophyta	Ulvophyceae	Ulvales	unclassified (derived from Ulvales)	Pseudendoclonium	*Pseudendoclonium akinetum*	1
Eukaryota	Eustigmatophyceae	unclassified (derived from Eustigmatophyceae)	Eustigmatales	Monodopsidaceae	Nannochloropsis	*Nannochloropsis granulata*	5
Eukaryota	Phaeophyceae	unclassified (derived from Phaeophyceae)	Fucales	Fucaceae	Fucus	*Fucus vesiculosus*	6
Eukaryota	Streptophyta	Anthocerotopsida	Anthocerotales	Anthocerotaceae	Anthoceros	*Anthoceros formosae*	26
Eukaryota	Streptophyta	Coleochaetophyceae	Coleochaetales	Chaetosphaeridiaceae	Chaetosphaeridium	*Chaetosphaeridium globosum*	4
Eukaryota	Streptophyta	Coniferopsida	Coniferales	Pinaceae	Pinus	*Pinus taeda*	2
Eukaryota	Streptophyta	Isoetopsida	Selaginellales	Selaginellaceae	Selaginella	*Selaginella uncinata*	1

Eukaryota	Streptophyta	Liliopsida	Alismatales	Araceae	Lemna	*Lemna minor*	219
Eukaryota	Streptophyta	Liliopsida	Poales	Poaceae	Agrostis	*Agrostis stolonifera*	1
Eukaryota	Streptophyta	Liliopsida	Poales	Poaceae	Festuca	*Festuca ovina*	17
Eukaryota	Streptophyta	Polypodiopsida	Schizaeales	Lygodiaceae	Lygodium	*Lygodium japonicum*	1
Eukaryota	Streptophyta	Sphagnopsida	Sphagnales	Sphagnaceae	Sphagnum	*Sphagnum palustre*	1
Eukaryota	Streptophyta	Zygnemophyceae	Desmidiales	Desmidiaceae	Staurastrum	*Staurastrum punctulatum*	3
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Apiales	Apiaceae	Daucus	*Daucus carota*	2
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Arabidopsis	*Arabidopsis thaliana*	4
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Capsella	*Capsella bursa-pastoris*	4
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Crucihimalaya	*Crucihimalaya wallichii*	4
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Brassicales	Brassicaceae	Olimarabidopsis	*Olimarabidopsis pumila*	4
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Ericales	Ericaceae	Vaccinium	*Vaccinium corymbosum*	880
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Fabales	Fabaceae	Phaseolus	*Phaseolus vulgaris*	2

Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Psilotales	Psilotaceae	Psilotum	*Psilotum nudum*	2
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Solanales	Solanaceae	Solanum	*Solanum bulbocastanum*	5
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Solanales	Solanaceae	Solanum	*Solanum lycopersicum*	5
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Solanales	Solanaceae	Solanum	*Solanum tuberosum*	5
Eukaryota	Streptophyta	unclassified (derived from Streptophyta)	Vitales	Vitaceae	Vitis	*Vitis hybrid cultivar*	1892
Eukaryota	unclassified (derived from Eukaryota)	Florideophyceae	Ceramiales	Ceramiaceae	Antithamnionella	*Antithamnionella spirographidis*	11
Eukaryota	unclassified (derived from Eukaryota)	Florideophyceae	Ceramiales	Ceramiaceae	Euptilota	*Euptilota fergusonii*	1
Eukaryota	unclassified (derived from Eukaryota)	Florideophyceae	Ceramiales	Ceramiaceae	Euptilota	*Euptilota molle*	1
Eukaryota	unclassified (derived from Eukaryota)	Florideophyceae	Gelidiales	Gelidiaceae	Capreolia	*Capreolia implexa*	1
Eukaryota	unclassified (derived from Eukaryota)	Florideophyceae	Gelidiales	Gelidiaceae	Gelidium	*Gelidium serrulatum*	4
Eukaryota	unclassified (derived from Eukaryota)	Pelagophyceae	unclassified (derived from Pelagophyceae)	unclassified (derived from Pelagophyceae)	unclassified (derived from Pelagophyceae)	*Aureoumbra lagunensis*	1

Table 17. Taxonomic category 'unassigned' obtained in sample ONM

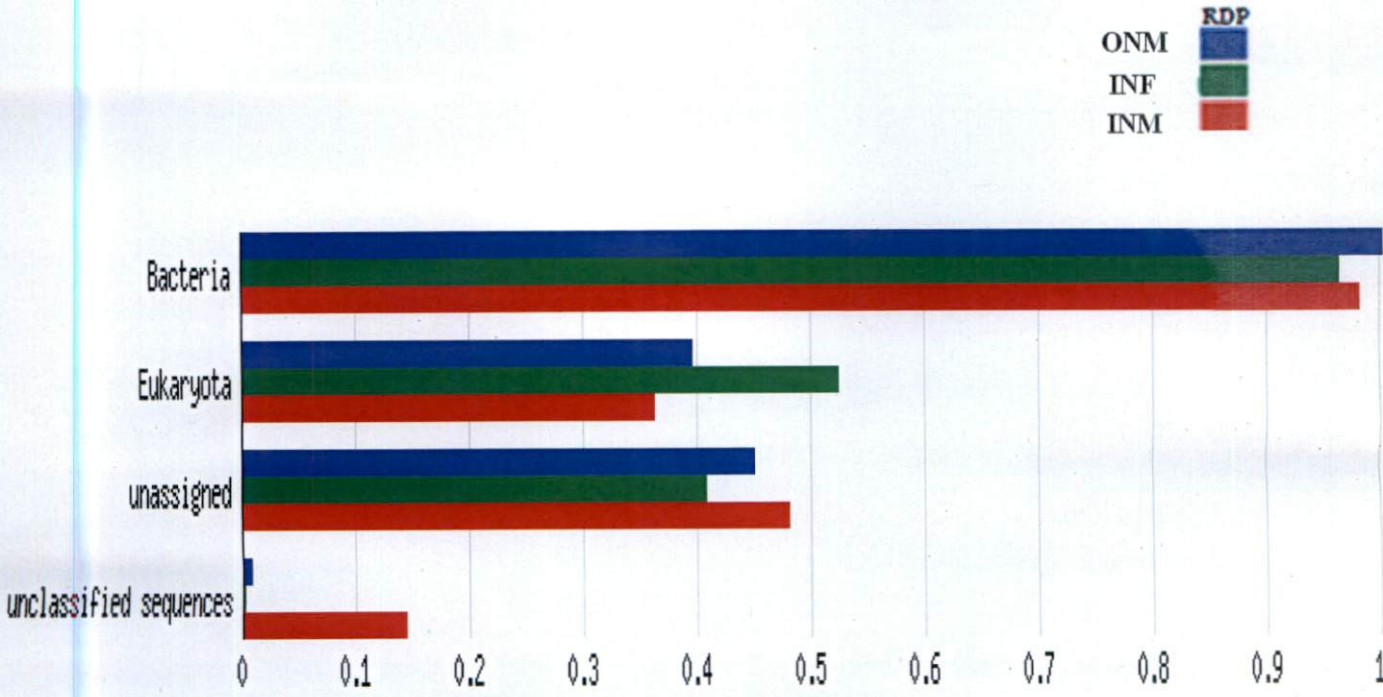
Domain	Phylum	Class	Order	Family	Genus	Species	Abundance
Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	2042
Unclassified sequences	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Uncultured marine microorganism	44
Unclassified sequences	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Uncultured organism	40

Table 18. Taxonomic category 'unassigned' obtained in sample INM

Domain	Phylum	Class	Order	Family	Genus	Species	Abundance
Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	2524
Unclassified sequences	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Uncultured organism	225

Table 19. Taxonomic category 'unassigned' obtained in sample INF

Domain	Phylum	Class	Order	Family	Genus	Species	Abundance
Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	1503
Unclassified sequences	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Uncultured marine microorganism	51
Unclassified sequences	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Unclassified (derived from unclassified sequences)	Uncultured organism	29



(data normalized to values between 0 and 1 to allow for comparison of differently sized samples).

PLATE 17. Barchat of the samples at domain level

Table 21. Abudance of major 20 taxonomic category from phylum to species level of bacteria in sample ONM

Phylum	Class	Order	Family	genus	species
Actinobacteria (54.39%)	Actinobacteria (class) (54.39%)	Actinomycetales (49.67%)	unclassified (derived from bacteria) (17.78%)	unclassified (derived from bacteria) (17.78%)	*uncultured bacterium* (16.52%)
unclassified (derived from Bacteria) (17.78%)	unclassified (derived from Bacteria) (17.78%)	unclassified (derived from Bacteria) (17.78%)	Frankiaceae (9.89%)	*Frankia* (9.89%)	*Frankia* sp. (9.44%)
Acidobacteria (10.31%)	Solibacteres (8.47%)	Solibacterales (8.47%)	Solibacteraceae (8.47%)	*Candidatus Solibacter* (8.47%)	*Candidatus Solibacter usitatus* (8.08%)
Proteobacteria (7.05%)	Clostridia (3.80%)	Coriobacteriales (4.20%)	Micromonosporaceae (7.70%)	*Saccharopolyspora* (6.00%)	*Micromonospora chokoriensis* (4.77%)
Firmicutes (6.76%)	Deltaproteobacteria (2.90%)	Clostridiales (3.00%)	Pseudonocardiaceae (6.82%)	*Micromonospora* (5.73%)	*Arthrobacter aurescens* (4.58%)
Bacteroidetes (2.81%)	Bacilli (2.85%)	Bacillales (2.65%)	Nocardiaceae (6.80%)	*Arthrobacter* (4.86%)	*Arthrobacter nitroguajacolicus* (4.58%)
Verrucomicrobia (0.53%)	Bacteroidia (2.00%)	Bacteroidales (2.00%)	Micrococcaceae (4.97%)	*Nocardia* (3.82%)	*Nocardia cyriacigeorgica* (2.98%)
Chlorobi (0.12%)	Betaproteobacteria (1.88%)	Acidobacteriales (1.53%)	Coriobacteriaceae (4.20%)	*Rhodococcus* (2.98%)	*Saccharopolyspora rectivirgula* (2.84%)
Chloroflexi (0.11%)	Acidobacteriia (1.53%)	Burkholderiales (1.35%)	Nocardioidaceae (3.64%)	*Nocardioides* (2.57%)	*Saccharopolyspora hirsute* (2.69%)
Cyanobacteria (0.03%)	Alphaproteobacteria (0.79%)	unclassified (derived from Deltaproteobacteria) (1.30%)	Promicromonosporaceae (2.24%)	*Cellulosimicrobium* (2.24%)	*Cellulosimicrobium cellulans* (2.14%)

Phylum	Class	Order	Family	genus	species
Spirochaetes (0.01%)	Gammaproteobacteria (0.71%)	Desulfovibrionales (1.21%)	Bacillaceae (2.01%)	*Atopobium* (1.93%)	*Gordonibacter pamelaeae* (1.80%)
Nitrospirae (0.0163%)	Epsilonproteobacteria (0.66%)	Thermoanaerobacterales (0.79%)	Thermomonosporaceae (1.67%)	*Bacillus* (1.91%)	*Atopobium minutum* (1.74%)
Thermotogae (0.014%)	Flavobacteriia (0.58%)	Flavobacteriales (0.58%)	Acidobacteriaceae (1.53%)	*Gordonibacter* (1.88%)	*Rhodococcus opacus* (1.71%)
Synergistetes (0.0108%)	Verrucomicrobiae (0.46%)	Verrucomicrobiales (0.46%)	unclassified (derived from Deltaproteobacteria) (1.30%)	*Acidobacterium* (1.53%)	*Acidobacterium capsulatum* (1.46%)
Gemmatimonadetes (0.005%)	unclassified (derived from Acidobacteria) (0.30%)	unclassified (derived from Epsilonproteobacteria) (0.45%)	Clostridiaceae (1.23%)	unclassified (derived from *Deltaproteobacteria)* (1.30%)	uncultured *delta proteobacterium* (1.24%)
Chlamydiae (0.004%)	Sphingobacteriia (0.16%)	Bifidobacteriales (0.41%)	Corynebacteriaceae (1.15%)	*Actinomadura* (1.21%)	*Desulfovibrio indonesiensis* (1.03%)
Fusobacteria (0.003%)	Chlorobia (0.12%)	unclassified (derived from Betaproteobacteria) (0.41%)	Desulfovibrionaceae (1.14%)	*Corynebacterium* (1.15%)	*Nocardioides albus* (0.97%)
Deinococcus-Thermus (0.002%)	unclassified (derived from Proteobacteria) (0.093%)	Xanthomonadales (0.41%)	Mycobacteriaceae (1.11%)	*Desulfovibrio* (1.14%)	*Corynebacterium simulans* (0.84%)
Tenericutes (0.001%)	Erysipelotrichi (0.09%)	Rhizobiales (0.38%)	Prevotellaceae (1.08%)	*Mycobacterium* (1.11%)	*Pelomonas saccharophila* (0.82%)
Dictyoglomi (0.0009%)	Chloroflexi (class) (0.07%)	Desulfuromonadales (0.30%)	Comamonadaceae (0.86%)	*Prevotella* (1.06%)	*Actinomadura namibiensis* (0.80%)

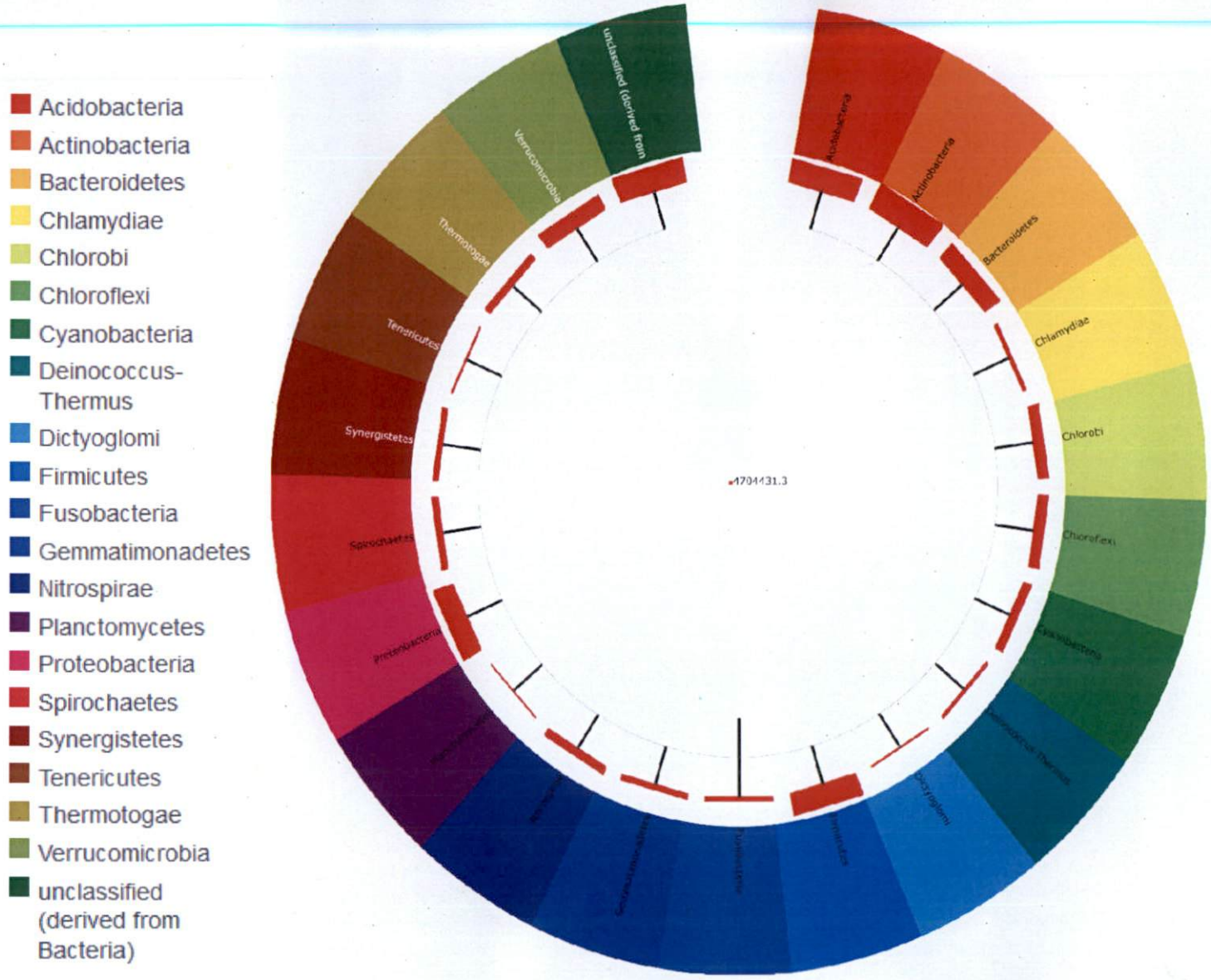


PLATE 18. Phylogenetic tree of bacteria at phylum level constructed in MG-RAST with Illumina sequence data set of ONM sample

one per cent abundance included Verrucomicrobia, Chlorobi, Chloroflexi, Cyanobacteria, Spirochaetes, Nitrospirae, Thermotogae, Synergistetes, Gemmatimonadetes, Chlamydiae, Fusobacteria, Deinococcus-Thermus, Tenericutes, Dictyoglomi and Planctomycetes.

A total of 40 classes were discovered in organic treated soil and phylogenetic tree which represents bacterial diversity from phylum to the class level is given in Plate 19. Among the different classes, Actinobacteria (54.39%) dominated, followed by unclassified (derived from bacteria, 17.78%), Solibacteres (8.47%), Clostridia (3.80%), Deltaproteobacteria (2.90%), Bacilli (2.85%), Bacteroidia (2.00%), Betaproteobacteria (1.88%) and Acidobacteria (1.53%). Other classes of less than one per cent abundance included Alphaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria, Flavobacteria, Verrucomicrobiae, unclassified (derived from Acidobacteria), Sphingobacteria, Chlorobia, unclassified (derived from Proteobacteria), Erysipelotrichi, Chloroflexi, Spartobacteria and Cytophagia.

Analysis at order level revealed that a total of 82 orders were present and phylogenetic tree which represents bacterial diversity from phylum to the order level is given in Plate 20. Among them, Actinomycetales (49.67%) belonging to the Actiobacteria phylum was the dominant order followed by unclassified (derived from bacteria, 17.78%), Solibacterales (8.47%), Coriobacteriales (4.20%), Clostridiales (3.00%), Bacillales (2.65 %), Bacteroidales (2.00%), Acidobacteriales (1.53%), Burkholderiales (1.35%), unclassified (derived from Deltaproteobacteria) (1.30%), Desulfovibrionales (1.21%). Order comprising of less than one per cent in the sample were Thermoanaerobacterales, Flavobacteriales, Verrucomicrobiales, unclassified (derived from Epsilonproteobacteria), Bifidobacteriales, unclassified (derived from Betaproteobacteria), Xanthomonadales, Rhizobiales, Desulfuromonadales, unclassified (derived from Acidobacteria) and Campylobacterales.

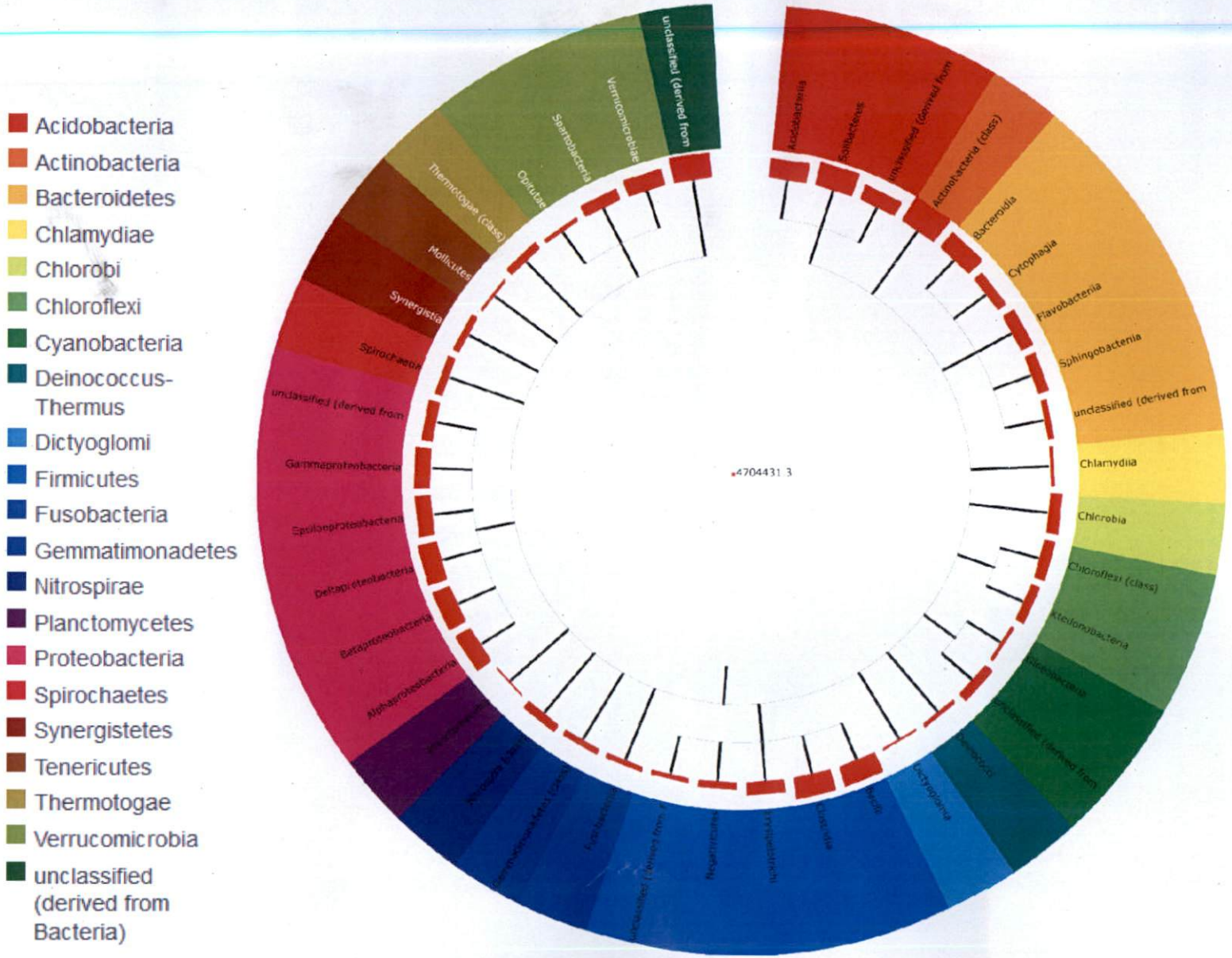


PLATE 19. Phylogenetic tree of bacteria at class level constructed in MG-RAST with Illumina sequence data set of ONM sample

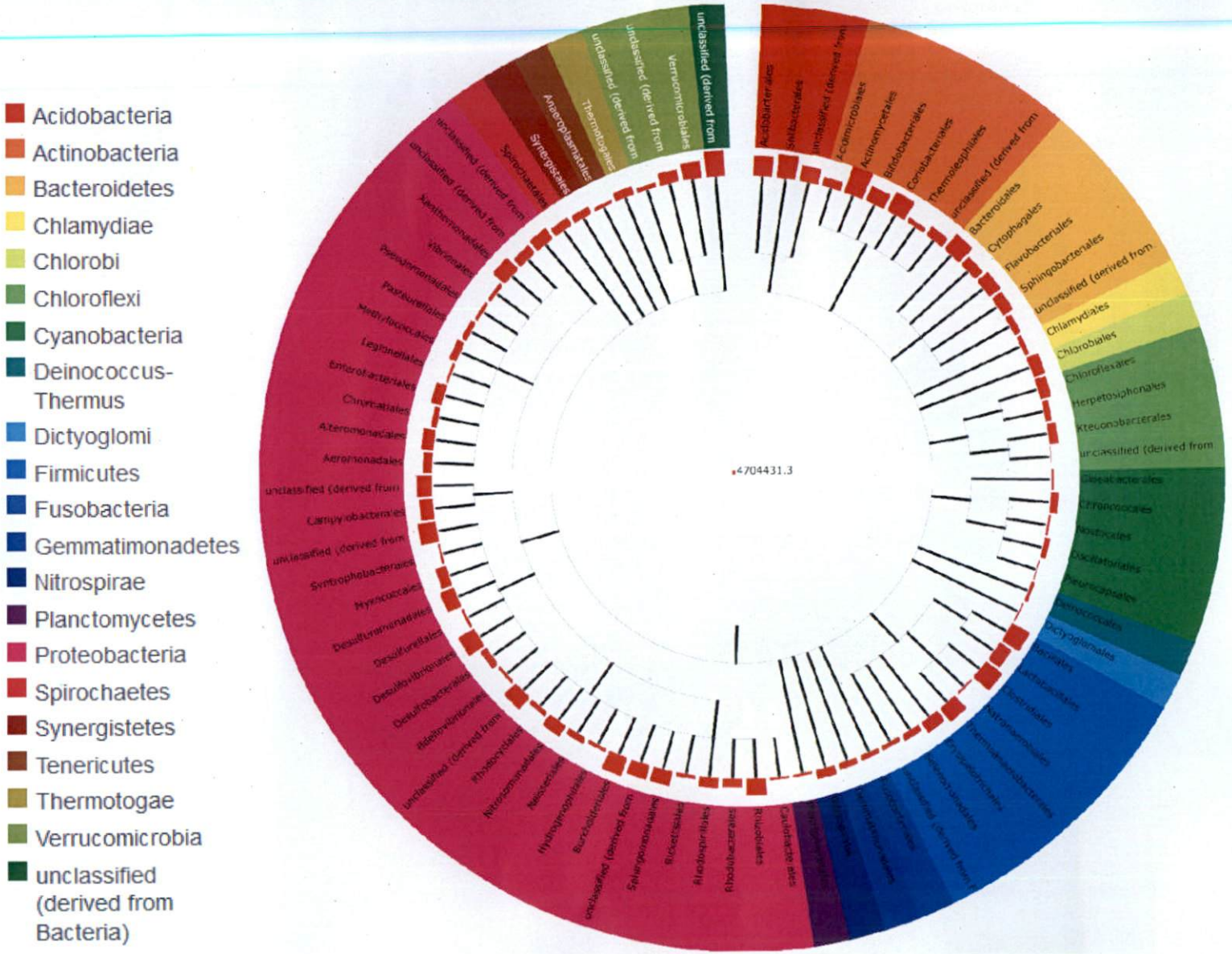


PLATE 20. Phylogenetic tree of bacteria at order level constructed in MG-RAST with Illumina sequence data set of ONM sample

Bacteria present in organic treated soil belonging to the family Comamonadaceae, Geodermatophilaceae, Lachnospiraceae and Propionibacteriaceae were present less than one per cent in the sample. Family unclassified (derived from bacteria) (17.78%) was most abundant and this was followed by Frankiaceae (9.89%), Solibacteraceae (8.47%), Micromonosporaceae (7.70%), Pseudonocardiaceae (6.82%), Nocardiaceae (6.80%), Micrococcaceae (4.97%), Coriobacteriaceae (4.20%), Nocardioidaceae (3.64%), Promicromonosporaceae (2.24%), Bacillaceae (2.01%), Thermomonosporaceae (1.67%), Acidobacteriaceae (1.53%), unclassified (derived from Deltaproteobacteria) (1.30%), Clostridiaceae (1.23%), Corynebacteriaceae (1.15%), Desulfovibrionaceae (1.14%), Mycobacteriaceae (1.11%), Prevotellaceae (1.08%). Altogether a total of 169 families were found in the sample and phylogenetic tree at family level is given in Plate 21.

Analysis at genus level yielded a total of 352 genera. Among them, the following genera were present above one per cent in ONM sample: unclassified (derived from Bacteria, 17.78%), followed by *Frankia* (9.89%), *Candidatus Solibacter* (8.47%), *Saccharopolyspora* (6.00%), *Micromonospora* (5.73%), *Arthrobacter* (4.86%), *Nocardia* (3.82%), *Rhodococcus*(2.98%), *Nocardioides* (2.57%), *Cellulosimicrobium* (2.24%), *Atopobium* (1.93%), *Bacillus* (1.91%), *Gordonibacter* (1.88%), *Acidobacterium* (1.53%), unclassified, derived from *Deltaproteobacteria* (1.30%), *Actinomadura* (1.21%), *Corynebacterium* (1.15%), *Desulfovibrio* (1.14%), *Mycobacterium* (1.11%), *Prevotella* (1.06%), and *Clostridium* (1.03%).

A total of 853 species were found in the organic inputs received soil. The most dominant was uncultured bacterium with 16.52 per cent abundance, followed by *Frankia* sp. (9.44%) belonging to *Actinobacteria*, *Candidatus Solibacter usitatus* (8.08%), *Micromonospora chokoriensis*(4.77%), *Arthrobacter aurescens* (4.58%), *Arthrobacter nitroguajacolicus* (4.58%), *Nocardiacyriacigeorgica* (2.98%), *Saccharopolyspora rectivirgula* (2.84%), *Saccharopolyspora hirsute* (2.69%), *Cellulosimicrobium* . .. (2.14%),

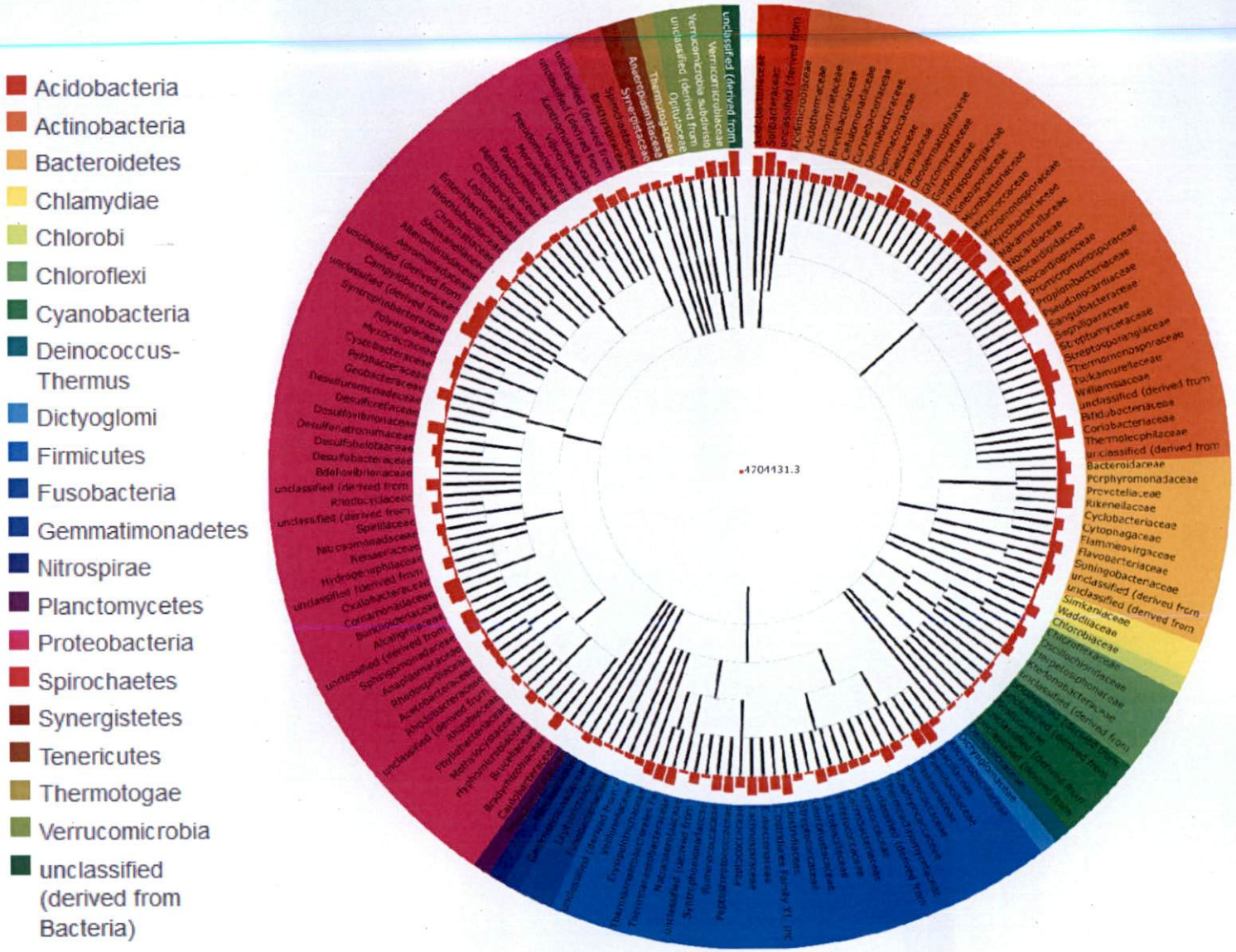


PLATE 21. Phylogenetic tree of bacteria at family level constructed in MG-RAST with Illumina sequence data set of ONM sample

Gordonibacterpamelaeae (1.80%), *Atopobium minimum* (1.74%), *Rhodococcusopacus* (1.71%), *Acidobacterium capsulatum* (1.46%), uncultured *Delta proteobacterium* (1.24%), *Desulfovibrio indonesiensis* (1.03%), *Nocardioides albus* (0.97%), *Corynebacteriumsimulans* (0.84%), *Pelomonassaccharophila* (0.82%), *Actinomadur anamibiensis*(0.80%), *Geodermatophilus obscures* (0.76%) and *Bacillus flexus* (0.75%).

4.11.2 Integrated nutrient management

The composition of bacteria was analysed and were grouped into taxonomic categories from phylum to species level, which is given in Table 22. Sequence data representing bacterial domain and abundance of phyla are given in Plate 22. A total of 18 phyla were found in the sample. Among these, phylum Actinobacteria consisted of 57.95 per cent followed by unclassified (derived from bacteria) (16.42%). Similarly, phylum Acidobacteria constituted (12.92%), followed by Proteobacteria (5.62%), Firmicutes (4.80%) and Bacteroidetes (1.67%). Phyla such as Gemmatimonadetes, Cyanobacteria, Chlorobi, Chloroflexi, Synergistetes, Verrucomicrobia, Thermotogae, Nitrospirae, Deinococcus-Thermus, Fusobacteria, Spirochaetes and Chlamydiae were present less than one per cent in INM sample.

A total of 35 classes were detected in soil under integrated nutrient management and phylogenetic tree is depicted in Plate 23. Among these, class Actinobacteria was the most dominant, consisting of 57.95 per cent followed by unclassified bacteria (16.42%), Solibacteres (10.74%) and Clostridia (4.72%). Bacteria belonging to class Deltaproteobacteria consisted of 3.45 per cent followed by Acidobacteriia (1.52%) and Alphaproteobacteria (1.00%). A total of 15 classes consisted of less than one per cent and these include Bacteroidia, unclassified Acidobacteria, Epsilonproteobacteria, Betaproteobacteria, unclassified Bacteroidetes, Gemmatimonadetes (class), Flavobacteria, Gammaproteobacteria, Sphingobacteria, Chlorobia, Bacilli, unclassified Cyanobacteria), Gloeobacteria, Cytophagia and Chloroflexi.

Table 22. Abundance of major 20 taxonomic category from phylum to species level of bacteria in sample INM

Phylum	Class	Order	Family	Genus	Species
Actinobacteria (57.95%)	Actinobacteria (class) (57.95%)	Actinomycetales (54.42%)	Nocardiaceae (21.81%)	*Rhodococcus* (16.98%)	uncultured bacterium (16.22%)
unclassified (derived from Bacteria) 16.42%	unclassified (derived from Bacteria) (16.42%)	unclassified (derived from Bacteria) (16.42%)	unclassified (derived from Bacteria) (16.42%)	*unclassified (derived from Bacteria)* (16.42%)	*Rhodococcus opacus* (13.70%)
Acidobacteria (12.92%)	Solibacteres (10.74%)	Solibacterales (10.74%)	Solibacteraceae (10.74%)	*Candidatus Solibacter* (10.74%)	*Candidatus Solibacter usitatus* (10.73%)
Proteobacteria (5.62%)	Clostridia (4.72%)	Thermoanaerobacterales (3.19%)	Pseudonocardiaceae (8.91%)	*Saccharopolyspora* (8.16%)	*Saccharopolyspora hirsute* (4.23%)
Firmicutes (4.80%)	Deltaproteobacteria (3.45%)	Coriobacteriales (2.11%)	Nocardioidaceae (4.58%)	*Nocardia* (4.82%)	*Saccharopolyspora rectivirgula* (3.64%)
Bacteroidetes (1.67%)	Acidobacteriia (1.52%)	Clostridiales (1.53%)	Frankiaceae (3.50%)	*Frankia* (3.50%)	*Frankia* sp. (3.48%)
Gemmatimonadetes (0.26%)	Alphaproteobacteria (1.00%)	Acidobacteriales (1.52%)	Thermoanaerobacteraceae (3.18%)	*Nocardioides* (3.26%)	*Moorella thermoacetica* (3.18%)
Cyanobacteria (0.11%)	Bacteroidia (0.86%)	Desulfuromonadales (1.41%)	Micrococcaceae (2.31%)	*Moorella* (3.18%)	*Nocardia pseudobrasiliensis* (2.61%)
Chlorobi (0.09%)	unclassified (derived from Acidobacteria) (0.65%)	unclassified (derived from Deltaproteobacteria) (1.41%)	Mycobacteriaceae (2.20%)	*Arthrobacter* (2.25%)	*Arthrobacter* sp. scl-2 (2.22%)
Chloroflexi (0.04%)	Epsilonproteobacteria (0.49%)	Acidimicrobiales (1.02%)	Coriobacteriaceae (2.11%)	*Mycobacterium* (2.20%)	*Acidobacterium capsulatum* (1.52%)

Phylum	Class	Order	Family	Genus	Species
Synergistetes (0.02%)	*Betaproteobacteria* (0.46%)	*Bacteroidales* (0.86%)	*Corynebacteriaceae* (2.01%)	*Corynebacterium* (2.01%)	*uncultured delta proteobacterium* (1.41%)
Verrucomicrobia (0.01%)	unclassified (derived from *Bacteroidetes*) (0.36%)	unclassified (derived from *Acidobacteria*) (0.65%)	*Micromonosporaceae* (1.98%)	*Acidobacterium* (1.52%)	*Verrucosispora gifhornensis* (1.38%)
Thermotogae (0.009%)	*Gemmatimonadetes* (class) (0.26%)	*Rhizobiales* (0.58%)	*Thermomonosporaceae* (1.91%)	unclassified (derived from *Deltaproteobacteria*) (1.41%)	*Geoalkalibacter ferrihydriticus* (1.34%)
Nitrospirae (0.008%)	*Flavobacteriia* (0.25%)	*Desulfovibrionales* (0.52%)	*Acidobacteriaceae* (1.52%)	*Verrucosispora* (1.38%)	*Nocardia nova* (1.27%)
Deinococcus-Thermus (0.007%)	*Gammaproteobacteria* (0.18%)	unclassified (derived from *Bacteroidetes*) (0.36%)	*Geobacteraceae* (1.41%)	*Geoalkalibacter* (1.34%)	*Nocardioides* sp. DN36 (1.25%)
Fusobacteria (0.002%)	*Sphingobacteriia* (0.14%)	*Bifidobacteriales* (0.35%)	unclassified (derived from *Deltaproteobacteria*) (1.41%)	*Geodermatophilus* (1.12%)	*Geodermatophilus obscures* (1.12%)
Spirochaetes (0.001%)	*Chlorobia* (0.09%)	unclassified (derived from *Alphaproteobacteria*) (0.32%)	*Geodermatophilaceae* (1.12%)	*Actinomadura* (1.06%)	*Nocardioides albus* (1.01%)
Chlamydiae (0.001%)	*Bacilli* (0.07%)	*Campylobacterales* (0.27%)	*Microbacteriaceae* (1.09%)	*Acidimicrobium* (1.04%)	*Acidimicrobium ferrooxidans* (1.01%)
-	unclassified (derived from *Cyanobacteria*) (0.06%)	*Gemmatimonadales* (0.26%)	*Acidimicrobiaceae* (1.02%)	*Microbacterium* (0.92%)	*Mycobacterium* sp. JS624 (0.89%)
-	*Gloeobacteria* (0.05%)	unclassified (derived from *Betaproteobacteria*) (0.25%)	*Propionibacteriaceae* (0.87%)	*Atopobium* (0.84%)	*Aeromicrobium erythreum* (0.81%)

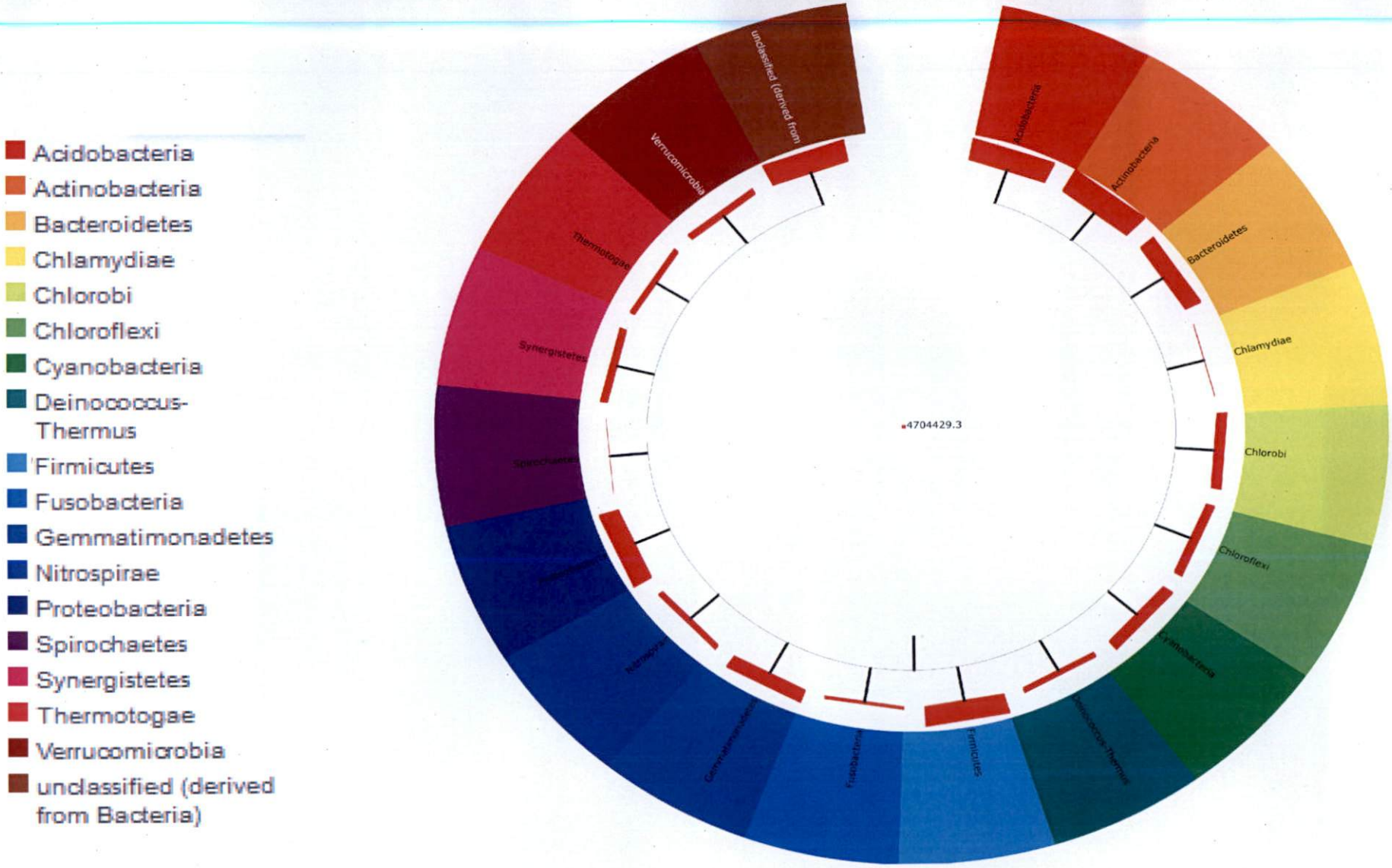


PLATE 22. Phylogenetic tree of bacteria at phylum level constructed in MG-RAST with Illumina sequence data set of INM sample

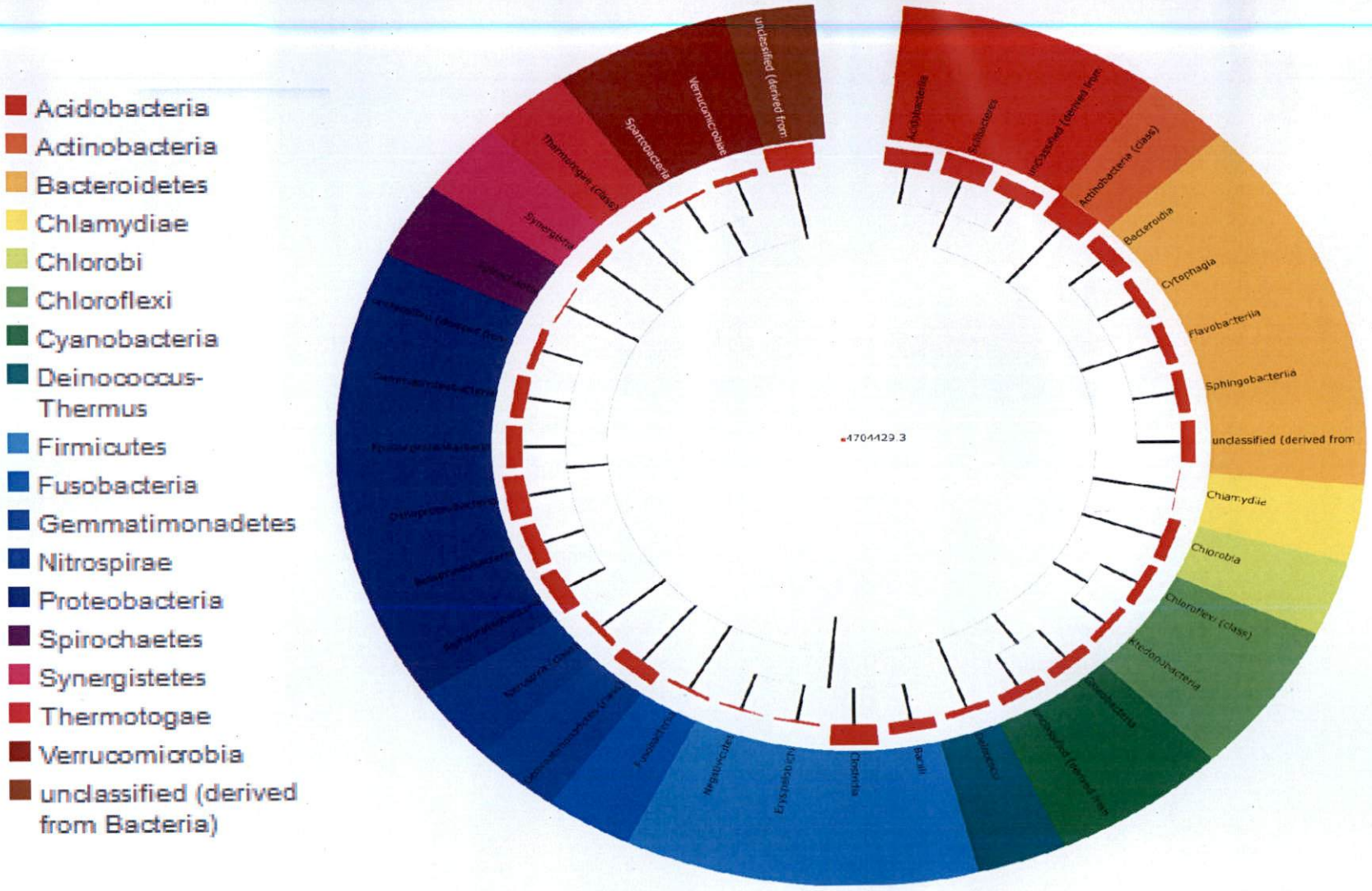


PLATE 23. Phylogenetic tree of bacteria at class level constructed in MG-RAST with Illumina sequence data set of INM sample

Altogether 66 orders were obtained in soil under integrated nutrient management and phylogenetic tree is given in Plate 24. Among theses, Actinomycetales were the most abundant (54.42%), followed by unclassified bacteria (16.42%) and Solibacterales (10.74%). Bacteria belonging to the order Thermoanaerobacterales constituted 3.19 % followed by Coriobacteriales (1.53 %) and Acidobacteriales (1.52 %). In addition to this, order Desulfuromonadales and unclassified Deltaproteobacteria consisted of 1.41% followed byAcidimicrobiales (1.02%). Orders of bacteria present less than one per cent included Bacteroidales, unclassified Acidobacteria, Rhizobiales, Desulfovibrionales, unclassified Bacteroidetes, Campylobacterales, Gemmatimonadales, unclassified (derived from Betaproteobacteria), Flavobacteriales and unclassified Epsilonproteobacteria).

A total number of 134 familes were detected in the soil under integrated nutrient management and phylogenetic tree is given in Plate 25. Family Nocardiaceae was the most dominant family (21.81 %), followed by unclassified bacteria (16.42 %), Solibacteraceae (10.74%), Pseudonocardiaceae (8.91%), Nocardioidaceae (4.58%), Frankiaceae (3.50%) and Thermoanaerobacteraceae (3.18%). Other families present were Micrococcaceae (2.31%), Mycobacteriaceae (2.20%), Coriobacteriaceae (2.11%) and Corynebacteriaceae (2.01%). Families between two and one per cent abundance included: Micromonosporaceae (1.98%), Thermomonosporaceae (1.91%), Acidobacteriacea (1.52%), Geobacteraceae (1.41%) unclassified Deltaproteobacteria (1.41%), Geodermatophilaceae (1.12%), Microbacteriaceae (1.02 %) and Acidimicrobiaceae (1.02%). However, abundance of Propionibacteriaceae, Clostridiaceae and unclassified Acidobacteria was less than one per cent. Altogether 272 bacterial genera were identified in sample INM. Among these, *Rhodococcus* was found to be the most dominat genus constituting 16.98% followed by unclassified bacteria (16.42%). In addition to this, genus *Candidatus Solibacter* constituted 10.74% followed by *Saccharopolyspora* (8.16%), *Nocardia* (4.82%), *Frankia* (3.50%), *Nocardioides* (3.26%), *Moorella* (3.18%),

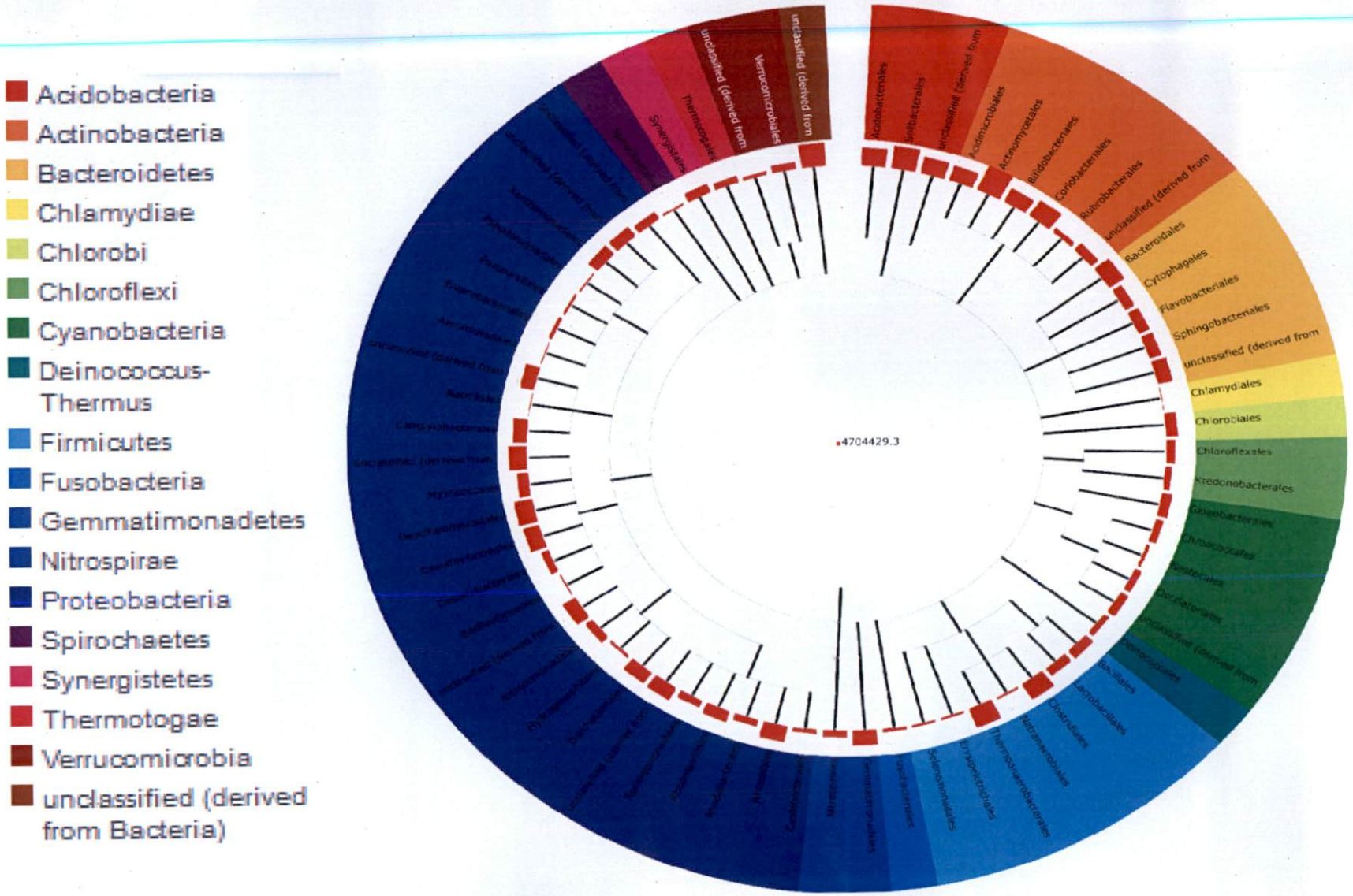


PLATE 24. Phylogenetic tree of bacteria at order level constructed in MG-RAST with Illumina sequence data set of INM sample

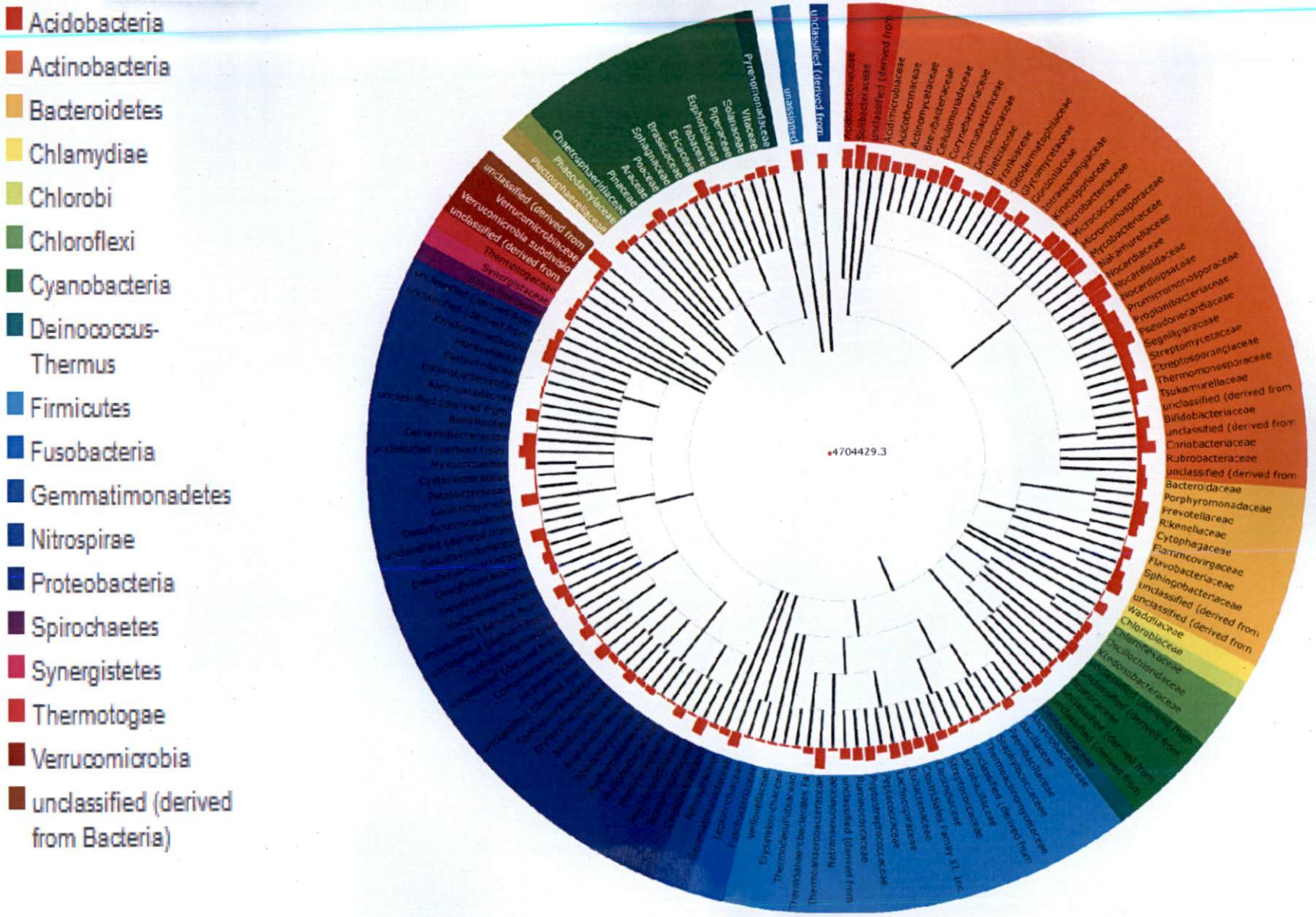


PLATE 25. Phylogenetic tree of bacteria at family level constructed in MG-RAST with Illumina sequence data set of INM sample

Arthrobacter (2.25%), *Mycobacterium* (2.20%) and *Corynebacterium* (2.01%). Bacteria belonging to the genera *Acidobacterium* consisted of 1.52 % followed by unclassified *Deltaproteobacteria* (1.41%), *Verrucosispora* (1.38%), *Geoalkalibacter* (1.34%), *Geodermatophilus* (1.12%), *Actinomadura*(1.06%), *Acidimicrobium* (1.04%). The genera *Microbacterium*, *Atopobium*, *Aeromicrobium* and *Propionibacterium* consisted of less than one per cent.

A total of 670 species were discovered, among which, uncultured bacterium was the majority (16.22%) followed by *Rhodococcusopacus* (13.70%), *Candidatus Solibacter usitatus* (10.73%), *Saccharopolyspora hirsute* (4.23%), *Saccharopolyspora rectivirgula* (3.64%), *Frankia* sp. (3.48%), *Moorella thermoacetica* (3.18%), *Nocardia pseudobrasiliensis* (2.61%), *Arthrobacter* sp. scl-2 (2.22%), *Acidobacterium capsulatum* (1.52%), uncultured *deltaproteobacterium* (1.4%), *Verrucosispora gifhornensis* (1.38%), *Geoalkalibacter ferrihydriticus* (1.34%), *Nocardia nova* (1.27%), *Nocardioides* sp. DN36 (1.25%), *Geodermatophilus obscurus* (1.12%), *Nocardioidesalbus* (1.01%) and *Acidimicrobium ferrooxidans* (1.01%). Species such as *Mycobacterium* sp. JS624, *Aeromicrobium erythreum*, *Microbacterium lacticum* and *Actinomadura namibiensis* consisted of less than one per cent.

4.11.3 Inorganic inputs treated soil

Composition of bacteria was analysed in inorganic inputs treated soil (Table 23) and sequence data representing domain bacteria and abundance of phyla are given in Plate 26. A total of 21 phyla were reported in the sample and among these, unclassified bacteria was found to be dominant one (33.57 %), followed by Actinobacteria (25.80%), Proteobacteria (14.55%) and Acidobacteria (14.13%). Phylum Firmicutes was also present (6.41 %) followed by Bacteroidetes (4.12%). Bacteria belonging to Verrucomicrobia, Cyanobacteria, Chlorobi, Synergistetes, Spirochaetes, Nitrospirae, Chloroflexi, Deinococcus-Thermus, Fusobacteria, Thermotogae, Chlamydiae, Dictyoglomi,

Table 23. Abundance of major 20 taxonomic category from phylum to species level of bacteria in sample INF

Phylum	Class	Order	Family	Genus	Species
unclassified (derived from Bacteria) (33.57%)	unclassified (derived from Bacteria) (33.57%)	unclassified (derived from Bacteria) (33.57%)	unclassified (derived from Bacteria) (33.57%)	unclassified (derived from Bacteria) (33.57%)	uncultured bacterium (31.80%)
Actinobacteria (25.80%)	Actinobacteria (class) (25.80%)	Actinomycetales (24.34%)	Solibacteraceae (10.62%)	*Candidatus Solibacter* (10.62%)	*Candidatus Solibacter usitatus* (10.52%)
Proteobacteria (14.55%)	Solibacteres (10.63%)	Solibacterales (10.63%)	Nocardiaceae (6.70%)	*Rhodococcus* (6.24%)	*Rhodococcus opacus* (5.75%)
Acidobacteria (14.13%)	Deltaproteobacteria (5.78%)	unclassified (derived from Betaproteobacteria) (3.43%)	Pseudonocardiaceae (4.55%)	*Saccharopolyspora* (3.80%)	uncultured *beta proteobacterium* (3.39%)
Firmicutes (6.41%)	Betaproteobacteria (4.36%)	unclassified (derived from Deltaproteobacteria) (3.20%)	unclassified (derived from Betaproteobacteria) (3.43%)	unclassified (derived from *Betaproteobacteria)* (3.43%)	uncultured *delta proteobacterium* (3.16%)
Bacteroidetes (4.12%)	Clostridia (3.11%)	Bacteroidales (3.09%)	unclassified (derived from Deltaproteobacteria) (3.20%)	unclassified (derived from *Deltaproteobacteria)* (3.20%)	*Acidobacterium capsulatum* (2.71%)
Verrucomicrobia (0.47%)	Bacteroidia (3.09%)	Clostridiales (2.89%)	Acidobacteriaceae (2.73%)	*Acidobacterium* (2.73%)	*Saccharopolyspora hirsute* (2.14%)
Cyanobacteria (0.36%)	Bacilli (2.90%)	Acidobacteriales (2.73%)	Frankiaceae (2.49%)	*Frankia* (2.49%)	*Frankia* sp. (1.61%)
Chlorobi (0.27%)	Acidobacteriia (2.73%)	Bacillales (2.10%)	Prevotellaceae (1.96%)	*Prevotella* (1.96%)	*Cellulosimicrobium cellulans* (1.54%)
Synergistetes (0.06%)	Gammaproteobacteria(2.19%)	Xanthomonadales (1.27%)	Bacillaceae (1.64%)	*Bacillus* (1.57%)	*Saccharopolyspora rectivirgula* (1.48%)

Phylum	Class	Order	Family	Genus	Species
Spirochaetes (0.04%)	Epsilonproteobacteria(0.92%)	Coriobacteriales (1.20%)	Promicromonosporaceae (1.56%)	*Cellulosimicrobium* (1.56%)	uncultured *soil bacterium* (1.21%)
Nitrospirae (0.03%)	unclassified (derived from Acidobacteria) (0.76%)	Desulfovibrionales (0.94%)	Xanthomonadaceae (1.27%)	*Stenotrophomonas* (1.27%)	*Stenotrophomonas maltophilia* (1.21%)
Chloroflexi (0.03%)	unclassified (derived from Proteobacteria) (0.72%)	Desulfuromonadales (0.90%)	Coriobacteriaceae (1.25%)	*Corynebacterium* (1.15%)	*Arthrobacter aurescens* (0.98%)
Deinococcus-Thermus (0.02%)	Flavobacteriia (0.61%)	Burkholderiales (0.82%)	Corynebacteriaceae (1.15%)	*Arthrobacter* (1.04%)	*Arthrobacter nitroguajacolicus* (0.98%)
Fusobacteria (0.01%)	Alphaproteobacteria (0.55%)	Lactobacillales (0.80%)	Micrococcaceae (1.14%)	*Geodermatophilus* (0.96%)	*Geodermatophilus obscures* (0.95%)
Thermotogae (0.01%)	Verrucomicrobiae (0.34%)	unclassified (derived from Acidobacteria) (0.76%)	Nocardioidaceae (1.05%)	*Mycobacterium* (0.91%)	*Frankia* sp. CcI3 (0.84%)
Chlamydiae (0.003%)	Chlorobia (0.27%)	unclassified (derived from Epsilonproteobacteria) (0.76%)	Geodermatophilaceae (0.96%)	*Desulfovibrio* (0.83%)	*Prevotella stercorea* (0.78%)
Dictyoglomi (0.003%)	Sphingobacteriia (0.27%)	unclassified (derived from Proteobacteria) (0.72%)	Lachnospiraceae (0.94%)	*Candidatus Koribacter* (0.76%)	*Candidatus Koribacter versatilis* (0.76%)
Gemmatimonadetes (0.002%)	Gloeobacteria (0.23%)	Flavobacteriales (0.61%)	Mycobacteriaceae (0.91%)	unclassified (derived from *Epsilonproteobacteria* (0.76%)	uncultured *epsilon proteobacterium* (0.75%)
Planctomycetes (0.002%)	Negativicutes (0.21%)	Enterobacteriales (0.50%)	Geobacteraceae (0.89%)	*Alistipes* (0.76%)	*Prevotella maculosa* (0.73%)

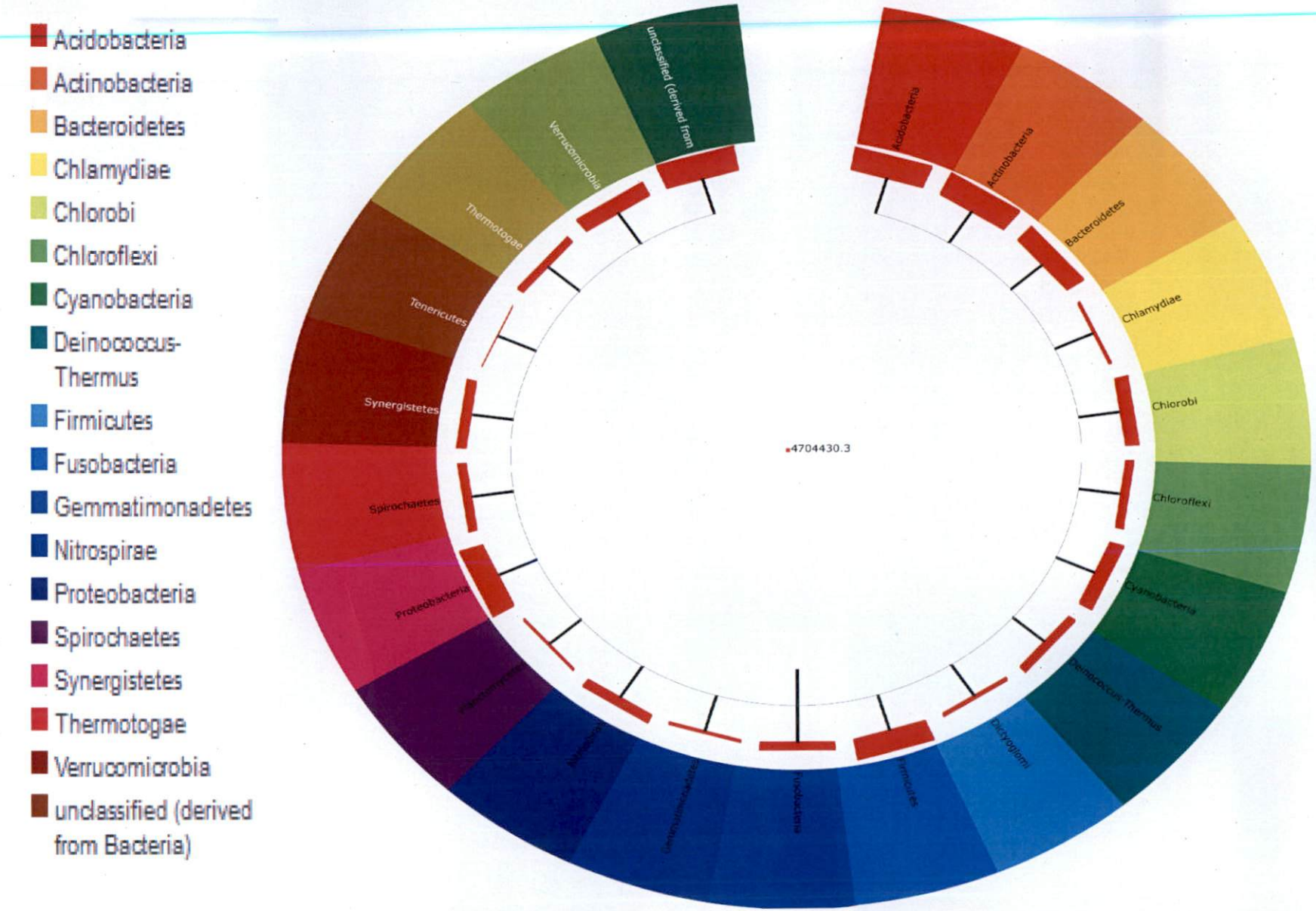


PLATE 26. Phylogenetic tree of bacteria at phylum level constructed in MG-RAST with Illumina sequence data set of INF sample

Gemmatimonadetes, Planctomycetes and Tenericutes constituted less than one per cent.

Total number of classes detected in the sample was 40 and the phylogenetic tree is given in Plate 27. Among them, unclassified (derived from bacteria) comprised of 33.57 per cent and was dominant over the following classes; Actinobacteria (25.80%), Solibacteres (10.63%), Deltaproteobacteria (5.78%), Betaproteobacteria (4.36%), Clostridia (3.11%), Bacteroidia (3.09%), Bacilli (2.90%), Acidobacteriia (2.73%) and Gammaproteobacteria (2.19%). Classes consisting of less than one per cent included Epsilonproteobacteria, unclassified Acidobacteria, unclassified Proteobacteria, Flavobacteriia, Alphaproteobacteria, Verrucomicrobiae, Chlorobia, Sphingobacteriia, Gloeobacteria, Negativicutes, Erysipelotrichi, Cytophagia and unclassified Cyanobacteria.

A total of 85 orders were detected in the sample and the phylogenetic tree is given in Plate 28. Among these, unclassified (derived from bacteria) was reported to be the highest (33.5 %) followed by Actinomycetales (24.3 %), Solibacterales (10.6%), unclassified (derived from Betaproteobacteria) (3.43%), unclassified (derived from Deltaproteobacteria) (3.20%), Bacteroidales (3.09%), Clostridiales (2.89%), Acidobacteriales (2.73%), Bacillales (2.10%), Xanthomonadales (1.27%), Coriobacteriales (1.20%). Bacteria belonging to orders Desulfovibrionales, Desulfuromonadales, Burkholderiales, Lactobacillales, unclassified Acidobacteria, unclassified Epsilonproteobacteria, unclassified Proteobacteria, Flavobacteriales, Enterobacteriales, Myxococcales and Verrucomicrobiales were found to be less than one per cent.

Altogether, 173 families were detected in the sample and phylogenetic tree is given in Plate 29. Among these, unclassified bacteria) constituted 33.57% per cent and dominated other families such as Solibacteraceae (10.62%), Nocardiaceae (6.70%), Pseudonocardiaceae (4.55%), unclassified Betaproteobacteria) (3.43%), unclassified Deltaproteobacteria (3.20%), Acidobacteriaceae (2.73%), Frankiaceae (2.49%), Prevotellaceae (1.96%),

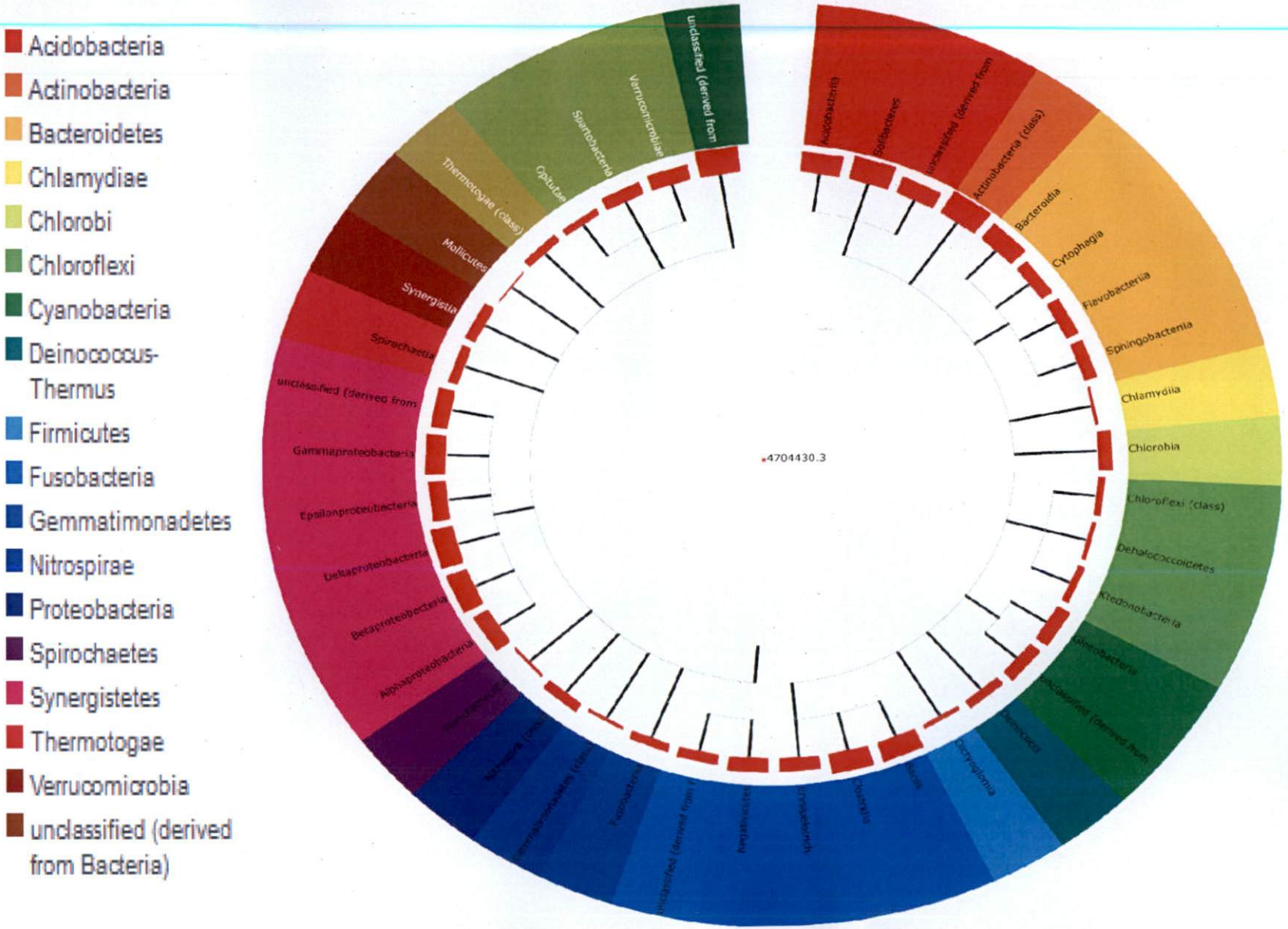


PLATE 27. Phylogenetic tree of bacteria at class level constructed in MG-RAST with Illumina sequence data set of INF sample

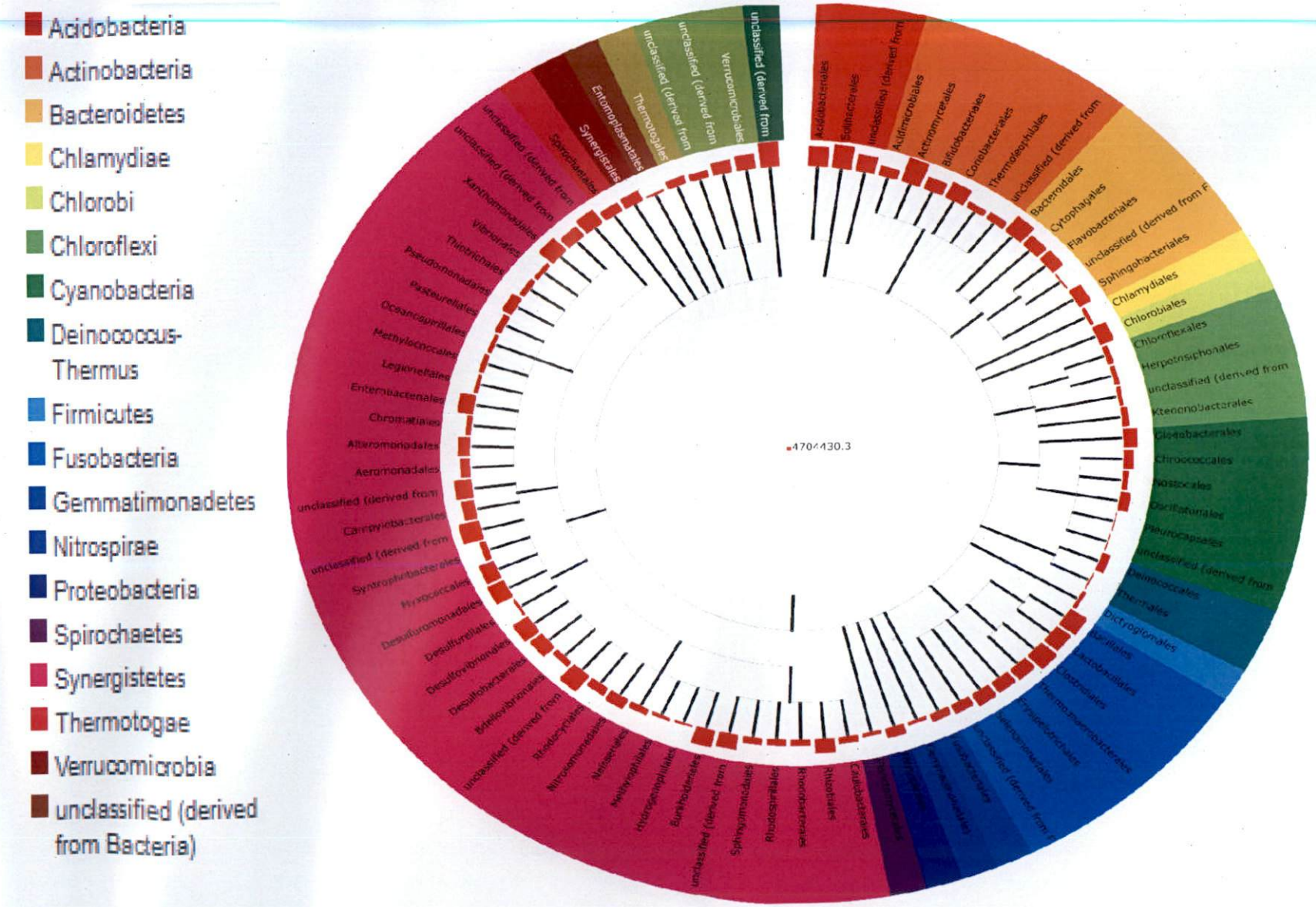


PLATE 28. Phylogenetic tree of bacteria at order level constructed in MG-RAST with Illumina sequence data set of INF sample

Bacillaceae (1.64%), Promicromonosporaceae (1.56%), Xanthomonadaceae (1.27%), Coriobacteriaceae (1.25%), Corynebacteriaceae(1.15%), Micrococcaceae (1.14%) and Nocardioidaceae (1.05%). Bacteria belonging to families Geodermatophilaceae, Lachnospiraceae, Mycobacteriaceae, Geobacteraceae, Desulfovibrionaceae and Thermomonosporaceae were found to be less than one per cent.

The analysis also revealed that a total of 365 genera were present in the sample. Dominant genera were found to be unclassified bacteria (33.57 %) followed by *Candidatus Solibacter* (10.62%), *Rhodococcus* (6.24%), *Saccharopolyspora* (3.80%), unclassified *Betaproteobacteria* (3.43%), unclassified *Deltaproteobacteria* (3.20%), *Acidobacterium* (2.73%), *Frankia* (2.49%), *Prevotella* (1.96%), *Bacillus* (1.57%), *Cellulosimicrobium* (1.56%), *Stenotrophomonas* (1.27%), *Corynebacterium* (1.15%), and *Arthrobacter* (1.04%). Bacteria belonging to genera *Geodermatophilus*, *Mycobacterium*, *Desulfovibrio*, *Candidatus Koribacter*, unclassified *Epsilonproteobacteria*, *Alistipes*, *Nocardioides* and unclassified *Proteobacteria*) were present less than one per cent.

Altogether, 868 species were identified in the sample. Among these, uncultured bacterium was the major species (31.80%), followed by *Candidatus Solibacter usitatus* (10.52%), *Rhodococcus opacus* (5.75%), uncultured *Betaproteobacterium* (3.39%), uncultured *Deltaproteobacterium* (3.16%), *Acidobacterium capsulatum* (2.71%), *Saccharopolyspora hirsute* (2.14%), *Frankia* sp. (1.61%). *Cellulosimicrobium cellulans* (1.54%), *Saccharopolyspora rectivirgula* (1.48%), uncultured soil bacterium (1.21%) and *Stenotrophomonas maltophilia* (1.21%). Least occurrence of species (those constituting less than one per cent) in soil receiving inorganic inputs were as follows: *Arthrobacter nitroguajacolicus* (0.98%), *Arthrobacter aurescens* (0.98%), *Geodermatophilus obscures* (0.95%), *Frankia* sp. CcI3 (0.84%), *Prevotella stercorea* (0.78%) and *Candidatus Koribacter versatilis* (0.76%).

4.12. Comparison of bacterial diversity in the samples at different taxonomy levels

Taxonomic category of bacteria present in the samples was compared using the table data in per cent, available under MG-RAST automated tools. Nine most abundant phyla (Table 24) were considered for comparison. Phylum Actinobacteria was highest in INM (57.95%) followed by ONM (54.39%) and INF (25.80%). Unclassified bacteria were found highest in INF (33.57%) followed by ONM (17.78%) and INM (16.42%). Acidobacteria and Cyanobacteria were most abundant in INF 14.13% and 0.36% respectively followed by INM 12.92% and 0.11% respectively. The presence of Proteobacteria, Chlorobi and Bacteroidetes was found to be maximum in INF (14.55%, 0.27% and 4.12 % respectively) and minimum in INM. Firmicutes was most abundant in ONM (6.76%) followed by INF (6.41%) and INM (4.80%). Phylum Cyanobacteria was highest in INF (0.36 %), followed by INM (0.11 %) and ONM (0.03 %).

The abundance of phyla was also graphically represented as bar chart, with data normalized to values between 0 and 1 to allow for comparison of differently sized samples (Plate 30) and heat map (Plate 36). Heat map is a graphical representation where abundance is indicated with different colours. High intensity of green colour indicates more abundance of microbes.

The abundance of predominant classes under each phylum was compared (Table 25). In phylum Actinobacteria, Class Actinobacteria was found highest in INM (57.95%) followed by ONM (54.39%) and INF (25.80%). Unclassified bacteria were most in INF (33.57%) followed by ONM (17.78%) and INM (16.42%). Solibacteres were found to be highest in INM (10.74%) followed by INF (10.63%) and ONM (8.49%). Acidobacteria was found highest in number in sample INF, followed by INM (4.72%) and by INF (2.73%) and ONM (1.53%). Under phylum Proteobacteria, Class Deltaproteobacteria were found to be present highest in INF (5.78%) followed by INM (3.45%) and ONM (1.53%),

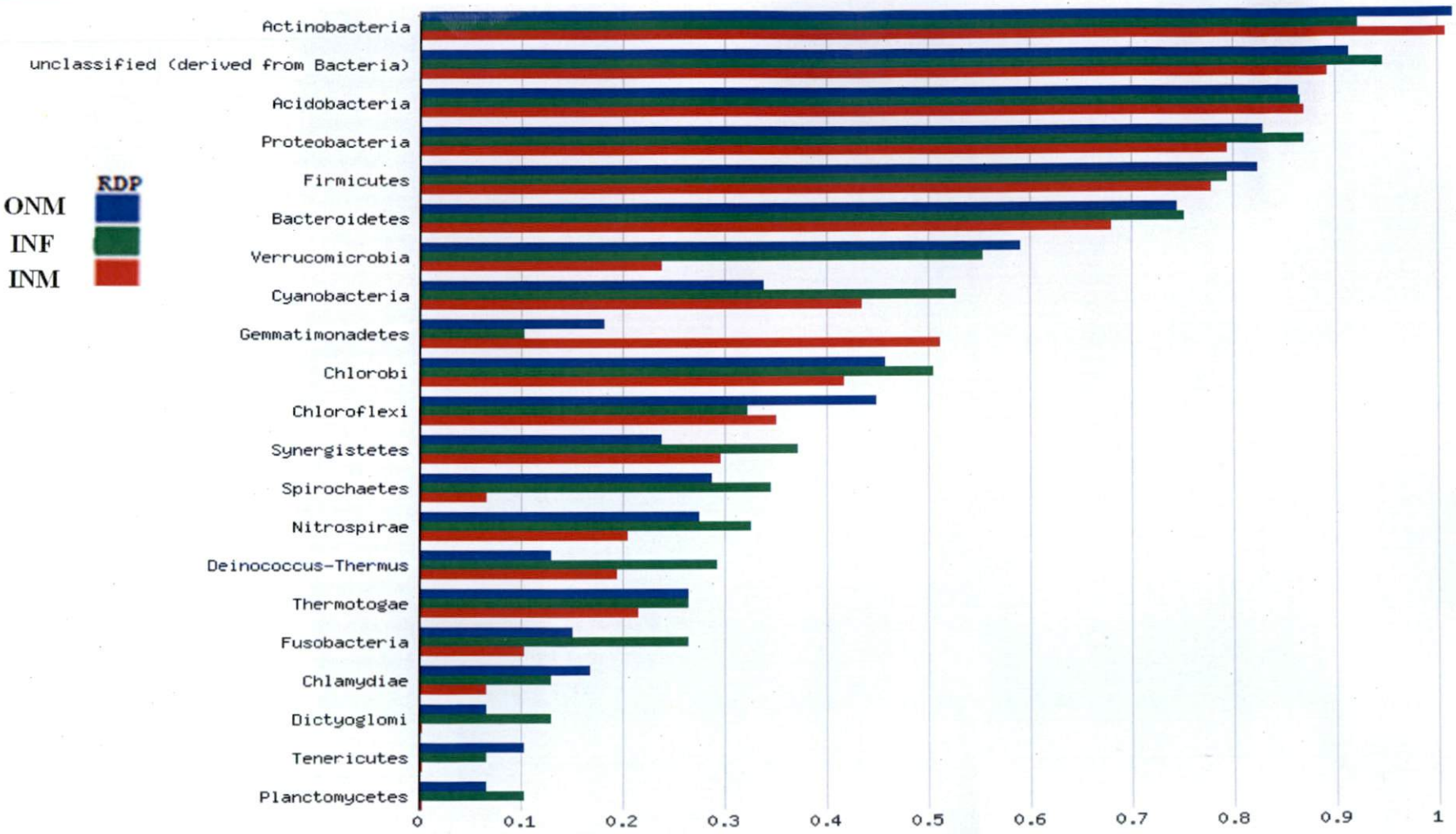


PLATE 30. Barchart of the samples at phylum level (data normalized to values between 0 and 1 to allow for comparison of differently sized samples)

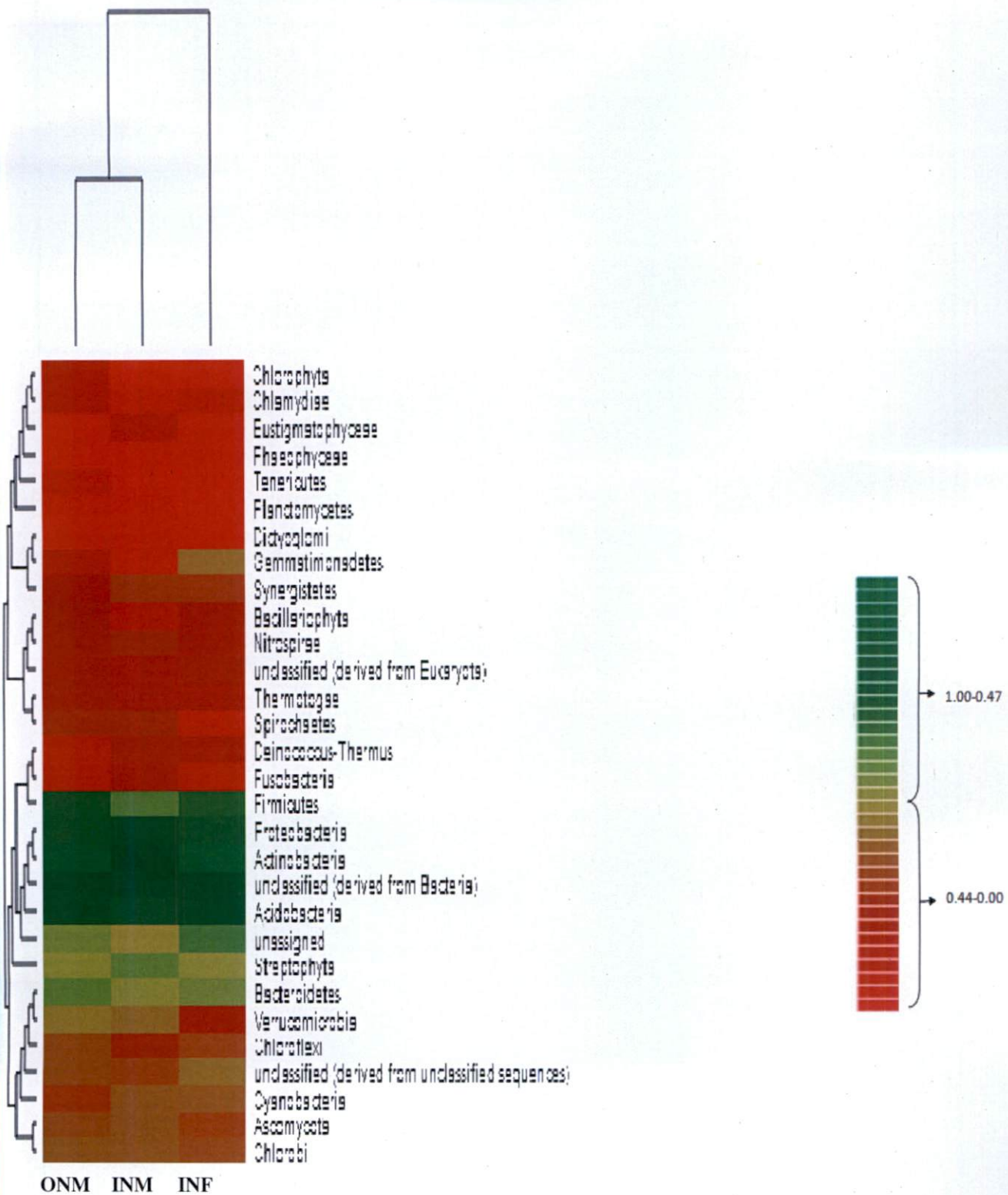


PLATE 36. Heatmap of the samples clustering using ward with Euclidean distance metric at phylum level constructed in MG-RAST with Illumina sequencing data set (heat map with colours represent the intensity of phylum (0-1) present in three samples.

Table 24. Abundance of phyla in the three samples

Phylum	ONM (%)	INM (%)	INF (%)
Actinobacteria	54.39	57.95	25.80
unclassified (derived from Bacteria)	17.78	16.42	33.57
Acidobacteria	10.31	12.92	14.13
Proteobacteria	7.05	5.62	14.55
Firmicutes	6.76	4.80	6.41
Bacteroidetes	2.81	1.67	4.12
Cyanobacteria	0.03	0.11	0.36
Chlorobi	0.12	0.09	0.27
Chloroflexi	0.11	0.04	0.03

Table 25. Abundance of class in the three samples

Class	ONM (%)	INM (%)	INF (%)
Actinobacteria	54.39	57.95	25.80
Unclassified(derived from bacteria)	17.78	16.42	33.57
Solibacteres	8.49	10.74	10.63
Acidobacteria	1.53	4.72	2.73
Deltaproteobacteria	2.90	3.45	5.78
Betaproteobacteria	1.88	0.46	4.36
Gammaproteobacteria	0.71	0.18	2.19
Alphaproteobacteria	0.79	1.00	0.55
Epsilonproteobacteria	0.66	0.49	0.92
Clostridia	3.80	4.72	3.11
Bacilli	2.85	0.07	2.90
Negativicutes	0.007	0.001	0.21
Erysipelotrichi	0.09	0.001	0.13
Bacteroidia	2.00	0.86	3.09
Shingobacteriia	0.16	0.14	0.27
Flavobacteriia	0.58	0.25	0.61
Unclassified (derived from Bacteroidetes)	0.009	0.36	-
Cytophagia	0.05	0.04	0.13
Verrucomicrobiae	0.46	0.01	0.34
Spartobacteria	0.06	0.002	0.11
Gloeobacteria	0.002	0.05	0.23
Unclassified (derived from Cynobacteria)	0.03	0.06	0.12
Chlorobia	0.12	0.09	0.27

Betaproteobacteria was found to be most abundant in INF (4.36%) followed by ONM (1.88%) and INM (0.46%), Gammaproteobacteria most abundant in INF (2.19%) followed by ONM (0.71%) and INM (0.46%). In Phylum Firmicutes, Class Clostridia was found to be highest in INM (4.72%) followed by ONM (3.80%) and INF (3.11%), class Bacilli and Negativicutes were found to be highest in INF (2.85% and 0.21% respectively).

Under phylum Bacteroidetes, classes Bacteroidia and Shingobacteria were found to be highest in INF (3.09%) and (0.27%) respectively followed by ONM (2.00%) and (0.16%) respectively and in INM (0.86%) and (0.14%).

Distribution of classes present in each phylum in the three samples were also visualized by bar chart tool (Plates 31-35) and heat map (Plate 37).

Unclassified (derived from Cyanobacteria) was highest in INF (0.12%) followed by INM (0.06%) and ONM (0.03%). class Gloeobacteria was highest in INF (0.23%) followed by INM (0.05%) and ONM (0.002%), Class Chlorobia under phylum Chlorobi found highest in INF (0.27%) followed by ONM (0.12%) and INM (0.09%). Abundance at different taxonomic categories such as order, family and genus of three samples is given in Tables 26, 27, 28 and heat maps is given in Plates 38-40.

At genus level, Unclassified bacteria was most abundant in INF followed by ONM and INM. Genus *Frankia* belonging to phylum Actinobacteria, was found highest in ONM (9.89%) followed by INM (3.50%) and INF (2.49%), *Rhodococcus* was highest in INM (16.98%) followed by INF (6.24%) and ONM (2.98%), *Saccharopolyspora* was highest in INM (8.16 %) followed by ONM (6.00%) and INF (3.80 %). *Nocardia* was highest in INM (4.82%) followed by ONM (3.82%) and INF (0.46%), *Cellulosimicrobium* was highest in ONM (2.24%) followed by INF (1.56%) and INM (0.61%), *Arthrobacter* was highest in ONM (4.86%) followed by INM (2.25%) and INF (1.04%), *Actinomadura* was highest in ONM (1.21%) followed by INM (1.01%) and CEH (0.45%), *Mycobacterium* was highest in INM (2.20%) followed by ONM (1.11%) and INF

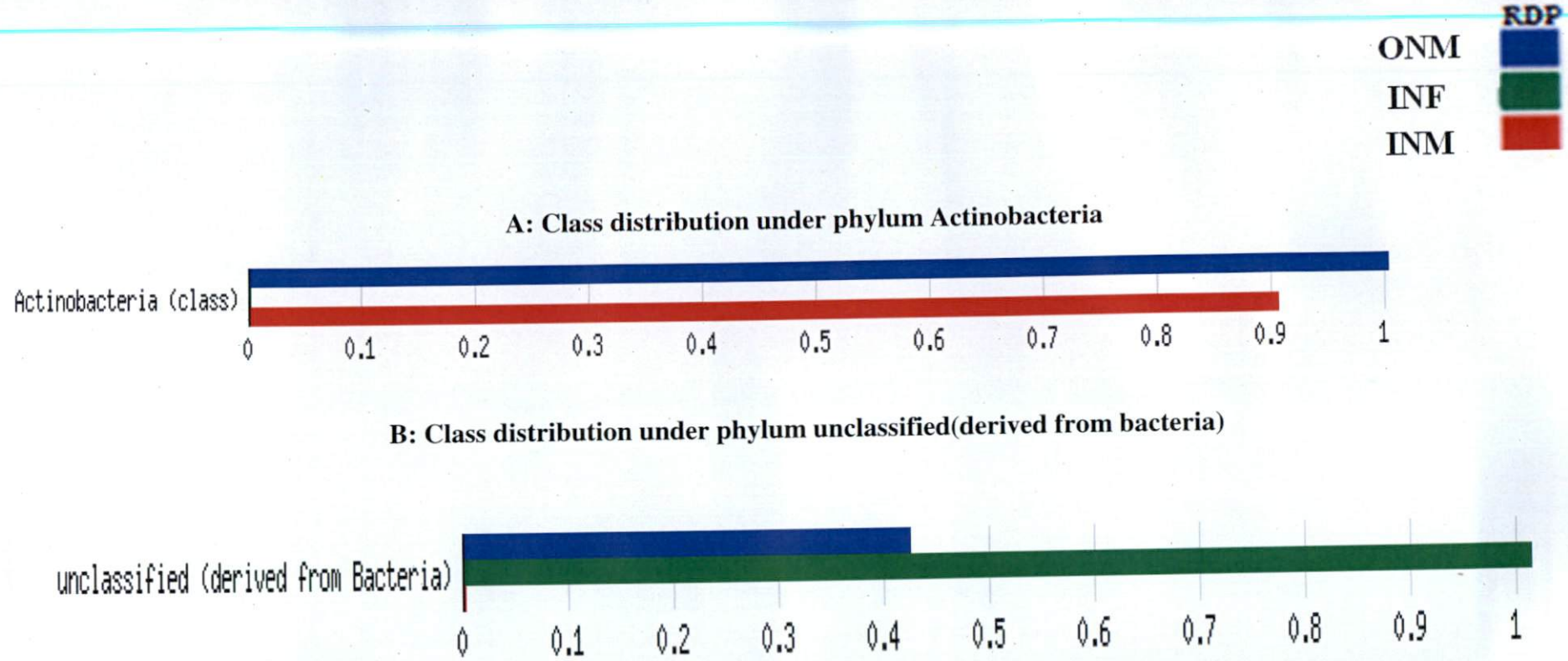


PLATE 31. Barchart of the samples showing class distribution under phylum (data normalized to values between 0 and 1 to allow for comparison of differently sized samples).

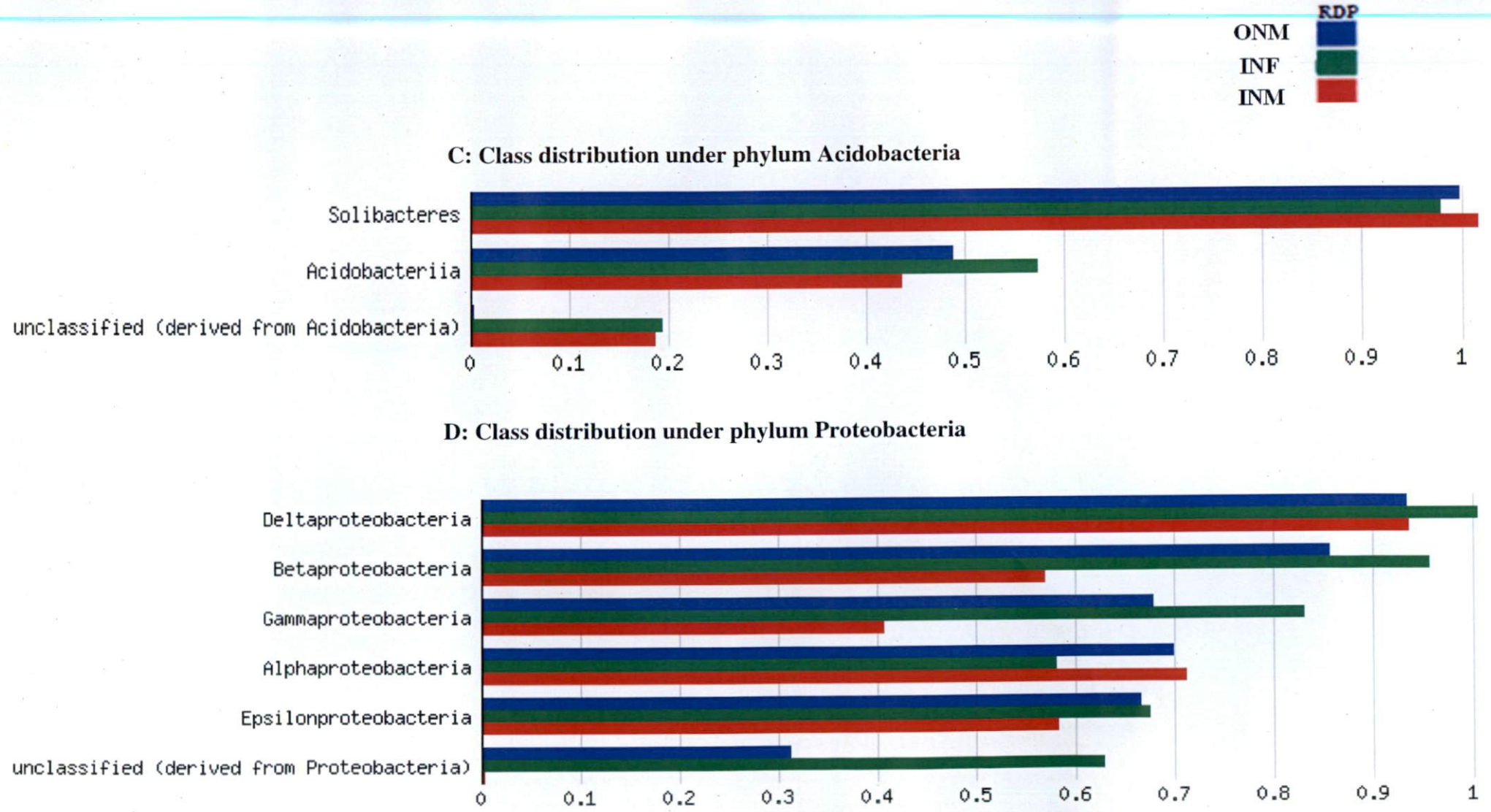


PLATE 32: Barchart of the samples showing class distribution under phylum (data normalized to values between 0 and 1 to allow for comparison of differently sized samples)

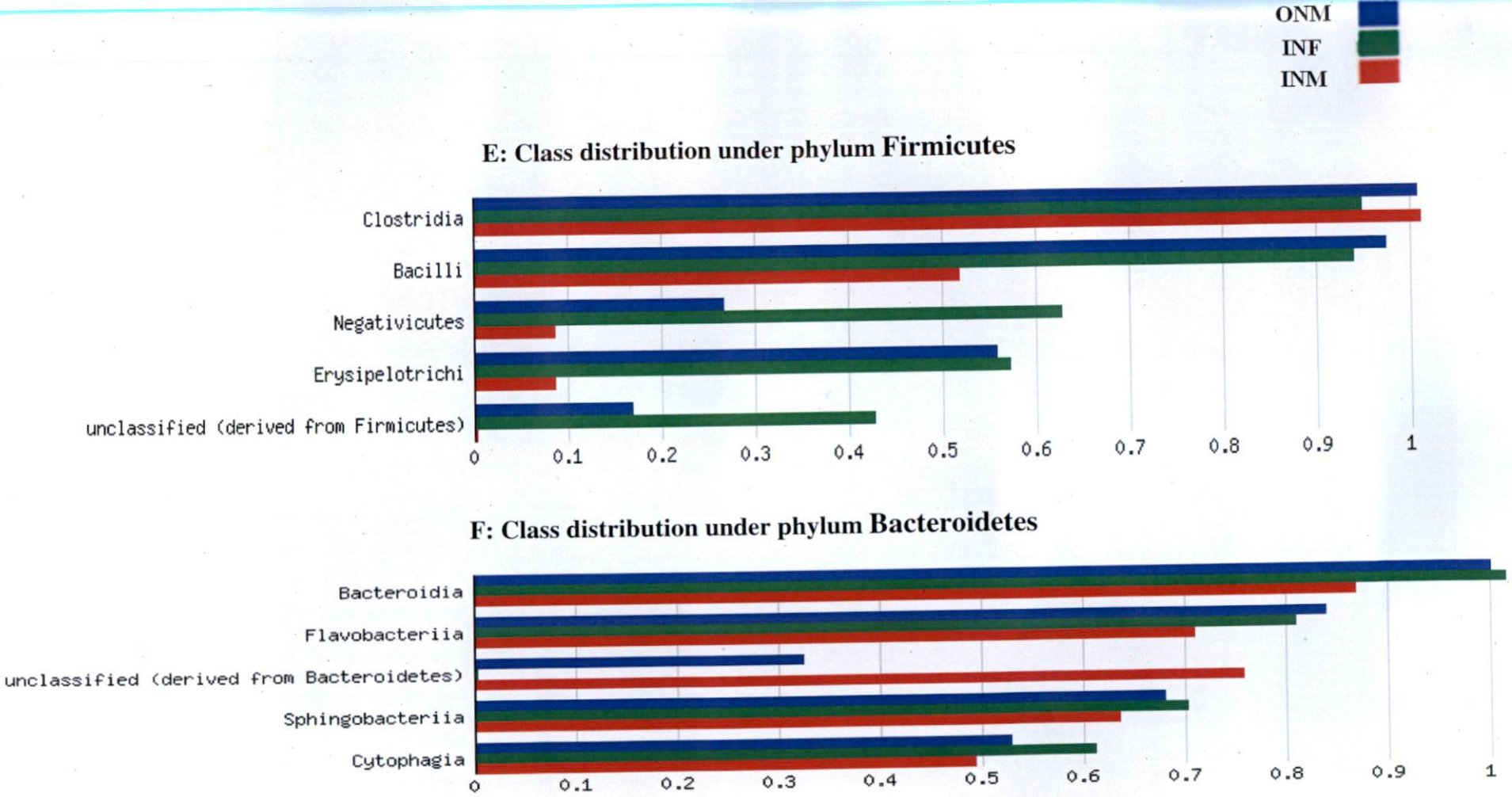
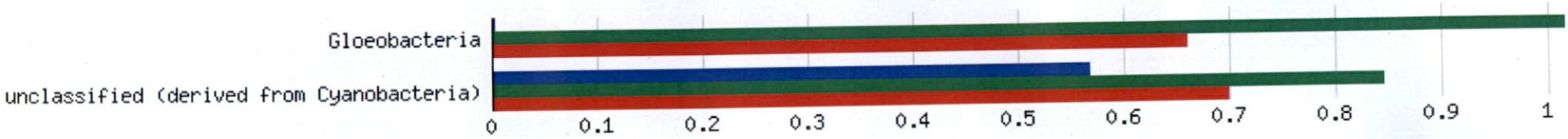


PLATE 33. Barchart of the samples showing class distribution under phylum (data normalized to values between 0 and 1 to allow for comparison of differently sized samples)

G: Class distribution under phylum Cyanobacteria



H: Class distribution under phylum Chlorobi

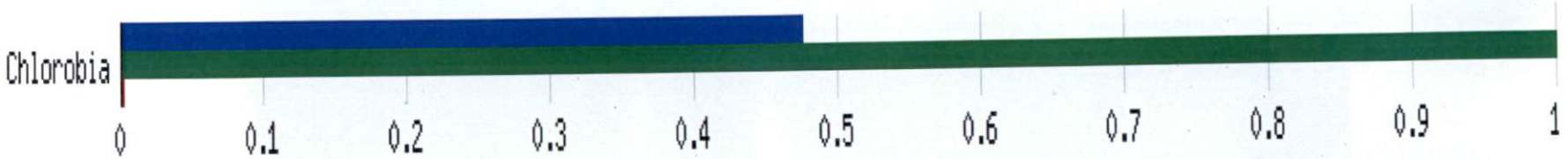


PLATE 34. Barchart of the samples showing class distribution under phylum (data normalized to values between 0 and 1 to allow for comparison of differently sized samples).

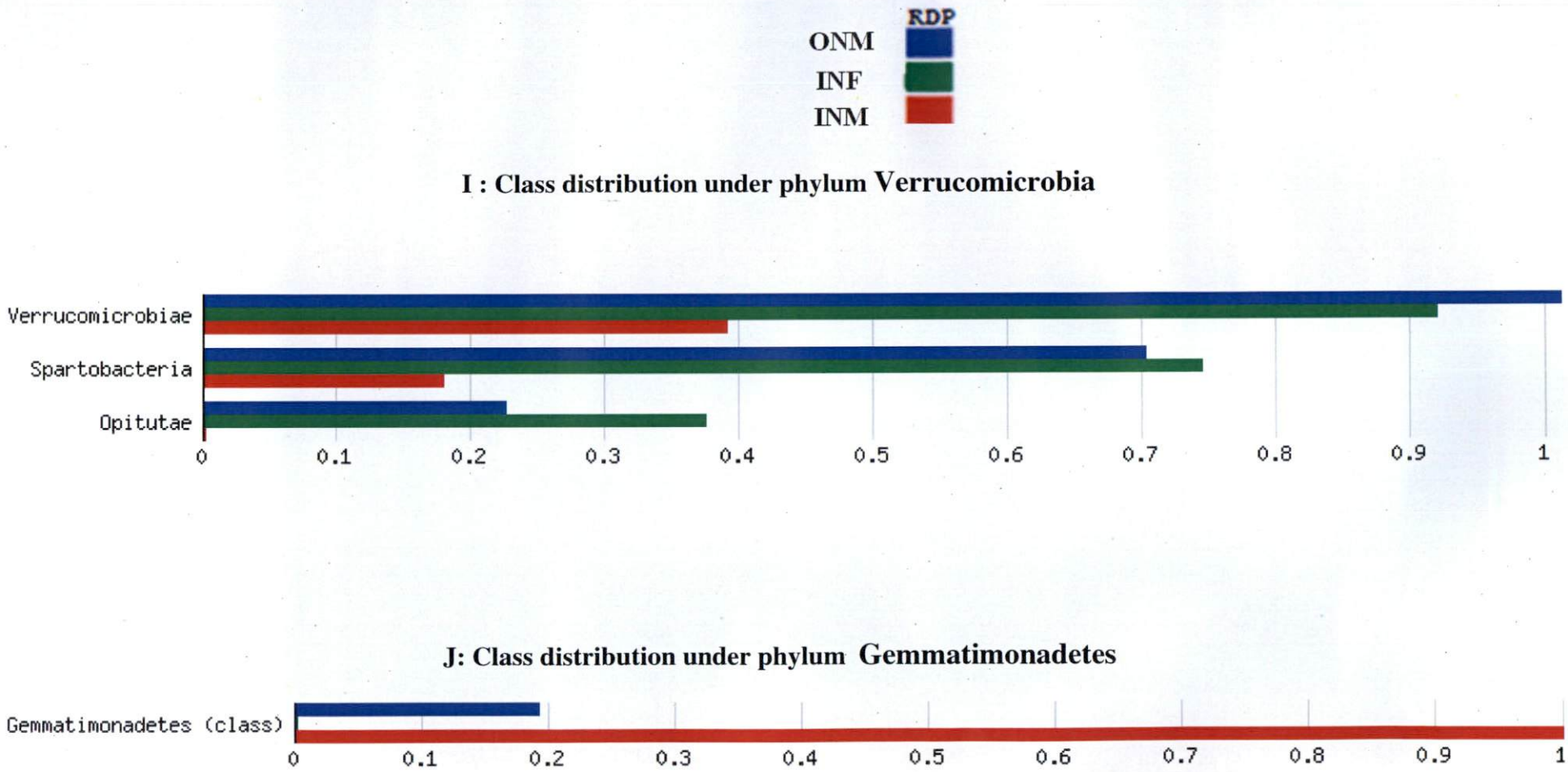


PLATE 35. Barchart of the samples showing class distribution under phylum (data normalized to values between 0 and 1 to allow for comparison of differently sized samples).

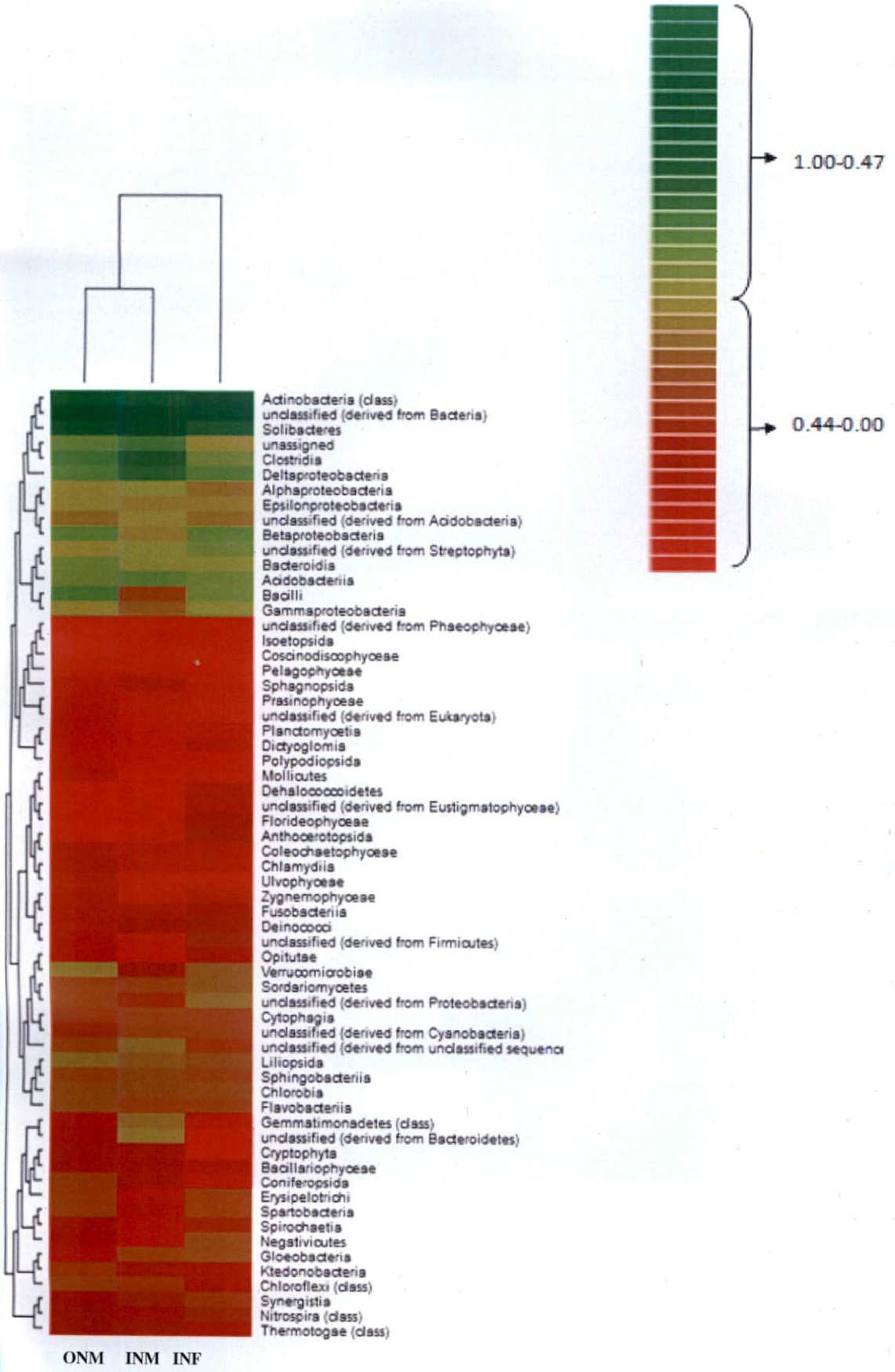


PLATE 37. Heatmap of the samples clustered using ward with Euclidean distance metric at class le constructed in MG-RAST with Illumina sequencing data set (heat map with colours represent the Intensity of classes (0-1) present in three samples).

Table 26. Abundance of order in the three samples

Order	ONM (%)	INM (%)	INF (%)
unclassified (derived from Bacteria)	17.78	16.42	33.57
Actinomycetales	49.67	54.42	24.34
Solibacterales	8.47	10.74	10.62
unclassified (derived from Betaproteobacteria)	0.41	0.25	3.43
unclassified (derived from Deltaproteobacteria)	1.30	1.41	3.20
Bacteroidales	2.00	0.80	3.00
Clostridiales	3.00	1.53	2.89
Acidobacteriales	1.53	1.52	2.73
Bacillales	2.65	0.04	2.10
Xanthomonadales	0.41	0.08	1.27
Coriobacteriales	4.20	2.11	1.20
Desulfovibrionales	1.21	0.52	0.94
Desulfuromonadales	0.30	1.41	0.90
Burkholderiales	1.35	0.17	0.82
Thermoanaerobacterales	0.79	3.19	0.22

Table 27. Abundance of family in the three samples

Family	ONM (%)	INF (%)	INM (%)
unclassified (derived from Bacteria)	17.78	16.42	33.57
Frankiaceae	9.89	3.50	2.49
Solibacteraceae	8.47	10.74	10.62
Micromonosporaceae	7.70	1.98	0.64
Pseudonocardiaceae	6.82	8.91	4.55
Nocardiaceae	6.80	4.58	6.70
Micrococcaceae	4.97	2.31	1.14
Coriobacteriaceae	4.20	2.11	1.25
Nocardioidaceae	3.64	4.58	1.00
Promicromonosporaceae	2.24	0.61	1.56
Bacillaceae	2.01	0.03	1.64
Thermomonosporaceae	1.67	3.18	0.82
Acidobacteriaceae	1.53	1.52	2.73
unclassified (derived from Deltaproteobacteria)	1.30	1.41	3.20
Corynebacteriaceae	1.15	2.01	1.15

Table 28. Abundance of genera in the three samples

Genus	ONM (%)	INM (%)	INT (%)
unclassified (derived from bacteria)	17.78	16.42	33.57
Frankia	9.89	3.50	2.49
Candidatus Solibacter	8.47	10.74	10.62
Rhodococcus	2.98	16.98	6.24
Saccharopolyspora	6.00	8.16	3.80
Nocardia	3.82	4.82	0.46
Acidobacterium	1.53	1.52	2.73
Arthrobacter	4.86	2.25	1.04
Nocardioides	2.57	3.26	0.75
unclassified (derived from *Betaproteobacteria)*	0.43	0.25	3.43
unclassified (derived from *Deltaproteobacteria)*	1.30	1.41	3.20
Bacillus	1.91	0.01	1.57
Cellulosimicrobium	2.24	0.61	1.56
Actinomadura	1.21	1.01	0.45
Desulfovibrio	1.14	0.45	0.83
Mycobacterium	1.11	2.20	0.91
Moorella	0.43	3.18	0.20
Acidimicrobium	0.05	1.01	0.05

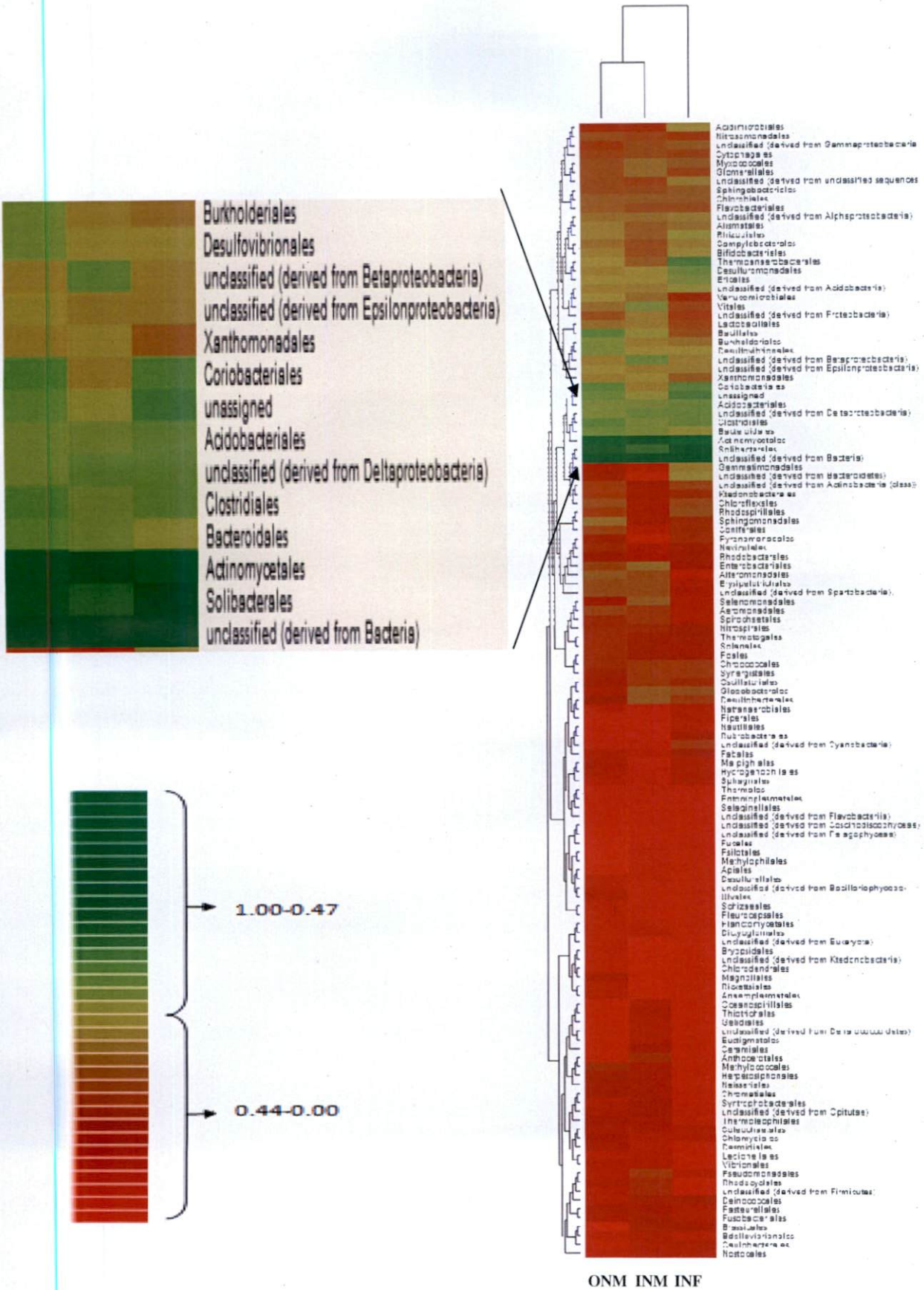


PLATE 38. Heatmap of the samples clustered using ward with Euclidean distance metric at order level constructed in MG-RAST with Illumina sequencing data set (heat map with colours represent the intensity orders (0-1) present in three samples.

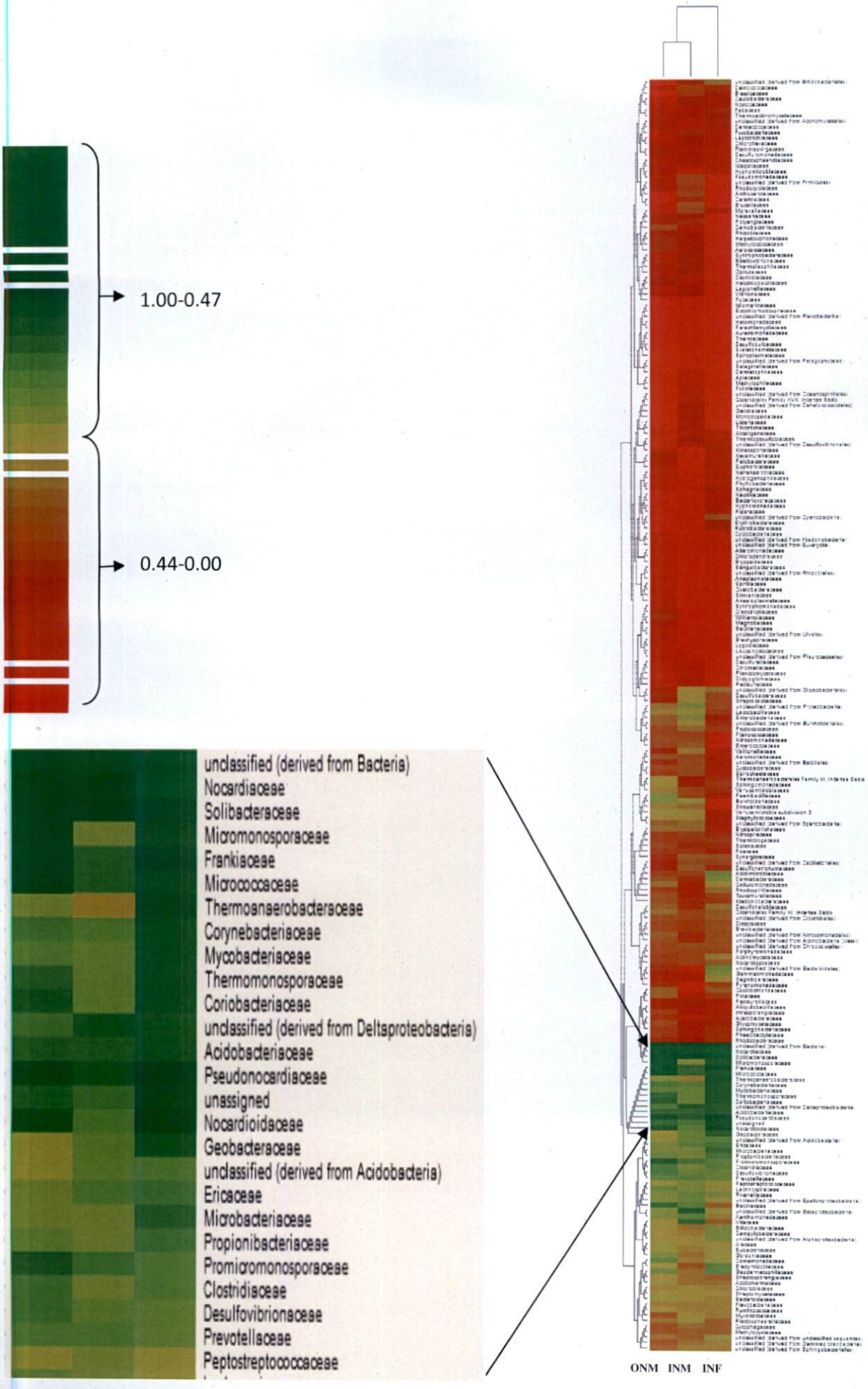


PLATE 39. Heatmap of the samples clustered using ward with Euclidean distance metric at family level constructed in MG-RAST with Illumina sequencing data set

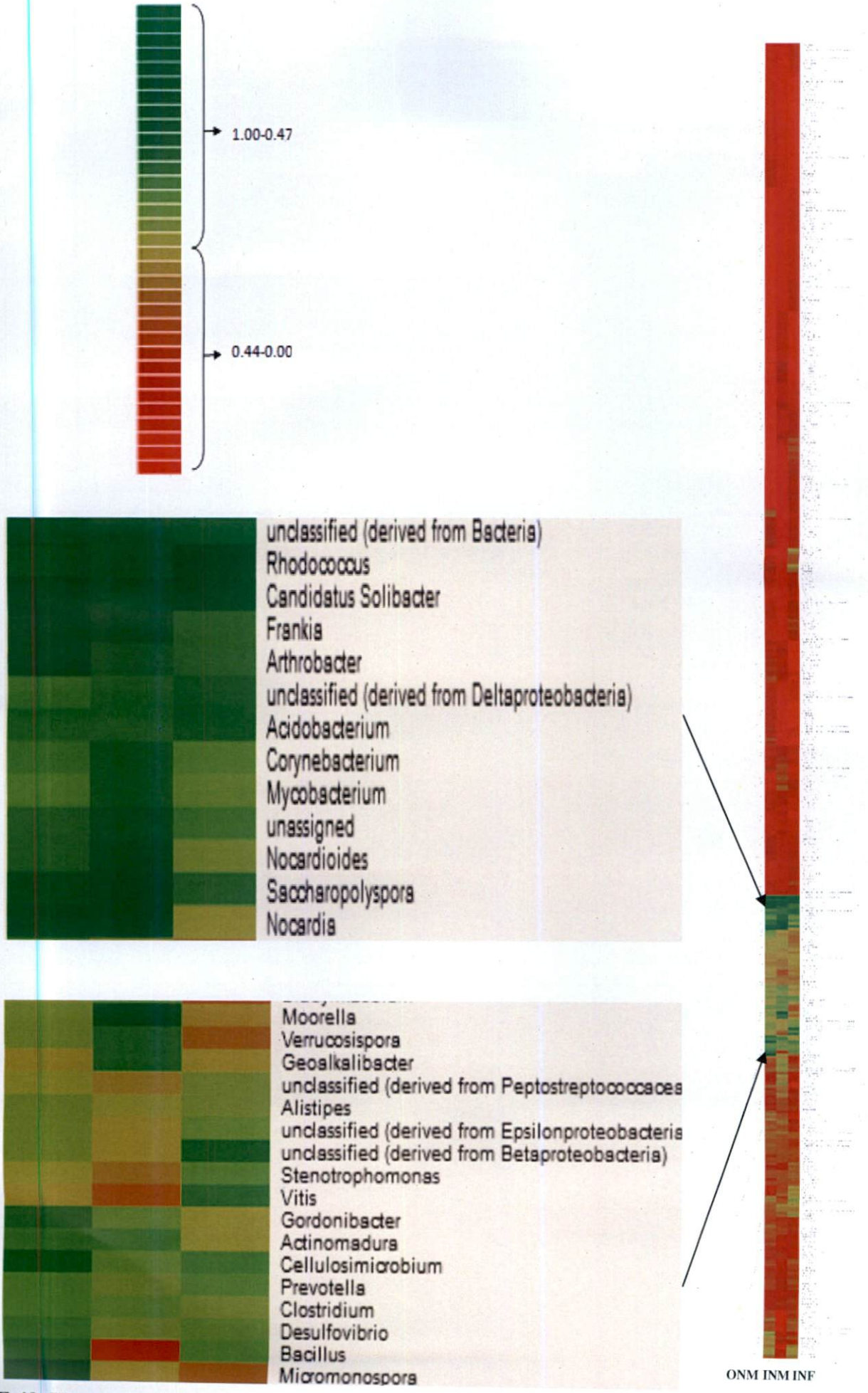


PLATE 40. Heatmap of the samples clustered using ward with Euclidean distance metric at genus level constructed in MG-RAST with Illumina sequencing data set

(0.91%), *Nocardiodes* was highest in INM (3.26%) followed by ONM (2.57%) and INF (0.75%) and *Acidomicrobium* was highest in INM (1.01%) followed by ONM (0.05%) and INM (0.05%).

The genus *Candidatus Solibacter* was highest in INM (10.74%) and followed by INF (10.62%) and ONM (8.47%); *Acidobacterium* was highest in INF (2.73%) followed by ONM (1.53%) and INM (1.52%). Genera belonging to Proteobacteria included unclassified *Betaproteobacteria,* which was highest in INF (3.43%), unclassified *Deltaproteobacteria,* which was highest in INF (3.20%) followed by INM (1.41%) and ONM (1.30%); *Desulfovibrio* was highest in ONM (1.14%) followed by INF (0.83%) and INM (0.45%). Genera belonging to Firmicutes included *Bacillus* and *Clostridium. Bacillus* was highest in ONM (1.91%) followed by INF (1.57%) and INM (0.01%). *Desulfovibrio* was highest in ONM (1.14%) followed by INF (0.83%) and INM (0.45%).

The total number of species present in ONM was 853, INM was 670 and INF was 867. For comparison study, only top 10 speices were considered (Table 29) and heatmap is given in Plate 41. Uncultured bacterium (unclassified) was observed to be most abundant in INF followed by ONM and INM, *Rhodococcus opacus* was highest in INM followed by INF and ONM. *Frankia* sp. was found to be maximum in ONM followed by INM and INF, *Candidatus Solibacterus itatus* was observed to be highest in INM followed by INF and ONM. *Saccharopolyspora hirsute* was recorded highest in INM followed by ONM and INF, *Arthrobacte raurescens* population was maximum in ONM followed by INF, *Saccharopolyspora rectivirgula* was present highest in INM followed by ONM and INF, *Moorella thermoacetica* was most abundant in INM followed by INF and ONM, *Nocardia pseudobrasiliensis* was highest in INM followed by ONM and INF. *Acidobacterium capsultam* was most abundant in INF followed by INM and ONM; *Arthrobacter nitroguajacolicus* in ONM followed by INF; *Nocardia cyriacigeONMica* in ONM followed by INM and INF and *Cellulosimicrobium cellulans* in ONM followed by INF. Uncultured *Betaproteobacterium* species

Table 29. Abundance of species in the three samples

Species	ONM (%)	INM (%)	INT (%)
Uncultured bacterium	16.52	16.22	31.80
Rhodococcus opacus	1.71	13.70	5.75
Frankia sp.	9.44	3.48	1.61
Candidatus solibacter usitatus	8.08	10.73	10.52
Saccharopolyspora hirsute	2.69	4.23	2.14
Arthrobacter aurescens	4.58	-	0.98
Saccharopolyspora rectivirgula	2.84	3.64	1.48
Moorella thermoacetica	0.41	3.18	0.19
Nocardia pseudobrasiliensis	0.007	2.61	0.003
Arthrobacter sp. scl-2	-	2.22	-
Acidobacterium capsultam	1.46	1.52	2.71
Arthrobacter nitroguajacolicus	4.58	-	0.98
Nocardia cyriacigeorgica	2.98	0.16	0.008
Cellulosimicrobium cellulans	2.14	-	1.54
uncultured *Betaproteobacterium*	-	-	3.39
Uncultured *Delta-roteobacterium*	1.24	1.41	3.16

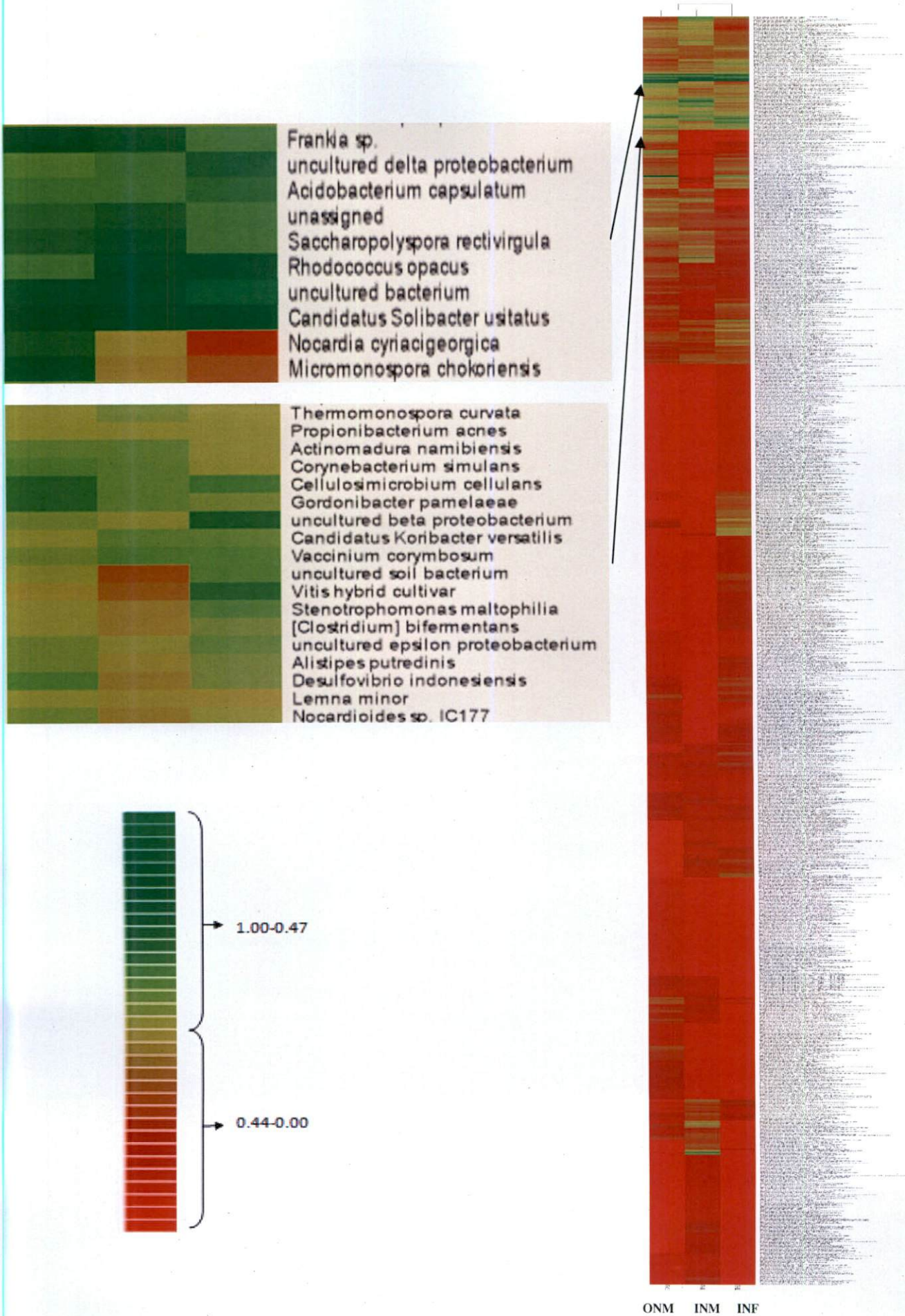


PLATE 41. Heatmap of the samples clustered using ward with Euclidean distance metric at species level constructed in MG-RAST with Illumina sequencing data set (heat map with colours represent the intensity of species (0-1) present in three samples.

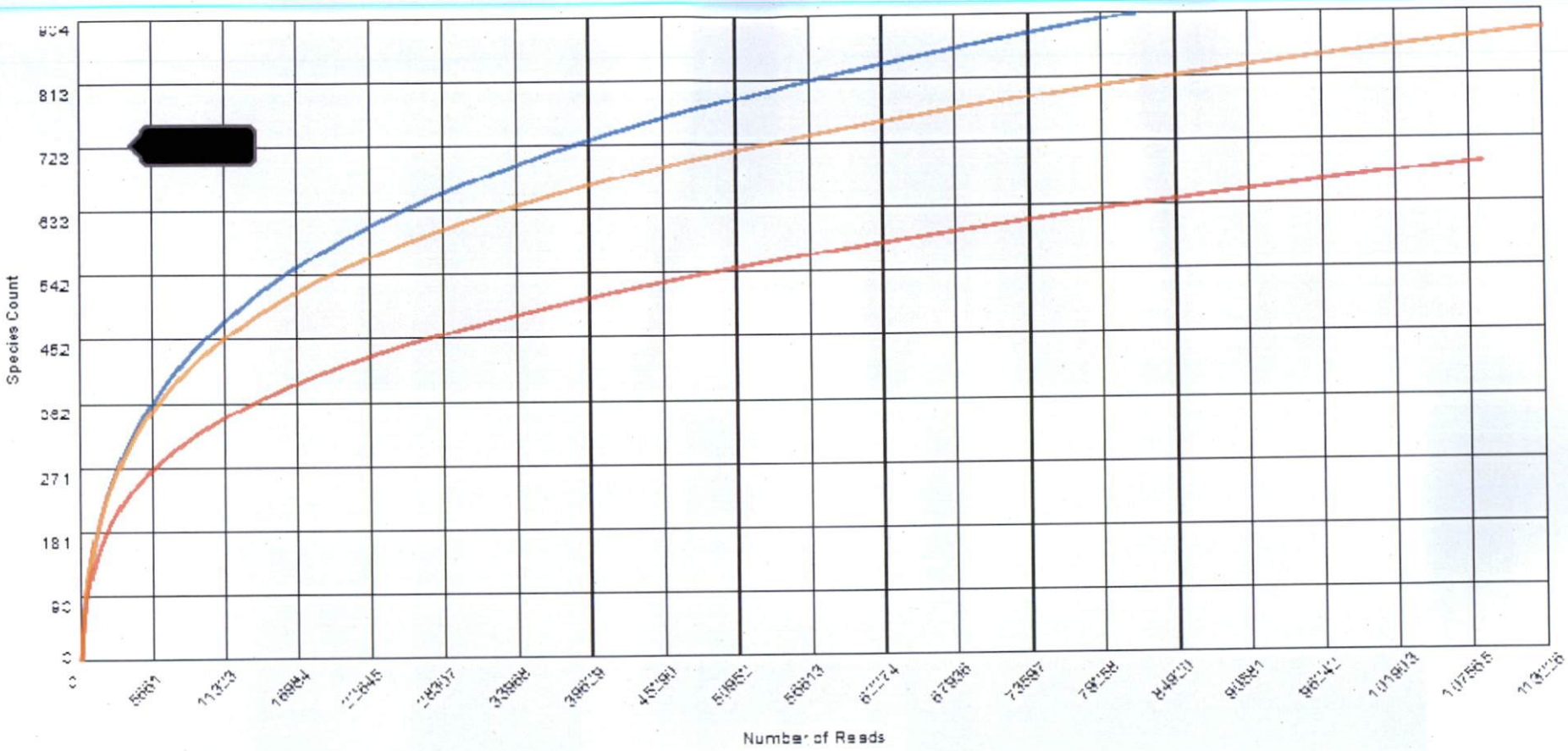
was highest in INF, uncultured *Delta-proteobacterium* was highest in INF followed by INM and ONM.

4.13. Alpha diversity within samples using MG-RAST

The data viualization tool 'rarefraction' was used to analyse alpha diversity and graph was generated using three metagenomes and the diversity of ONM was highest (54.52) followed by INM (44.13) and INF (38.86), as displayed in Plate 42.

4.14. Beta diversity between the sample

Beta diversity is the comparison of microbial communities between the samples and analysis revealed that microbial communities present in INM and ONM soil are similar with the value of 0.0335 and microbial communities present in INF sample differing from other two sample with the value of 0.0273, as displayed in Plate 43.



rarefaction curve	metagenome	alpha diversity
	INF	38.86
	INM	44.13
	ONM	54.52

PLATE 42. Rarefraction analyses of ONM, INM and INF by MG-RAST analysis platform

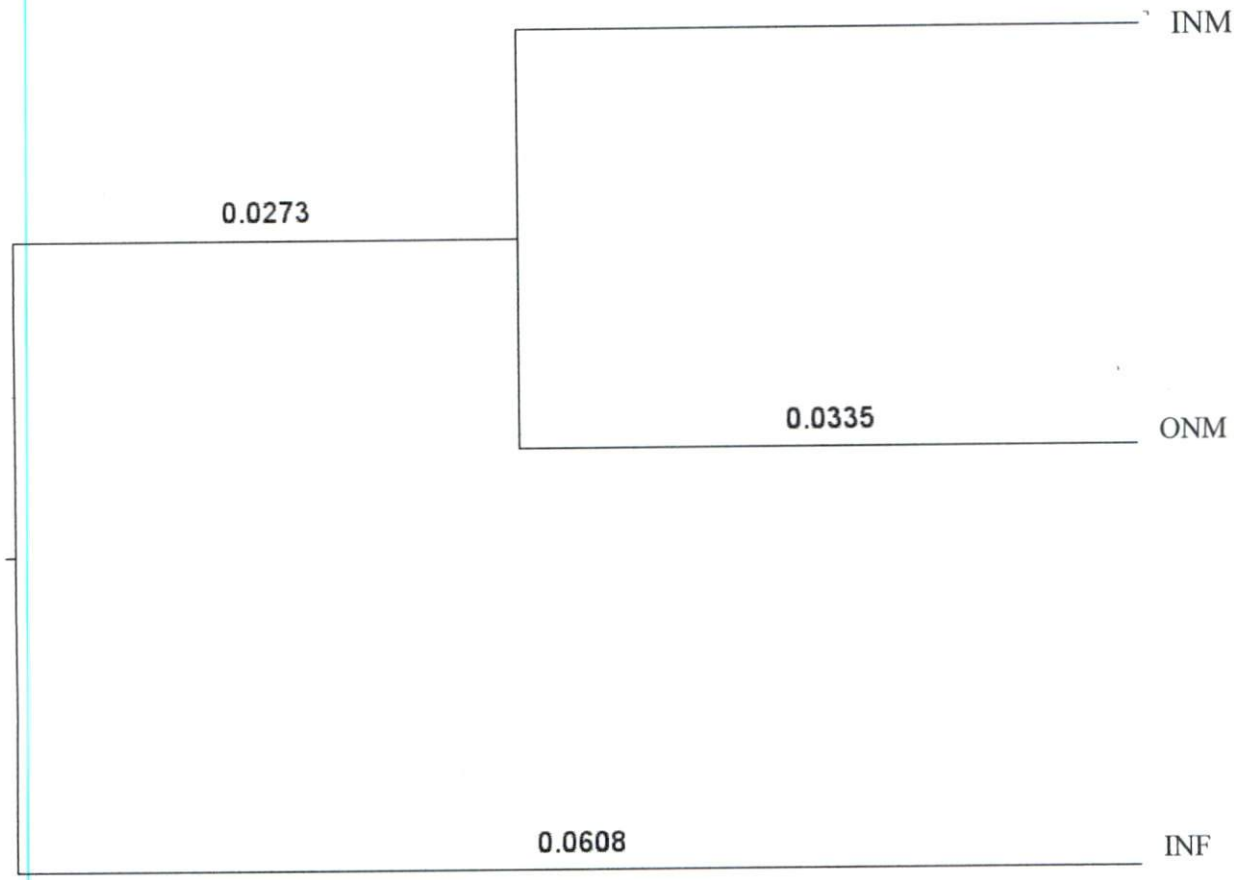


PLATE 43. Beta diversity between the samples

DISCUSSION

5. DISCUSSION

Rice rhizosphere soil is inhabitated by the complex microorganisms such as bacteria, fungi and actinomycetes. It plays an important role in all biochemical process and maintaining soil productivity (Lin *et al.*, 2004). Soil microorganisms also improve the soil physical and microbiological properties and probably represent the world's greatest reservoir of the biological diversity (Torsvik *et al.*, 1990). Understanding of the diversity is less known because traditional methods for the analysis of diversity of microbial communities in different environments are based on the cultivation of microorganisms on laboratory media. However, these methods are successful in isolating only less than 1 per cent of total population of bacteria from natural habitats (Ward *et al.*, 1992). It means that more than 99 per cent of genetic information and biotechnological potential of those cannot be studied or used by traditional methods.

To derive 99 per cent of microbes present in samples, metagenomics a DNA based approach was developed. It is a culture independent method, can be used to recover 16S rRNA gene directly from the environmental samples by isolation of total metagenomic DNA and targeting only V3 region for sequencing. Sequences obtained from V3 region of 16s rRNA gene by using Illumina MiSeq^{TM} high-throughput sequencing approach allowed researchers to identify several taxa (Lazarevic *et al.*, 2009) dwelling in the rhizosphere soil. Illumina platform is the benchtop version of next generation sequencing and it is based on sequencing by synthesis. It is mostly preferred because this approach generates several billion reads of nucleotide sequence (Bentley *et al.*, 2008) and allows sequencing of up to 500 bp lengths of read and through paired-end sequencing of 250 bp on their MiSeq platform. It has throughput of only 8 GB, and 34 million paired end reads and take approximately 39hto complete a 2 x 250 bp sequencing run. The Illumina MiSeq had the highest throughput and lowest error rates (Davis, 2013)

Soil rhizosphere is the region around the root and it is the most active site of microbial activity (Pathania *et al.*, 2014). It is well-established that root exudates

from plants exert a strong influence on the number and types of microorganisms present in the rhizosphere. Apart from this, soil management practices like tillage, mulching and application of organic and inorganic inputs also control microbial types and their functions (Lazcano *et al.*, 2013). The present study was aimed at assessing the diversity and abundance of bacteria in rhizosphere soil, as affected by organic and inorganic inputs, and a combination of both. Soil samples from rice rhizosphere were selected from Permanent Manurial Trial (PMT) plots of RARS, Pattambi, from soil that received organic inputs (ONM), soil under integrated nutrient management (INM) and soil which received only inorganic inputs (INF). These plots were selected because for over a period of 30 years, these soils have been receiving the same treatments, and hence would yield very good information on the effect of different soil management systems on the soil microflora. Sequence-based metagenomic approach was used in the study, so that information on unculturable microorganisms could also be obtained. This method has been widely used for assessing the diversity of microorganisms in various environments like the sea, soil, human and animal gut and pesticide-contaminated soils (Neelakanta and Sultana, 2013)

Physico-chemical and biological attributes of soil were assessed. Bulk density was graded in the 'normal' range in all the three soils. Among the three samples, lowest bulk density was recorded in ONM and the highest in INF. Similar results were earlier obtained by Valpassos *et al.* (2001), who opined that the low bulk density in organic matter applied soil may be due to the higher organic matter content in soil. Chaudhari *et al.* (2013) investigated the dependence of bulk density on different parameters such as texture, organic matter content and available micronutrients in soil and reported that, as the organic matter content increases, the bulk density of soil decreases and it also had a negative correlation with the available nutrients. Lower bulk density is required for the proper growth of the plants and, if the soil bulk density is higher then, soil strength increases and soil aeration decreases. This may lead to adverse effects on root growth, thereby a decrease in plant growth may also occur.

The pH ranged from 4.6 to 4.7 in all the three samples analysed. The acidic nature of the soil may be due to the repeated application of synthetic fertilizers that are with high proportions of total nitrogen, derived from ammonium sources and because of accumulation of organic matter and subsequent release of fulvic and humic acid. Laterite soils of Kerala have been reported to have acidic conditions (Chandran *et al.*, 2005). Liu *et al.* (2010) analysed the soil physico-chemical properties of arable soils treated with nitrogen and phosphorus fertilizers annually (NP), farmyard manure added annually (FYM), and farmyard manure plus N and P fertilizers added annually (NP+FYM) and reported that addition of FYM along with nitrogen and phosphorous lowered the pH of the soil. Microorganisms grow in specific environment, lower pH affect the activity of all the enzymes in the plant, availability of plant nutrients and growth of plant (Rengel, 2002).

Electrical conductivity reflects the total concentration of soluble salts and the extent of mineralization of organic matter in the soil. Electrical conductivity was found highest in INM followed by ONM and INF. Sarwar *et al.* (2008) reported lowest electrical conductivity in inorganic inputs treated soil than, in soil treated with integrated inputs (compost along with fertilizer).

The total nitrogen content of all the three samples was within the normal range, with ONM having the highest (0.19%), followed by INM and INF. Yilmaz and Alagoz (2010) also found the highest content of total nitrogen in soil treated with organic inputs (FYM). Soils which received integrated inputs (manure and synthetic fertilizer) gained more total nitrogen, as compared to soils that received only inorganic inputs (Liu *et al.*, 2010). Tadesse *et al.* (2013) also reported that total nitrogen content was significantly increased from 17 to 30 per cent when soil was treated with organic inputs.

In the present investigation, all the three soils recorded high content of available phosphorous, and among the samples, INM recorded maximum value. A high content of available P has been reported from most of the soils of Kerala

(GOK, 2016). Kaur *et al.* (2005) found that available P content was highest in soil which received both organic manures and synthetic fertilizers continuously for 7 years. Liu *et al.* (2010) reported that available phosphorus content of the soil was very less in the soil treated with N fertilizer at the rate of 4.98 mg kg^{-1}, compared with soil that received organic inputs (FYM at 21.17 mg kg^{-1}). A similar trend of high available phosphorous content in soil under integrated nutrient management was also reported by Tadesse *et al.* (2013), who stated that application of integrated inputs (FYM at 15 t ha^{-1} with 50 and 100 kg ha^{-1} P_2O_5 increased the available phosphorus content of the soil compared with the application of inputs.

Available potassium was graded as low in all the three samples and the highest value of 47.04 kg ha^{-1} was recorded in INF. The lowest available potassium was obtained in ONM. Hui *et al.* (2007) reported that NK fertilizer application to the soil has increased the available potassium 154 mg $kg^{-1}$and NP fertilizers application observed the least content of available potassium (66 mg kg^{-1}). With the above finding, it can be concluded that low content of available potassium in sample ONM was because of lack of external application of potassium fertilizer.

Highest available calcium and magnesium contents were recorded in ONM and minimum in INF. Similar results were obtained by Bulluck *et al.* (2002), who concluded in his experiment, that available calcium and magnesium content were highest in organic inputs (composted cotton-gin trash, cattle manure) treated soil and lowest in the inorganic inputs (fertilizer) treated soil.

Micronutrients are required in lower amounts than other essential nutrients. In the present study, available copper, zinc, manganese and boron was highest in the ONM sample. Bulluck *et al.* (2002) reported that available boron was found to be highest in the soil received organic inputs (cattle manure and yard waste compost). Similarly, Rathod *et al.* (2013) also reported highest micronutrient content in soil treated with the organic amendments such as farm yard maure (FYM).

In the present study, the lowest content of available copper, available zinc and available boron were recorded in INF. This may be due to the depletion of micronutrient by long-term applications of inorganic fertilizers, with no addition of organic matter. However, in case of available iron, maximum value was reported in INF and similar results were earlier reported by Fan *et al.* (2012).

Soil organic carbon (SOC) refers to the carbon component of organic compounds and carbon is a measureable component of soil organic matter. Organic matter has a critical role in the physical, chemical and biological function of agricultural soils. Estimated organic carbon status of the soil samples varied from 2.41 (ONM) to 1.69 (INF) per cent. Bulluck *et al.* (2002) reported that total carbon content in the soil was highest in organic inputs (cotton-gin trash, hay manure compost or yard-waste) treated soil (1.90 %) and lowest in commercial fertilizer treated soil (1.17 %). They concluded that application of organic inputs increases the organic matter. Similar results obtained by Kaur *et al.* (2005), who stated that organic inputs such as farm yard manure, poultry manure increased soil carbon content.

The biomass carbon was highest in INM followed by ONM and INF. As the microbial biomass carbon is the measure of carbon content of the living component present in the soil the highest content of biomass carbon in integrated nutrient management soil may be due to the abundance of microbes such as bacteria in INM. Similar finding was noticed by Dhull *et al.* (2010), who estimated the biomass carbon content in the soil of wheat crop and found that application of commercial fertilizer with green manure as organic inputs will increase the biomass carbon content.

Abundance and diversity of different groups of culturable microflora were assessed by serial dilution and plate technique. The soil sample INM harboured more number of bacteria and actinomycetes, compared to ONM and INF. Lowest population of bacteria and actinomycetes were noticed in INF. Similar results observed by Arbad *et al.* (2008), who reported that application of synthetic fertilizers along with the vermicompost observed increase in bacteria and

actinomycetes population in the soil of sweet sorghum as compared to the application of inorganic inputs. Gudadhe *et al.* (2015) stated that application of 10 tonnes of farm yard manure (FYM)/ha + recommended dose of fertilizer (RDF) to the soil under the cotton-chickpea cropping system showed positive trend on population of bacteria and actinomycetes. The positive effect of organic inputs on the population of bacteria and actinomycetes, could be because of nutrients provided during decomposition of organic substrates. The results of the present study also supports the finding that microbial biomass was also more in INM.

Among the different samples, maximum fungal population was observed highest in INF followed by INM and ONM. Population of fungi is directly correlated with the nitrogen content of the soil (Pratt and Tewolde, 2009). The application of synthetic fertilizers increases the ammonium nitrate in the soil. Ammonium nitrate application at 0.06M stimulated the growth of fungal species (*Aspergillus niger* and *Gaeumannomyce graminis*) (Veverka *et al.*, 2007).

Population of fluorescent pseudomonads was found to be highest in ONM and lowest in INM. Similar results were observed by Workneh and Van Bruggen (1994), who observed the highest population of fluorescent pseudomonads in the rhizosphere soil of tomato plant under organic farm (chicken manure and green manure) compared to the conventional farm (50 kg of nitrogen per hectare). In the present investigation, the sample ONM recorded maximum population of nitrogen fixers and this was followed by INM and INF. Orr *et al.* (2011) also observed highest population of nitrogen-fixing bacteria in the soil treated with the organic inputs. It has been reported that soils receiving high doses of fertilizer N may not promote biological nitrogen fixation and they are sensitive to the inorganic inputs (Omar and Abd-Alla, 1992).

The key factor for any metagenomic study is the isolation of quality environmental DNA in appreciable amount from a given environment. It is also one of the bottlenecks in metagenomic studies. The extracted DNA should be of high quality and in good yield, in order to pursue molecular biology applications

(Kauffmann *et al.,* 2004). Over the past 10 years, several techniques have been described for DNA extraction from environmental samples, in addition to commercial kits (Purohit and Singh, 2009). These protocols are broadly classified as direct and indirect methods. Indirect method involves bacterial cell extraction from the environmental sample followed by cell lysis and DNA recovery (Holben *et al.,* 1988). Direct extraction involves cell lysis within the sample matrix, followed by separation of DNA from cell debris (Ogram *et al.,* 1987). In the latter method, lysis can be achieved either by soft or harsh treatments. Soft lysis is based on the disruption of the microorganisms by enzymatic and chemical means, whereas harsh lysis approaches involve mechanical cell disruption by bead beating, sonication, freeze-thawing and grinding (Siddhapura *et al.,* 2010).

In the present study, DNA was isolated by the direct method, following the procedure of Siddhapura *et al.* (2010). Extraction buffer contained detergent (SDS) which helps in complete lysis of bacterial cells and release of DNA. The denatured contaminating molecules were precipitated and removed with the help of phenol and chloroform. Both phenol and chloroform denature proteins; get solubilised in organic phase or interphase, while nucleic acids remain in aqueous phase. Chloroform is mixed with phenol to increase the efficiency of nucleic acid extractions. The increased efficiency is due to the ability of chloroform to denature proteins. It helps in removal of lipids, thus improving separation of nucleic acids into the aqueous phase. Chloroform: isoamyl alcohol improves deproteinization. DNA thus extracted in aqueous phase was precipitated with sodium acetate and ethanol. Final DNA pellet was dissolved in sterile distilled water. Upon agarose gel electrophoresis, a single sharp band was observed.

Metagenomic DNA isolated from all the three samples was used for next generation sequencing, targeting the V3 region of the 16S rRNA gene (Fig 1), at the Sequencing Facility of SciGenom, Cochin using Illumina Miseq^{TM} sequencing system. The steps followed in library preparation are depicted in Fig. 2. Schmalenberger *et al.* (2001) reported that parallel analysis of 3 different hypervariable regions of 16S rDNA sequence (V2–V3, V4–V5, and V6–V8

regions) was effective in determining the composition of bacterial consortia in maize rhizospheres. Chakravorty *et al.* (2007) studied different hypervariable regions and demonstrated different efficacies with respect to species calls in different genera, and the V2 and V3 regions were most effective for universal genus identification.

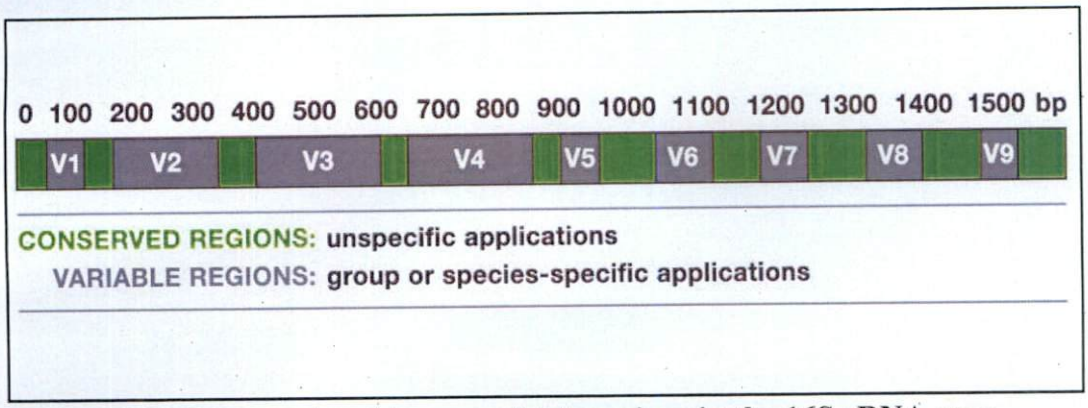


Fig.1. Conserved and hypervariable regions in the 16S rRNA gene

Total raw sequencing reads (paired end) of ONM, INM and INF DNA samples were 3, 74,632; 3, 35,666 and 3, 81,295 respectively. Raw sequences were filtered based on base quality score, average base content per read and GC distribution in the reads.

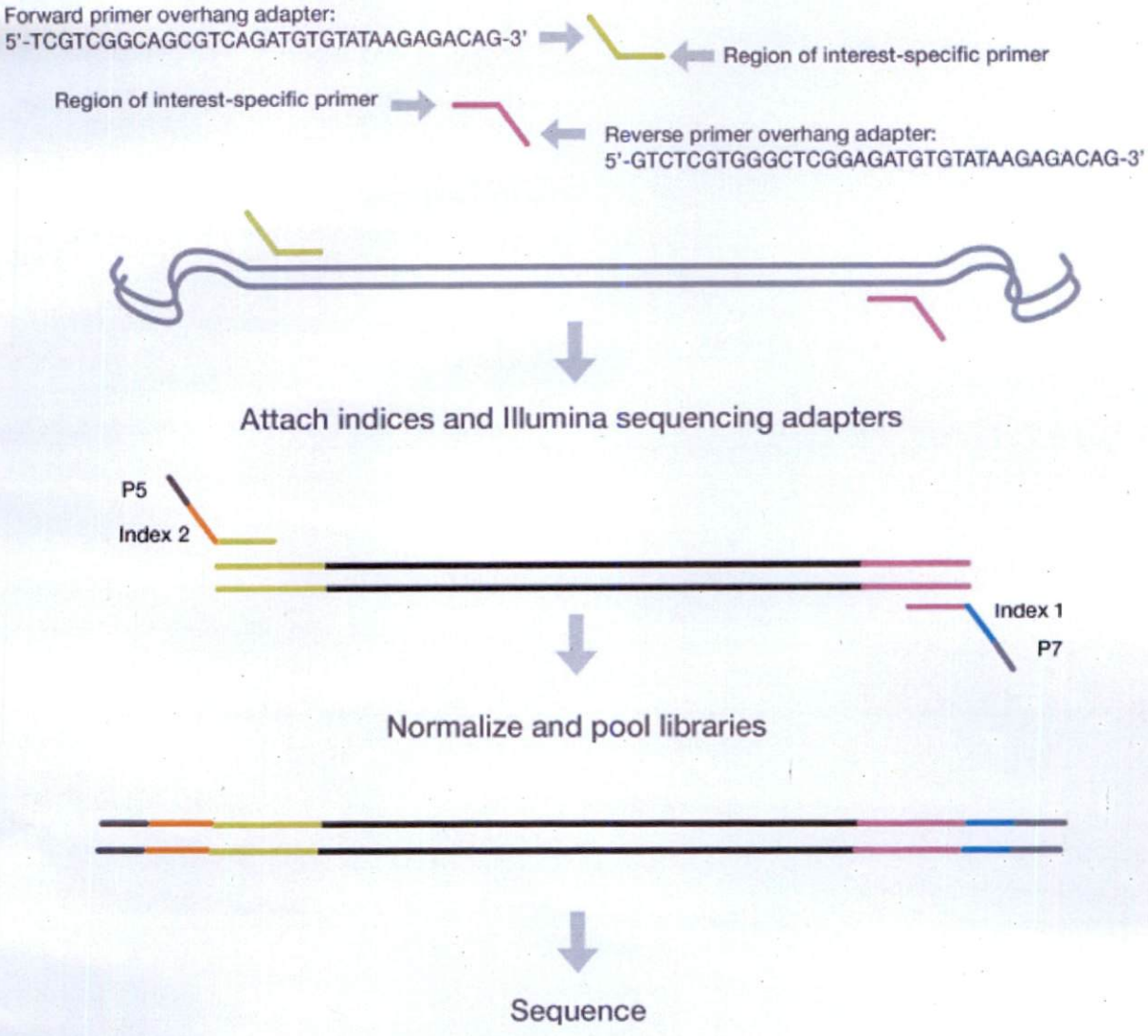


Fig2. 16s rRNA library preparation work flow

Reads that did not cluster with other sequences *i.e.* singletons were removed. Chimeras were also removed using UCHIME program. The pre-processed consensus V3 sequences were finally grouped into operational

taxonomic units (OTUs) using the clustering program UCLUST at a similarity threshold of 0.97. All the pre-processed reads were used to identify the OTUs using QIIME program and the representative sequences were aligned against the Greengenes core set reference database using PyNAST program. Representative sequence for each OTU was classified using RDP classifier and Greengenes OTU database. Sequence data were uploaded to the MG-RAST automated analysis server. It is an important bioinformatics analysis tool for phylogenetic and functional metagenomes. The current server version is 3.6. MG-RAST is an abbreviation of Metagenomic Rapid Annotations using Subsystems Technology and was launched in 2007. The pipeline automatically produces functional assignments to sequences that belong to the metagenome by performing sequence comparisons to Ribosomal Database Project (RDP) using a maximum e-value of $1e^{-5}$. A minimum identity of 90 per cent and a minimum alignment length of 15 bp were selected. MG-RAST also provides data visualization tools for comparing different metagenomes. The tool was useful in having quantitative insights of the microbial population present in each sample.

Domain bacteria was predominant in the samples, and among three samples; ONM showed high diversity of bacteria followed by INM and less diversity of bacteria was observed in INF. It is quite natural that addition of organic matter favours the growth of bacterial population. Eukaryota were also present in the samples. Microbes which were identified but not yet classified under each taxonomic category were also observed and called as "unclassified" sequences which were not identified. These were grouped under unclassified, because of lack of any similarity with current database.

The results from the sequence data analysis of three samples revealed that phylum Actinobacteria was dominant over other phyla, irrespective of management practices, with highest value in INM sample (57.95%) and lowest in INF (25.80%). It may be because the farm yard manure used as an organic input for organic nutrient management and integrated nutrient management was farm yard manure and greater population of actinomycetes was noticed in the soil

treated with the farm yard manure (Arbad *et al.*, 2014). Actinobacteria play an important role in the cycling of organic compounds and have been associated with soil organic matter and production of humic acid (Gomes *et al.*, 1996). Piao *et al.* (2008) reported that the actinobacterial communities detected in soil applied with organic and inorganic amendments did not significantly change the phylogenetic diversity, but did significantly change the community structure.

Organic nutrient management favoured bacterial growth and one of the most abundant phylum detected was Actinobacteria in soil containing good organic matter (Chinnadurai *et al.*, 2014). They are producers of antibiotics and therefore, may be playing key role in controlling many plant pathogens. Class Actinobacteria includes various genera and *Rhodococcus* was predominant in INM (16.98%) and lowest in ONM (2.98%). *Rhodococcus* is reported to be capable of catabolizing compounds to produce bioactive steroids and also are involved in fossil fuel biodesulfurization, It is a bacterium of commercial application in the field of environmental and industrial biotechnology, as it synthesizes products such as surfactants, flocculants, amides and polymers (Bell *et al.*, 1998). Genus *Saccharopolyspora* is known for the antibiotic production and was most abundant in INM (8.16%) and lowest in INF (3.80%). Genus *Frankia*, filamentous bacteria that fixes atmospheric nitrogen is also known for its capacity for decomposition of organic matter. It was most abundant in ONM (9.89%) and lowest in INF (2.49%),

Genus *Arthrobacter* population was observed highest in ONM (4.86%) and lowest in INF (1.04%). These bacteria are easily isolated, indigenous, aerobic bacteria that have ability to survive under harsh conditions (Mongodin *et. al.*, 2006). They can also survive temperature variations, starvation, ionizing radiation, toxic chemicals and oxygen radicals. They can metabolize a diverse group of chemicals and pollutants including nicotine, glyphosate and 2,4-D.

Phylum Acidobacteria was found to be highest in INF (14.13%) and lowest in ONM (10.31%). Reports indicate that Acidobacteria was dominant group (16.5%) in the soil applied with organic manure (Sun *et al.*, 2004).

Occurrence of Acidobacteria was more in soils with low pH (Griffiths *et al.*, 2011). Naether *et al.* (2012) reported that Acidobacteria were highly active and *in situ*. Though they are abundant in soils, their metabolic diversity and role in biogeochemical processes is still not clearly known, since most of them are unculturable. They have been adapted to low substrate availability and C availability was negatively correlated with Acidobacteria. In the phylum Acidobacteria, Class Solibacteres was most abundant in INM (10.74%) and lowest in ONM (8.47%). Under family Solibacteraceae,a species (*Candidatus Solibacter usitatus*) belonging to genus *Candidatus Solibacter* was obtained. This belongs to the category 'yet to be cultured', produces enzymes to break down organic carbon available in its environment and participate in the degradation of plant litter (Eichorst *et al.*, 2011) and acts as an ecosystem engineer in the soil. *Candidatus Solibacter* was found to be highest in INM (10.74%) and lowest in ONM (8.47%). Genus *Acidobacterium* plays major role in the fluxes of carbon, nitrogen and iron across microbial communities and the genus was highest in INF (2.73%) and lowest in INM (1.52%).

Phylum Proteobacteria was found highest in INF (14.55%) and lowest in INM (5.62%). This phylum is a metabolically diverse group of bacteria in several subphyla. It has been reported that β-Proteobacteria increased in response to both labile and chemically recalcitrant substances (Goldfarb *et al.*, 2011). Proteobacteria was observed to be the dominant phylum present in the rice rhizosphere soil (Arjun and Harikrishnan, 2011). Pisa *et al.* (2011) also reported that Proteobacteria was highest rhizosphere soil receiving inorganic fertilizers. Classes under phylum Proteobacteria were α, β, γ, δ, and ε Proteobacteria, among which δ-Proteobacteria was dominant in all the samples. Highest abundance was noticed in INF (5.78%) and lowest in ONM (2.90%). *Geoalkalibacter ferrihydriticus* which is alkaliphilic, new obligately anaerobic, iron-reducing bacterium classified under δ-Proteobacteria was reported in INM (1.34%). Genus *Desulfovibrio* is a Gram-negative, sulfate-reducing bacteria and it is commonly found in aquatic environments with high levels of organic material and also

proved to have bioremediation capacity. In the present study, highest abundance was observed in ONM (1.14%) and lowest in INF (0.83%).

Phylum Firmicutes was found highest in ONM (6.76%) and lowest in INM (4.80%). Class Bacilli was found highest (2.85%) in ONM and lowest in INM (0.07%). However, Class Clostridia was highest in INM (4.72%) and lowest in INF (3.11%). Similarly, Genus *Bacillus* was found highest in ONM (1.91%). Several species of the Genus *Bacillus* are used as Plant Growth Promoting Rhizobacteria (PGPR). Several species of this genus are capable of producing antibiotics, and *B. thuringiensis* produces a toxin called 'crystal protein', which kills insects. *Clostridium* was reported highest in ONM (1.03%) and lowest in INF (0.70%). David *et al.*, (2014) reported an increase in Firmicutes with addition of organic matter in soil.

Rokunuzzaman *et al.* (2016) reported that application of chloropicrin as soil fumigant has positive influence on the bacterial community and it was observed that phylum Firmicutes was dominant by occupying about 75 per cent.

Several agriculturally important bacteria were recorded in the study. The nitrogen-fixing *Frankia* was found highest in ONM (9.89%) followed by INM (3.50%) and INF (2.49%). Abundance of *Bacillus* was also recorded in ONM (1.91%). This bacterium can be used as a PGPR for boosting the growth of plants; as a biocontrol agent, in case of antibiotic producing strains; and as bioinsecticides against lepidopteran, dipteran and coleopteran pests. *Rhizobium* is another nitrogen fixing bacterium, which fixes N in symbiotic association with leguminous plants. Highest population was recorded in ONM (0.009%) and lowest in INF (0.004%). *Bradyrhizobium* is also a nitrogen fixing bacteria and was highest in INM (0.52%) followed by ONM (0.33%) and INF (0.02%). *Klebsiella* is the nitrogen fixer and was highest in ONM and INF (0.02%) and lowest in INM (0.002%). Mehnaz *et al.* (2014) reported that *Klebsiella* is a diazotrophic bacterium, which fixes N in association with plants. As organic matter favours the microbial growth (Bingeman *et al.*, 1953) application of

organic inputs increases the organic matter hence highest growth of microbes are observed in ONM.

The Genus *Azospirillum* was another nitrogen fixing bacteria, isolated from the root and above ground parts of a variety of crop plants and is known as the best plant growth promoting rhizobacteria. This genus was found highest in INM (0.02%) followed by ONM (0.011%). *Rhodopseudomonas* is a purple nonsulfur phototrophic bacterium, which can degrade and recycle several different aromatic compounds and found highest in INM (0.016%) followed by ONM (0.009%) and INF (0.004%). The abundance of microorganisms in INM sample could be because of application of organic inputs along with inorganic inputs, which favours the microbial growth (Anisa *et al.*, 2016).

Genus *Burkholderia* is a good biodegrader of polychlorinated biphenyls (PCBs) and was highest in INF (0.27%) followed by ONM (0.26%). This could be because of the use of weedicides and other chemicals in the treatment INF. Cyanobacteria are photosynthetic bacteria (blue green algae) and have been harnessed in rice and popularized as a biofertilizer for rice. This group was more abundant in INF (0.36%), followed by INM (0.11%) and ONM (0.03%). The data of beneficial microbes is given in Table 30.

The diversity of microorganisms at species level is generally described in terms of α-diversity and β-diversity. α-diversity or within group diversity refers to a group of organisms interacting and competing for the same resources or sharing the same environment. It is measured as number of species within a given area (Whittaker, 1967) and to measure the alpha diversity rarefaction tool of MG-RAST was used, rarefaction (α- diversity) is a technique to assess species richness from the results. Rarefaction allows the calculation of species richness for a given number of individual samples, based on the construction of so-called rarefaction curves. The highest α- diversity was found to be in ONM (54.52 %) followed by INM (44.13%) and INF (38.86%). Soil under long term fertilization of organic manure (with or without NPK application) leads to a shift in carbon

Table 30. Abundance of beneficial microbes in the three samples

Beneficial microbes	ONM (%)	INM (%)	INF (%)
Frankia	9.89	3.50	2.49
Bacillus	1.91	0.01	1.57
Rhizobium	0.009	-	0.004
Azospirillum	0.011	0.02	-
Bradyrhizobium	0.33	0.52	0.02
Pseudomonas	0.002	-	0.03
Rhodopseudomonas	0.009	0.016	0.004
Burkholderia	0.26	-	0.27
Klebsiella	0.02	0.002	0.02
Cyanobacteria	0.03	0.11	0.36

utilization pattern and increased soil microbial diversity (Zhong *et al.*, 2010). Kamaa *et al.* (2011) reported a decrease in microbial diversity in soil treated with synthetic fertilizers.

β-diversity refers to the response of organisms to spatial heterogeneity. High beta-diversity implies low similarity between species composition of different habitats. It is usually expressed in terms of similarity index between communities (or species turnover rate) between different habitats in same geographical area (Whittaker, 1967). In the present investigation, microbial communities present in INM and ONM soil were found to be similar, with a β-diversity index of 0.0335 and microbial communities present in INF sample were different from other two sample with a β-diversity index of 0.0273 (Plate 41).

Some of the bacteria are unculturable; the possible reasons are that a required nutrient is not present in the culture medium, the culture medium itself is toxic, or that other bacteria in the sample produce substances inhibitory to the target organism and another reason for non-culturability *in vitro* may be the disruption of networks involved in bacterial cytokine. In the present investigation some of the unculturable bacteria were also detected in the soil samples to identify the unculturable bacteria advanced strategies required.

The present investigation was of structural deriving strategy to assess the bacterial diversity of the soil samples. The soil samples were acidic in nature and some of the bacteria which are resistant to acidic nature of soil; the genes related to the resistant may be identified using function-derived strategy.

The present investigation brings out the importance of following integrated nutrient management, rather than complete dependence on either organic or inorganic inputs alone.

SUMMARY

6. SUMMARY

The study on "Metagenomics to assess bacterial diversity in the soil as influenced by organic and chemical inputs" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Agricultural Microbiology, College of Horticulture, Vellanikkara, during the period 2014-2016. The main objective of the study was to assess the bacterial community in the soil, as affected by the organic inputs (Cattle manure + green manure @ 9 t ha^{-1} each) as well as inorganic inputs (Synthetic fertilizers to supply 90:45:45 kg ha^{-1} N, P_2O_5 and K_2O each), using a metagenomic approach. The salient findings of the study are summarized below:

- Soil physical properties such as bulk density, electrical conductivity were found to be normal, and soil was acidic in nature.
- Total nitrogen content was normal in all soil samples. Available phosphorous content was high whereas the available potassium was low.
- Available calcium and sulphur were deficient in soil under integrated management (INM) and sufficient in soil that received only organic inputs (ONM) and only inorganic inputs INF. Available magnesium and boron were deficient in all the samples. Available copper, iron, zinc, manganese were sufficient in all the samples.
- The population of culturable microorganisms was estimated in three different soil samples. Population of fluorescent pseudomonads (36×10^5 cfu g^{-1}) and nitrogen fixers (22.25×10^3 cfu g^{-1}) were highest in ONM. The population of bacteria (33.22×10^6 cfu g^{-1}) and actinomycetes (57.50×10^4 cfu g^{-1}) was highest in INM. The population of fungi (93.00×10^2 cfu g^{-1}) population was highest in INF.
- Good concentration of DNA was isolated from ONM (80.40 ng/µl), INM (36.01 ng/µl) and INF (65.11 ng/µl), using the soft lysis method. Electrophoresis on 0.8 per cent agarose gel revealed presence of a single band, without any shearing.

- Next Generation Sequencing of the hypervariable region (V3) using Illumina Miseq™ system was carried out at the NGS facility of SciGenom and the reads, after quality check and filtering, were uploaded to MG-RAST software for analysis.
- The diversity of bacterial taxonomic category was assessed at different Operational Taxonomic Unit (OTU) levels using Ribosomal Database Project (RDP) pipeline and MG-RAST. At phylum level, Actinobacteria was dominant over other phyla in all the samples, with highest value in INM sample (57.95%) and lowest in INF (25.80%). Unclassified bacteria was most abundant in INF (33.57%) and lowest in INM (16.42%). Acidobacteria was found highest in INF (14.13%) and lowest in ONM (10.31%). INF (14.55%) recorded highest and INM (5.62%) the lowest abundance of Proteobacteria. Firmicutes was found highest in ONM (6.76%) and lowest in INM (4.80%).
- At Class level also, Actinobacteria was most predominant, followed by unclassified, Solibacteres and Acidobacteria in all the three samples. Acidobacteria was abundant in INM (2.73%) and lowest in INM (1.52%). Under Proteobacteria, Delta- proteobacteria was dominant in all the samples, being highest in INF (5.78%) and lowest in ONM (2.90%).
- A total of 365 genera were reported in INF, in which *Candidatus Solibacter* (10.62%), *Rhodococcus* (6.24%), *Saccharopolyspora* (3.80%), unclassified (derived from *Betaproteobacteria*) (3.43%), unclassified (derived from *Deltaproteobacteria*) (3.20%), *Acidobacterium* (2.73%), *Frankia* (2.49%), *Prevotella* (1.96%), *Bacillus* (1.57%), *Cellulosimicrobium* (1.56%), *Stenotrophomonas* (1.27%), *Corynebacterium* (1.15%), *Arthrobacter* (1.04%) were present.
- In ONM sample, 352 genera were identified and *Frankia* (9.89%), *Candidatus Solibacter* (8.47%), *Saccharopolyspora* (6.00%), *Micromonospora* (5.73%), *Arthrobacter* (4.86%), *Nocardia* (3.82%), *Rhodococcus* (2.98%), *Nocardioides* (2.57%), *Cellulosimicrobium* (2.24%), *Atopobium* (1.93%), *Bacillus* (1.91%), *Gordonibacter* (1.88%),

Acidobacterium (1.53%), unclassified (derived from *Deltaproteobacteria*) (1.30%), *Actinomadura* (1.21%), *Corynebacterium* (1.15%), *Desulfovibrio* (1.14%), *Mycobacterium* (1.11%), *Prevotella* (1.06%), *Clostridium* (1.03%) and *Clostridium* (1.03%) genera were present.

- Sample INM recorded 272 genera, among which *Rhodococcus* (16.98%) was highest followed by unclassified bacteria (16.42%). In addition to this, *Candidatus Solibacter* (10.74%), *Saccharopolyspora* (8.16%), *Nocardia* (4.82%), *Frankia* (3.50%), *Nocardioides* (3.26%), *Moorella* (3.18%), *Arthrobacter* (2.25%), *Mycobacterium* (2.20%) and *Corynebacterium* (2.01%) were also identified.
- The highest α-bacterial diversity was reported in sample ONM, followed by INM and INF.
- The study revealed that highest diversity was observed in ONM (54.52), followed by INM (44.13) and INF (38.86).
- The study therefore, highlights the importance of metagenomic approach in assessing the diversity of microbial communities in specific environments. This approach was useful in detecting the presence of unculturable communities also, in rice rhizosphere soils, which would not have been possible with conventional methods.
- The study also revealed the importance of Actinomycetes, which were the most abundant phyla in rice soils, irrespective of management practices. The results clearly bring about the relevance of integrated nutrient management to maintain high population of actinomycetes in soil. Beneficial bacteria included N-fixers like *Azospirillum*, *Cellulosimicrobium*, and PGPR like *Bacillus* and fluorescent pseudomonads. Prolonged use of inorganic inputs leads to dominance of Acidobacteria, capable of utilizing inorganic chemical compounds as C and energy sources.
- In future, more detailed studies may be taken up to assess diversity of microorganisms at various growth stages of the crop and with different varieties. The diversity of plant microbiome-rhizosphere as a continuum, may also be assessed, as both are dependent on each other. Attempts could

be made to culture the bacteria grouped under 'unculturable' or 'yet to be cultured'. A function-derived strategy could also be used for identification of genes related to desirable traits like PGPR activities, acid tolerance, biological control potential and so on.

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ANNEXURE

ANNEXURE I

Equipment used in present study

1. Sterilization of culture media - Autoclave Equitron-7440 SLEFA (Eutech Instruments India).
2. Incubation of cultures - Incubator-shaker (Merck-Genei-OS2, Merck , India Ltd.).
3. Centrifugation - Centrifuge (Eppendorf-5804R, Eppendorf, Germany).
4. Bulk density - Hot air oven (B & C Industries Rotak, Kerala).
5. pH of culture media - pH meter (Cyberscan-Eutech, Eutech Instruments, India).
6. Visualization of the gel - UV transilluminator (UVP-Benchtop Transilluminator, USA).
7. Microbial cultures and soil metagenomic DNA stored in refrigerator.
8. Vision works LS software was used to visualize the gel and UVP GelDoc-IT^{TM} imaging system (USA) was used for imaging the gel.

ANNEXURE II

Chemicals used in direct method of metagenomic DNA extraction by soft lysis

1. Extraction buffer

A. 100mM Tris HCl	-	10 ml
1M Tris HCl (pH - 8.0)	-	1ml
Distilled water	-	100 ml
B. 100mM EDTA	-	0.372g
C. 1.5M NaCl		
1M NaCl	-	0.75 ml
Distilled water	-	100 ml
2. Lysis buffer	-	4 ml
A. 20% SDS	-	0.8 g
B. Lysozyme	-	20 mg/ ml
C. Proteinase K	-	10 mg/ml
D. N-lauroyl sarcosine	-	10 mg/ml
E. 1% CTAB	-	4 g

4. Phenol: chloroform: isoamylalcohol (25:24:1)

To 25 parts of phenol, 24 parts of chloroform, 1 part of isoamylalcohol was added and mixed properly. The mixture was stored in refrigerator before use.

5. Chloroform: isoamylalcohol (24:1)

To 24 parts of chloroform, 1 part of isoamylalcohol was added and mixed properly. The mixture was stored in refrigerator before use.

6. Potassium acetate 7.5M

Potassium acetate	-	20.412 g
Distilled water	-	50 ml

7. Chilled ethanol (70%)

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

8. Sterile distilled water - 20–50 µl

ANNEXURE III

Chemicals used in direct method of metagenomic DNA extraction by direct lysis

1. Extraction Buffer

200 mM Tris (pH- 8.0)	- 0.2 ml
25 mM EDTA (pH- 8.0)	- 0.5 ml
250 mM NaCl	- 0.375 ml
0.5 % SDS.	– 0.005 ml

2. Phenol: chloroform: isoamylalcohol (25:24:1)

To 25 parts of phenol, 24 parts of chloroform, 1 part of isoamylalcohol was added and mixed properly. The mixture was stored in refrigerator before use.

3. Ice-cold isopropanol

Equal volume of isopropanol

4. 70 percent chilled ethanol

ANNEXURE IV

Materials used for agarose gel electrophoresis

1. 6x Loading/ tracking dye

Bromophenol blue	-	0.25%
Xylene cyanol	-	0.25%
Glycerol	-	30%

The dye was prepared and kept in fridge at 4^0C

2. Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg/ ml in water and was stored at room temperature in a dark bottle.

3. 50x TAE buffer (pH 8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5M EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature

ANNEXURE V

Media used and composition

a) Jensen's agar

Sucrose	20.00 g
Dipotassium phosphate	1.00 g
Magnesium sulphate	0.50 g
Sodium chloride	0.50 g
Ferrous sulphate	0.10 g
Sodium molybdate	0.005 g
Calcium carbonate	2.00 g
Agar	20.00 g
Distilled water	1000 ml

b) Kenknight & Munaierers agar

Dextrose:	1.00 g
Monopotassium dihydrogen phosphate	0.10 g
Sodium nitrate	0.10 g
Potassium chloride	0.10 g
Magnesium sulphate	0.10 g
Agar	20.0 g
Distilled water	1000 ml

c) King's medium B Base

Proteose peptone	20.00 g
Dipotassium hydrogen phosphate	1.50 g
Magnesium sulphate heptahydrate	1.50 g
Agar	20.00 g
Glycerol	15 ml
Distilled water	1000 ml
pH	7.2 ± 0.2

d) Martin rose Bengal agar

Papaic digest of soyabean meal	5.00 g
Dextrose	10.00g
Monopotassium phosphate	1.00 g
Magnesium sulphate	0.50 g
Rose Bengal	0.05 g
Agar	20.00g
Distilled water	1000 ml
pH	7.2 ± 0.2

e) Nutrient agar

Beef extract	3.00g
Peptone	5.00 g
NaCl	5.00 g
Agar	20.00 g
Distilled water	1000 ml

f) Pikovskaya's agar

Glucose:	10.00 g
$Ca_3 (PO_4)_2$	5.00 g
$(NH_2)_4SO_4$	0.50 g
NaCl	0.20 g
$MgSO_4.\ 7H_2O_4$	0.10 g
KCl	0.20 g
Yeast extract	0.50 g
$MnSO_4.H_2O_4$	0.002 g
$FeSO_4.7H_2O$	0.002 g
Distilled water	1000 ml
pH	7.0

g) *Trichoderma* selective media

$MgSO_4$. $7H_2O$	0.2 g
K_2HPO_4	0.9 g
KCl	0.15 g
NH_4NO_3	1.0 g
Glucose	3.0 g
Chloramphenicol	0.25 g
p- dimethylaminobenzenediazo sodium sulfonate	0.3g
Pentachloronitrobenzene	0.2 g
Rose Bengal	0.15 g
Agar	20 g

ABSTRACT

METAGENOMICS TO ASSESS BACTERIAL DIVERSITY IN THE SOIL AS INFLUENCED BY ORGANIC AND CHEMICAL INPUTS

By

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ABSTRACT OF THE THESIS

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Abstract

The rhizosphere region of soil is the dwelling place for many microorganisms. The rich microbial activity supports many biological processes in the soil. The abundance of Plant Growth Promoting Rhizobacteria (PGPR) in rhizosphere assumes natural significance from agronomic point of view. Knowledge about the total diversity of these bacterial communities is less understood as the conventional methods for the study of microbes has their own limitations. It has been estimated that 99 per cent of microbes cannot be cultured easily. Metagenomics is the culture-independent genomic analysis of microbes that has been developed to overcome the drawbacks of culture-based analysis of microbial communities.

An attempt was made to analyse the diversity of bacterial community using metagenomic approach in three different rice rhizosphere soils that received organic inputs (ONM), inorganic inputs (I) and soil under integrated management (INM) from permanent manurial trial plots at RARS, Pattambi. Metagenomic DNA was isolated from the soils by direct lysis method and sequencing of V3 region of 16S rRNA gene was carried out by using Illumina Miseq™ platform, at SciGenom, Cochin.

The diversity of bacterial taxonomic category was assessed at different Operational Taxonomic Unit (OTU) levels using Ribosomal Database Project (RDP) pipeline and MG-RAST. At phylum level, Actinobacteria was the most dominant in all the three soils, and abundance was highest in INM (57.95%) and lowest in INF (25.80%). Actinomycetes play a major role in organic matter decomposition and their presence is an indicator of soil health. Bacteria under 'unclassified derived from bacteria' was highest in INF (33.57%). These bacteria could be novel ones, since no homology was observed with any sequence in database. Acidobacteria was found highest in INF (14.13%) and lowest in ONM (10.31%). Acidobacteria are metabolically and genetically diverse. Members of Proteobacteria increased in response to chemically recalcitrant substances and were dominant in INF (14.55%) and lowest in INM (5.62%). Phylum Firmicutes comprised *Bacillus* and *Clostridium* and was found highest in ONM (6.76%) and lowest in INM (4.80%).

Among Actinobacteria, genus *Frankia* belongs to family Frankiaceae was predominant and was highest in ONM (9.89%) and lowest in INF (2.49%). *Frankia* is an actinomycete, involved in nitrogen fixation. Genus *Rhodococcus* was predominant in INM (16.98%) and lowest in ONM (2.98%). *Rhodococcus* is capable of catabolizing compounds to produce bioactive steroids also involved in fossil fuel biodesulfurization. Genus *Arthrobacter* was observed highest in ONM (4.86%) and lowest in INF (1.04%). This is resistant to desiccation and starvation, degrades agricultural pesticides, reduces hexavalent chromium and may also be useful in bioremediation.

Among Acidobacteria, genus *Candidatus Solibacter* was found to be highest in INM (10.74%) and lowest in ONM (8.47%). *Candidatus Solibacter* is reported to be involved in the production of biofilm and acts as an ecosystem engineer in the soil. Genus *Acidobacterium* was found highest in INF (2.73%) and lowest in INM (1.52%) and members of this genus play major role in the fluxes of carbon, nitrogen and iron across microbial communities.

Among Proteobacteria, genus *Geoalkalibacter* and genus *Desulfovibrio* were abundant and these are associated with iron-reduction and sulfate-reduction, respectively. These are commonly found in aquatic environments with high levels of organic material and also proved to have bioremediation capacity. Genus *Bacillus* was found highest in ONM (1.91%) and lowest in INM (0.01%) and it is used as PGPR, as it is capable of producing natural antibiotics and producing toxin which kills insects. Alpha diversity within the sample was found to be highest in ONM (54.52) followed by INM (44.13) and INF (38.86).

The analysis of the metagenome provided quantitative insight into microbial populations, as affected by management practices. Sequence based screening of metagenomic DNA libraries can be exploited to identify 'unculturable' bacteria. The presence of beneficial flora like actinobacteria in soil which received both organic as well as inorganic inputs reveals the advantage of integrated nutrient management practices. A function-derived strategy could be used for bioprospecting of gene related to desirable traits.