METAGENOMIC ANALYSIS OF BACTERIAL DIVERSITY IN THE RICE RHIZOSPHERE OF KOLE LANDS OF THRISSUR

By,

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(2018-11-004)

DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLANIKKARA,THRISSUR-680 656
KERALA, INDIA

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THESIS

Submitted in partial fulfillment of the requirements for the degree of

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KERALA, INDIA

2021

DECLARATION

I, hereby declare that this thesis entitled **"Metagenomic analysis of bacterial diversity in the rice rhizosphere of Kole lands of Thrissur"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellanikkara

Date: 26.02.2021

ATHIRA KRISHNAN, L. R.

(2018-11-004)

CERTIFICATE

Certified that this thesis entitled **"Metagenomic analysis of bacterial diversity in the rice rhizosphere of Kole lands of Thrissur"** is a record of research work done independently by **Ms. Athira Krishnan, L. R.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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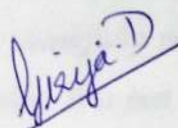

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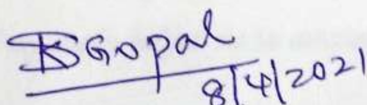

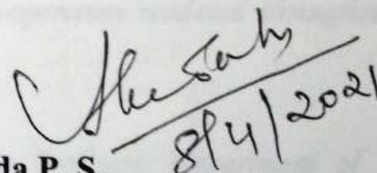

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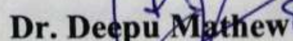

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ABBREVIATIONS

CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxy ribonucleic acid
g	Grams
h	Hours
ha	Hectre
cm	Centimeter
m	Meter
kg	Kilo gram
nm	Nano meter
kb	Kilo base
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 liter
µM	Micro molar
SDS	Sodium Dodecyl Sulfonate
cfu	Colony forming unit
OTU	Operational Taxonomic Unit

Introduction

1. INTRODUCTION

The Kole lands are low lying tracts which form part of the Vembanad-Kole wetland ecosystem, spread over an area of 1,51,250 ha. Within the Vembanad-Kole wetland ecosystem, the Kole lands cover an area of 13,632 ha in the central region of Kerala, spread over Thrissur and Malappuram districts. These tracts are located half to one metre below mean sea level. Thrissur Kole is geographically situated in Mukundapuram, Chavakkad and Thrissur taluks. Kole wetlands were once a contiguous land in Central Kerala. However, land reclamation for urbanization resulted in the wetlands being limited to a few fragments. These wetland fragments are now connected by a canal and the lands are seasonally used for rice cultivation. The rivers that feed the Thrissur Kole wetlands include Karuvannur, Kecheri and Puzhakkal. Kole lands have immense potential in achieving enhanced crop yields. The Kole land of Thrissur is fertile with alluvial soil deposited by Kechery and Karuvannoor rivers during monsoons.

Rice is the major cultivated crop of this region. Though several investigations were conducted at Kole wetlands for exploring the diversity of butterflies, fishes, birds and plants, no systematic studies have been carried out on soil microbial diversity. Plant rhizosphere microbiomes are shaped most probably by root exudates and the microbes develop various adaptations to thrive in the rhizosphere niche. Since rice is cultivated in flooded soil, it results in oxic and anoxic zones within the rice rhizosphere leading to selection of organisms of specific functional group with either aerobic, anaerobic or facultative metabolism (Brune *et al*., 2000).

The rhizosphere microbial community is critical for plant health as well as for soil organic matter degradation. However, the exact mechanism of host-microbe interaction and the processes that drive the modifications of microbiomes are not well-known. In the last few years, a great advancement has been made in the knowledge of rhizosphere microbiome composition and their dynamics.

Traditional method of growing bacteria in microbiological media has enormous limitations due to plate count anomaly and it also restricts analysis to those microbes which can be cultivated in laboratory conditions (Rondon *et al*., 2000). Using the agar plate method only 0.1 to 10 per cent of bacterial cells of arable soil can be cultured whereas the major portion remains unrevealed (Torsvik *et al*., 1998).

Metagenomics is a culture-independent method meant for analyzing the microbial composition, by recovering DNA directly from the environmental sample (Handelsman *et al*., 1998). Direct microbial DNA extraction and characterization through PCR amplification and metagenomics was reported to be efficient in identifying the uncultivated microbial diversity (Tiedje *et al*., 1999). Metagenomic approaches provide insights into the role of previously unidentified members in nutrient cycling and the enhancement of plant growth. Metagenomics has been successfully applied to document microbial diversity, adaptation, evolution, and function in several ecosystems. The profiling of microbial communities can be revealed by high-throughput sequencing of targeted PCR amplification. The recent advancement of high-throughput sequencing techniques has led to better understanding of the soil and rhizosphere microbial diversity.

In this background, the present investigation entitled 'Metagenomic analysis of bacterial diversity in the rice rhizosphere of Kole lands of Thrissur' was taken up with an objective to assess the diversity of bacterial community in the rice rhizosphere of Thrissur Kole wetlands using metagenomics approach.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Kole lands

Kole lands are one of the largest and highly productive complex ecosystems with many interacting organisms which provide substantial advantage to human society. Even if it is under the threat of several anthropogenic activities, it has immense potential in crop improvement and enhanced crop production (Jeena, 2010). According to Cowardin *et al*. (1979) wetlands are defined as transitional lands between terrestrial and aquatic systems and the water table is mostly at or near the surface.

In Kerala, the Kole lands are spread over the central region majorly, Thrissur and Malappuram districts and is a part of the Vembanad-Kole wet land ecosystem (Johnkutty and Venugopal, 1993). Thrissur Kole Wetlands are wetlands that are deputed to be of international importance under the Ramsar Convention. The word 'Kole' is of Malayalam origin, which indicates a bumper yield with reference to the cultivation in these regions. Rice is the major cultivated crop of this region. In addition to rice cultivation, the Kole lands provide nesting place for several migratory birds (Jeena, 2010). According to Ramsar site information service, the Kole lands support a wide population of aquatic life and waterfowl.

2.1.1 Geography

The Kole wetlands are low lying areas, about half to one metre below mean sea level and are located between 10° 20' and 10° 40' N latitudes and between 75° 58' and 76° 11' E longitudes. It remains submerged for about six months in a year. The Kole lands of Thrissur are spread over eight blocks. The annual rainfall of this region is around 3200 mm and the temperature ranges from 28°C to 31.5°C (Johnkutty and Venugopal, 1993).

The Kole wetland ecosystem of Kerala covers an area of 13,632 ha over Thrissur and Malappuram districts. The area extends from south Chalakkudy river to north Bharathapuzha and to Ponnani taluk of Malappuram district. The area from Vellukkara of Mukundapuram taluk of Chalakkudy river bank to Mullassery of Chavakkad taluk and Tholur-Kaiparama of Thrissur taluk are considered as ‘Thrissur Kole’. During monsoon, these wetlands are submerged in water and during summer when the water is less, these fields are used for cultivation by raising the bunds (Johnkutty and Venugopal, 1993).

2.1.2 Soil condition

The soils of Kole wetlands are highly fertile, with alluvial soil brought down by the Kechery and Kuravannoor rivers during monsoon. The study by Johnkutty and Venugopal (1993), revealed that some portion of Kole contains black carbonaceous clay with many plant parts and withered tree trunks. Subsurface investigations have shown presence of fine sandy deposits. The soils of Kole land in the flood plain are acidic in nature and highly clayey in texture. The soil pH of these regions ranges from 2.6 to 6.3 (Johnkutty and Venugopal, 1993).

2.1.3 Uses

Kole lands owing to the presence of water and terrestrial related properties ensure several economic activities that support the livelihoods of the local population. In addition to paddy cultivation, Kole lands are also used for duck rearing, aquaculture and lotus farming. Kole lands support inhabitation of large number of migratory and endangered birds. Kole lands helps in flood control, nutrient retention, recharge of ground water, etc. and also act as carbon sink (Jeena, 2010).

2.2 Rice cultivation in Kole land

The rice cultivation in Kerala is tremendously decreasing over the past few years. The Kole lands are the region where rice cultivation is still continued with characterized increase in crop productivity. Here, the rice cultivation has been started from 18th century onwards. Kole land contributes around 41 per cent of rice production together from Thrissur and Malappuram districts. These two districts contribute about 19 per cent of total rice production of Kerala in 2015 – 2016 (Deepak, 2018). The studies by Johnkutty and Venugopal (1993) on Kole wetlands showed that the shallow lagoons of Kecheri and Kuravanoor river basins were reclaimed for the paddy cultivation in 18th century. Bunds were raised for the cultivation of rice in low-lying fields to prevent the entry of flood water. Later, the State government funded for the construction of permanent bunds to reduce flooding and to enhance rice cultivation in this region.

Rice cultivation starts with dewatering the low-lying fields by pumping out the water. The cultivation of rice is carried out during the period of October- May. In Thrissur, during summer, when the water is less, the irrigation water is released from the reservoirs of Chimmoni and Peechi irrigation projects. During June to October when the water levels are high, some farmers use the fields for aquaculture (Deepak, 2018).

2.3 Soil as a microbial habitat

Soil has a major role in maintaining biodiversity above and below ground. Both plants and microbes obtain their nutrients from soil. It is a diverse as well as complex ecosystem, which provides intricate habitat for wide variety of living organisms. The interaction of microbial community with minerals and organic compounds present in the soil makes the soil more complex and heterogenous, due to which it can serve as a platform for various bio-geochemical cycles which are required for the sustenance of life (Mocali and Benedetti, 2010). This complexity created by the soil microflora as it depends on the physical and chemical properties of the soil, results in a confined area of few millimeters square with numerous diversified microsites (Nunan *et al*., 2002). Even

if the microorganisms contribute only 0.5 per cent of the soil mass, their impact is higher in determining the soil properties and processes. This results in shaping the population dynamics of the living components in soil. The soil microbial community plays a major role in modifying the properties of soil by catalyzing the chemical reactions and it is possible because of the huge metabolic diversity present in the soil. All these details bring out the fact that the soil due to its complex and heterogeneous nature, eventually shape the flora of an area, which makes it ideal for agricultural practices (Tate, 1995).

Soil is known to conceal a wide range of genetic diversity. There are more microbes in a handful of soil than the people on the earth. The conventional enumeration by cultivating microbes on various media can reveal only about 0.1 per cent of the total microbial diversity whereas; the major fraction (99.9 per cent) of the soil microflora remains unexplored. These unexplored fractions can even represent another phylum, as they are phylogenetically separated from their culturable counterparts (Handelsman *et al.*, 1998). Estimation of soil bacterial diversity using a culture-independent method by Torsvik *et al.* (1990a) showed that one gram of soil encloses more than 10^{10} number of bacterial cells (Torsvik *et al.*, 1990b).

It is observed that the factors like solute concentration, nutrient transportation and other soil processes influence the spatial arrangements and distribution of the microbial communities. Spatial distribution studies on bacterial communities revealed that the aggregation of bacteria was more in the so called ‘hotspots’ of the subsoil than the top soil (Nunan *et al.*, 2002).

2.4 Plant - microbe interaction

The interaction between plants and soil microbes can be either direct or indirect. The direct interaction mechanisms are further categorized as positive and negative interactions. The symbiotic association between plants and microbes are considered to be positive effects while, the pathogenic interaction of microbes with the plant are considered to be negative interaction (Van Der Heijden *et al.*, 2008). The most common

example for positive symbiotic relation is the interaction between plants and diazotrophs, which assists the plant to obtain one of the most important elements that, facilitates the growth of the plant. Another classic example for the positive direct interaction is the symbiotic relationship of mycorrhizal fungi with the roots of higher plants. The symbiotic relationship between the plants and the microbes provide multiple benefits to the plant, including increasing the bioavailability of nutrients like nitrogen, phosphorous, zinc, copper, iron, etc. for the carbon rewarded by the plants (Shtark *et al*., 2010). Microbial activity boosts plant growth mainly by manipulating the signaling of plant hormones (Verbon *et al*., 2016), competing with pathogenic microbial strains (Mendes *et al*., 2013) and enhancing the bioavailability of soil nutrients (Van Der Heijden *et al*., 2008).

The root exudates released in response to the chemical signals produced by soil microflora facilitates the interaction of plants with the microbes. These exudates are specific for each species as well as subspecies (Uren, 2007). The root exudates usually contains a mixture of compounds like organic acids, amino acids, purines and pyrimidines, pentoses and hexoses, enzymes and vitamins. The specificity of microbes at the plant vicinity is determined by the make, ratio and chemical properties of these compounds (Tate, 1995).

2.4.1 Root exudates

Root exudates play a crucial role in creating a versatile platform for the interaction of soil microflora with the plant roots (Hirsch *et al*., 2003). In general, root exudates are composed of two groups of compounds – i) the high molecular weight compounds like polysaccharides and proteins that form the major fraction of the root exudates and ii) the low molecular weight compounds like amino acids, phenolics, organic acids and other secondary metabolites responsible for the chemical diversity of the root exudates of different plant species (Uren, 2007). This implies the role of plants in shaping the soil microbiome by altering the soil parameters and soil microflora. The T-RFLP study conducted by Micallef *et al.* (2009) in the rhizosphere soil of various accessions of *Arabidopsis thaliana* disclosed that the qualitative and quantitative

differences in the plant root exudates can cause differences in the pattern of rhizosphere microbiome, even if there is no visible correlation or direct link among the variations. Another investigation carried out by Cavaglieri *et al.* (2009) on the culturable diversity analysis of maize rhizosphere microflora revealed that the developmental stages of plant may have selective effect on specific groups of microorganisms. Other than root exudates, the mucilaginous secretions and disrupted root cells are also released by the plants to the rhizosphere. The rhizosphere soil also contains compounds like pectin, lignin, cellulose and other cell wall polymers in higher concentration, which makes the plant rhizosphere ideal for the decomposition of cellulose and other organic matters. The decomposed compounds provide suitable carbon source for the growth of microbial population. The α and β classes of Proteobacteria were found to be the most predominant class of bacterial community, apart from the major groups like Actinobacteria, Acidobacteria, Bacteriodetes, Planctomycetes, Firmicutes and Verrucomicrobia (Turner *et al*., 2013).

2.4.2 Plant mineral nutrition by microbes

Microbes are the key elements of soil nutrient cycling. In natural soil ecosystem, most of the nutrients such as nitrogen, phosphorous, sulphur etc. are bound in complex organic molecules and their availability is minimal for plants. Plants depend on soil microbes to access these nutrients. The recalcitrant forms of soil nutrients are metabolized by soil microbes to release these elements for plant nutrition (Richardson *et al.*, 2009). As a result, the inorganic N, P, S and their ionic forms like nitrate, ammonium, phosphate and sulfate are released in to the soil for plant nutrition (Van Der Heijden *et al*., 2008). Such nutrient transformations by the microbes are the main grounds for plant growth and sometimes it can act as a rate-limiting step in the ecological productivity (Schimel and Bennett, 2004). A study on plant growth enhancing *Pseudomonas* strain has shown that they are capable of organic sulphur mineralization by knocking out the sulfonate monooxygenase enzyme gene and thereby account for plant growth promotion (Kertesz *et al*., 2004).The arbuscular mycorrhizal fungus (AMF), *Glomus intraradices*

was found capable to transfer organic nitrogen to the plants which suggests that the future investigations can focus on exploring other fungal strains with such character (Thirkell *et al*., 2016). A similar study conducted by Ye *et al*. (2015) revealed that the colonization of AMF *Glomus versiforme* resulted in the enhancement of N and P uptake by the ryegrass.

2.4.3 Soil microbes and plant health

Soil microbes play a pivotal role in maintaining plant health. The microbial community aid plants in nutrient uptake and also acts as antagonists to wide range of phytopathogens (Matilla and Krell, 2018). There are various plant growth promoting bacteria as well as mycorrhizal fungi which can enhance the plant growth and development under stressful conditions. The plant growth enhancing microbes play a vital role in plant stress management by improving nutrient access, production of secondary metabolites, plant hormones and siderophores and also provide induced and acquired systemic resistance against biotic stress (Narusaka *et al*., 1999). The plants are associated with microorganisms through metabolic cooperation and the exchange of signals, hormones and nutrients (Berg *et al*., 2017).

The ecological association of microbes with plants can range from negative interactions by pathogens to neutralism to positive (beneficial) interactions by plant growth promoting rhizobacteria (PGPR) (Bais *et al*., 2006). The PGPR can effectively mitigate the effects of many abiotic stresses (Milosevic *et al*., 2012). The PGPR performs multiple functions like nutrient cycling, suppression of antagonistic microbes and seedling growth enhancement. (Barea *et al*., 2005). Based on their function, PGPR are grouped into two categories. The first group includes those which account for plant growth stimulation and nutrient acquisition and the second group includes the biocontrol agents that are capable of suppressing plant pathogens (Bashan and Holguin, 1998). As the PGPR can successfully colonize and thrive in peculiar conditions and can compete against pathogens, they are widely exploited in crop production to increase the yield and to provide biotic and abiotic stress tolerance (Chaparro *et al*., 2012).

Like improved crop production, the disease suppressiveness of PGPR is also considered to be a major contribution as it indirectly assists the crop productivity (Janvier *et al*., 2007). Certain antibiotics and antifungal compounds produced by the PGPR can provide disease suppressive property to the soil (Weller *et al*., 2002).

2.5 Disease suppressive soils

Disease suppressive soils have the inherent capacity to limit the growth of pathogens to a certain extent. The impact of diverse soil microbial community affects the proliferation of phytopathogens in the soil. The microflora of most soils is nutrient starved and hence the microbes present in the rhizosphere compete with each other for the plant-derived nutrients (Raaijmakers *et al*., 2009). The soil microflora compete with the saprophytic soil-borne pathogens for food and space which results in general disease suppression (Berendsen *et al*., 2012). Specific suppression occurs when the microorganisms confer resistance against a particular disease. The specific disease resistance can be imparted to disease affected soil by transferring 0.1 to 10 percent of disease suppressive soil (Garbeva *et al*., 2004).

Disease suppressiveness which influences the soil health is an outcome of diverse biological community and their collective interactions in the soil. A virulent pathogen, susceptible host and favourable environmental conditions are the three elements necessary for the disease development. In disease suppressive soil, even if the factors are favourable for pathogen infection, the disease incidence will be low (Hoper and Alabouvette, 1996).

2.5.1 General disease suppression

Out of the two types of disease suppressiveness, the general suppression is considered as a result of collective interaction, competition and antagonism among the microorganisms for food and space. This is evident in every natural soil (Workneh *et al*., 1993). The specific disease is a result of selective parasitism and antagonism imposed by one or more antagonists on specific pathogens (Hornby and Bateman, 1997).

Monocropping which results in the reduction of take-all disease caused by *Gaeumannomyces graminis* is a classic example for specific disease suppression (Cook and Baker, 1983).

The functional units of microbial community are considered to be responsible for the disease suppressive character of the healthy soil. The microbial community plays a pivotal role in organic matter decomposition and soil microbial succession which determine the disease suppressiveness character of the soil (Van Bruggen and Semenov, 2000). *Pseudomonas fluorescens* strains were found capable of reducing the take-all disease by producing a chemical, phloroglucinol that imparts negative effect on the pathogen (Raaijmakers and Weller, 1998).

2.5.2 Specific disease suppression

The specific disease suppression is considered to be much more efficient than the general disease suppression. The presence of specific microbial species or its related groups in the soil are involved in specific disease suppression (Weller *et al*., 2002). The investigation carried out by Weller (2006) showed that the property of specific disease suppression can be transferred to fields and green houses.

Most of the specific disease suppression investigations were carried out for the repression of take-all disease, as the disease was predominant in wheat mono-cropped area. The parasitic *Trichoderma* spp. and *Pseudomonas fluorescens* strains producing 2,4 diacetylphloroglucinol (2,4 DAPG) were found to be efficient in suppressing take-all disease (Simon and Sivasithamparam, 1989). A study revealed that the take-all suppressive soils housed remarkable population of 2,4-DAPG producers, while the population was absent or nearly negligible in disease-prone soil (Raaijmakers *et al*., 1997).

Another classic example for specific disease suppression is the control of *Fusarium* wilt. The intrageneric competition between pathogenic and non-pathogenic *Fusarium* species for carbon resulted in the suppression of pathogenic *Fusarium* sp.

(Alabouvette, 1986). Another study showed that the presence of siderophore producing *Pseudomonas* sp. in soil resulted in the suppression of *Fusarium* wilt by competing with the pathogen for iron (Alabouvette, 1999).

2.6 Rice rhizosphere and microbiota

Rice is a staple food which feeds over 50% of world's population (Ge *et al*., 2012). Rice roots form a unique niche with an oxic region, the rhizosphere enclosed by an anoxic bulk soil (Zhao *et al*., 2018). This oxic-anoxic interface acts as a hotspot for various biogeochemical cycles mediated by different functional groups of microorganisms (Li and Wang, 2013). It was observed that the rice plants have huge influence on the composition of soil microflora. The abundance of 16S rDNA was found to be twice in the rice rhizosphere than that in the unplanted bulk soil which indicates the effect of rhizosphere in microbial growth stimulation (Breidenbach *et al*., 2016). A 16S rDNA study for bacterial diversity analysis conducted in the rice rhizosphere soil revealed the presence of high diversity in the bacterial community with majority of microbes closely related to the class Proteobacteria (Arjun and Harikrishnan, 2011). Continuous monoculturing of rice results in compositional changes in the soil microbiota, which can negatively affect the plant performance and can enhance the growth of greenhouse gas emitting microbes (Edwards *et al*., 2019). The System of Rice Intensification (SRI) practices which make rice soil more aerobic and rich in soil organic matter, supports the proliferation of beneficial soil microflora (Anas *et al*., 2011).

2.6.1 Effect of beneficial rhizobacteria on rice

Beneficial rhizobacteria are well known to confer protection against various biotic and abiotic stresses in rice plant. Rice plants when primed with *Bacillus amyloliquefaciens* and *Aspergillus spinulosporus* showed protection against disease caused by *Xanthomonas oryzae* pv. *oryzae*, by reprogramming the host defense mechanism under pathogen challenge (Jain *et al*., 2020). The study conducted by Lucas *et al.* (2014) in the rice rhizospheric bacterial strains BaC1-13and BaC1-38 showed

systemic induction of resistance against salt stress and *Xanthomonas campestris* infection. The root-associated rhizobacteria *Pseudomonas* EA105 was observed to be efficient to suppress the growth and appressoria formation of *Magnaporthe oryzae*, a fungal pathogen which causes blast disease in rice plant (Spence *et al*., 2014). A similar study revealed that the rhizobacteria *Pseudomonas fluorescens* BRM 32111 and *Burkholderia pyrrocinia* BRM-32113 imparted allelochemical stress tolerance to upland rice plants (Marcela *et al*., 2018).

The indigenous PGPR collected from the rhizosphere of early stages of Thai jasmine rice *(Oryza sativa* L. cv. KDML105) were found to produce IAA which emphasizes the beneficial effects of PGPR isolates on the growth of rice plant (Saengsanga, 2018). An investigation by Ashrafuzzaman *et al.* (2010) using PGPR isolates (PGB4, PGG2 and PGT3) from the rice rhizosphere soil are found to induce IAA production, phosphorous solubilization and enhance the growth of rice plant, they can be used as inoculants of biofertilizers for rice cultivation. A similar study by Bal *et al.* (2013), suggested that the presence of diverse rhizobacteria with effective PGP traits in the rice rhizosphere may be exploited for sustainable crop management under field conditions.

2.7 Soil microbial diversity analysis

Soil has a major role in maintaining biodiversity above and below ground. It is a complex ecosystem and it harbors a variety of living organisms. It is obvious that the microorganisms play significant role in several ecosystem activities like plant nutrition, nutrient cycling and modification in soil structure (Lakshmanan *et al*., 2014). The soil microbial diversity is the function of all the activities occurring in the soil. Various human activities, including agriculture are responsible for shaping the microbial community (Kirk *et al*., 2004).

The phenotypic characterization of the isolated strains is the most traditional method for analyzing the microbial diversity. But it limits the analysis to characterization

of culturable microorganisms. About 99.5 to 99.9 per cent of soil bacteria which are detectable under fluorescent microscope are unculturable under *in vitro* condition which shows that only a minute fraction of soil microflora can be culturable on artificial microbiological media (Torsvik *et al*., 1998).

Hill *et al.* (2000) critically analyzed all the methods from phenotypic characterization to metabolic fingerprinting to culture-independent methods, which are still practiced to determine its efficacy in assessing the community-level interactions of soil microbes, species abundance and the change in microbial communities which influence soil health.

2.7.1 Dilution plating and enumeration

The conventional culture-based method using variety of microbiological media is used for the analysis of soil microbial composition. This method is widely used for the analysis of soil quality, soil organic matter decomposition and suppression of disease (De Leij *et al*., 1994). Even though 99 percent of the microbial population fails to grow on culture media (Rondon *et al*., 1999), this method can be used effectively to determine the heterotrophic portion of the microbial population (Kirk *et al*., 2004).

2.7.2 Metabolic fingerprinting

Garland and Mills (1991) developed a community level method which uses 96-well microtitre plates to characterize and categorize heterotrophic microbial communities based on the utilization of sole-carbon source. These BIOLOG plates comprised 95 distinct carbon sources and a control (Hayward, CA, USA, www.biolog.com). The analysis includes the use of tetrazolium dye which produces a range of colour intensities based on the utilization of carbon source (Becker and Stottmeister, 1998). Discrete plates were developed for Gram positive (GP), Gram negative (GN) and fungal specific (SFN2, SFP2) plate types (Classen *et al*., 2003).

2.7.3 Fatty acid methyl ester (FAME) analysis

Fatty acid methyl ester (FAME) is a culture independent method used to characterize soil microbial communities based on the presence of fatty acids in the soil (Ibekwe and Kennedy, 1998). Since the fatty acids are integral part of the microbial cell and have high specificity at species level, it can act as a specific compound that can represent the constituents of a microbial population (Kelly *et al*., 1999). The FAME analysis includes methylation of fatty acids extracted directly from the soil, followed by analysis using gas chromatography and the profile thus created gives the sample microbial composition. Phospholipid fatty acid (PLFA) analysis was also done to obtain a result corresponding to that obtained from metabolic fingerprinting using microtitre plates (Ibekwe and Kennedy, 1998).

The misrepresentation of microbial population caused by the direct influence of temperature and nutrition on the fatty acid composition of microbial cells is a pitfall of FAME analysis. For the study of fungal diversity, about 130 to150 spores are required and hence the minor portion in the fungal population may get eliminated (Graham *et al*., 1995).

2.7.4 Molecular-based approaches

2.7.4.1 Guanine plus Cytosine (G+C) content

The guanine plus cytosine content of microorganisms varies from one species to another and even in closely related species, it is observed that G+C content varies from 3 to 5 per cent (Tiedje *et al*., 1999). This difference in the G+C content among the taxa is considered as the base for the analysis of microbial diversity (Nusslein and Tiedje, 1999). This quantitative analysis helps to discover the rare members of the microbial population, but the analysis requires large quantities of DNA (50µg) which is a slight flaw of this method (Tiedje *et al*., 1999).

2.7.4.2 Nucleic acid hybridization

The genetic complexity and microbial diversity in a sample can be revealed by measuring the rate of hybridization after the extraction of total DNA, purification and denaturation (Torsvik *et al*., 1990a). Since the hybridization rate depends on the diversity, complexity and sequence similarity, as the complexity increases, the rate of hybridization decreases (Theron and Cloete, 2000).

Griffith *et al.* (1998) stated that the factors like half-time required for DNA re-association and the extent of DNA sequence similarity analyzed using hybridization kinetics determines the similarities between the microbial populations. Quantitative analysis using fluorescein or rhodamine fluorescent markers was used to identify the special distribution of microorganisms (Theron and Cloete, 2000). The diversity of microorganisms from environmental samples was studied successfully by practicing Fluorescent *in situ* hybridization (FISH), a modified method which uses fluorescent labeled primers that hybridize the DNA specifically (Schramm *et al*., 1996).

Van Elsas and Wolters in 1995 practiced polymerase chain reaction (PCR) with total DNA as template to avoid the exclusion of minor population. In order to determine the microbial population, the PCR product thus amplified was allowed to hybridize with the oligonucleotide probes or with the extracted total DNA of the environmental samples (Kirk *et al*., 2004).

2.7.4.3 PCR based techniques

According to Pace (1996), the diversity of prokaryotes can by estimated by practicing 16SrRNA gene amplification followed by phylogenetic analysis. For the study of fungal population, the analysis of 18S rDNA region and the internal transcribed spacer (ITS) are employed. The microbial communities are then identified by comparing the amplicons sequences against available database (Ko *et al*., 2011). Other modified methods like temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) are also used for the study of diversity of microbes. Here, the

amplicons are separated on polyacrylamide gel based on the increasing concentration gradient, and is exploited for the study of microbial diversity (Miller *et al*., 1999).

2.7.5 Application of 16S rRNA gene sequencing in identification and classification of bacteria

In the 1980's a new standard for identifying bacteria began to be developed. Woese *et al.* (1985) suggested that by comparing a stable part of the genetic code, the phylogenetic relationships of bacteria and indeed, all life-forms could be determined. And the candidates of this region of bacteria included 5S, 16S and 23S rRNA and the spaces between these genes. The region of the DNA now commonly used for taxonomic classification is the 16S rRNA gene (Kolbert and Persing, 1999). The 16S rRNA gene is a highly conserved component of the transcriptional machinery of all DNA-based life forms (Cox *et al.,* 2013) and hence they are highly suited as a target gene for the sequencing of DNA in samples containing thousands of different species. The 16S rRNA gene has both conserved and variable regions. Universal PCR primers can be used for the amplification of these conserved regions with highly conserved priming sites. While the conserved regions make universal amplification possible, the sequencing of variable regions can provide the species-species signature sequences useful for the identification of bacteria.

There are nine hypervariable regions (V1-V9) in 16S rRNA gene and it can vary dramatically between bacteria. Although no hypervariable region can accurately classify bacteria from domain to species, some can reliably predict specific taxonomic levels (Yang *et al*., 2016). Studies suggests that the V4 hypervariable region can provide resolution at phylum level (Yang *et al.,* 2016) while the V3 hyper variable region can identify bacteria at genus level (Chakravorty *at al*., 2007). Furthermore, the bacterial genomes house multiple 16S genes, with V1, V2, and V6 regions containing the greatest intraspecies diversity (Coenye and Vandamme, 2003).

2.7.6 Culture independent approaches

The foundation on which the analysis of microbial diversity has been created lies on the idea of recovering and culturing microorganisms under laboratory conditions (De Long, 1998). Rondon *et al.* (1999) discovered that the stained natural samples, when visualized under microscope showed twice or thrice the number of cells recovered by culturing. In 1998, McDougald *et al.,* stated that the variations in the physiological state of the organisms can obstruct them from being cultured even if they are phylogenetically similar to the cultivable counterpart. De Long (1998) put forth a contradictory statement that the unculturable portion can be phylogenetically dissimilar, indicating the presence of large unexposed microorganisms in the soil. The DNA hybridization study by Torsvik *et al.* (1990a) discovered that one gram of soil carries nearly 4000 different bacterial genomes which explain the heterogeneity of the soil bacterial content.

2.8 Metagenomics

The concept of metagenomics was introduced as a result of the adoption of Bacterial Artificial Chromosome (BAC) technology in bacterial genomics, which uses prokaryotic DNA as the insert (Rondon *et al*., 1999). The adaptation of BAC library preparation was thus adopted for the analysis of microbial communities from the environmental samples. The BAC studies combined with 16S rDNA sequences favored the use of phylogenetics in the analysis of environmental microflora (Rondon *et al*., 1999).

Handelsman (2004) introduced the term metagenomics to deeply understand the unculturable microorganisms that remains unexplored and unidentified using 16S rDNA sequences. In the year 1996, Pace suggested the probability of studying the phylotype distribution in the natural environment by isolating total DNA from the sample followed by the sequencing of 16S rDNA. Metagenomic analysis, generally used for gene expression studies and identification of phylogenetic markers involves total DNA isolation from the environmental sample followed by cDNA library preparation, by

cloning it into a vector (Voget *et al*., 2003). The open reading frames (ORFs) can be studied using the sequences thus obtained, by assigning it to the orthologous gene clusters procured from the databases like KEGG (Kyoto Encyclopedia of Genes and Genomes). Apart from all these possibilities, the method of sampling as well as techniques involved in the extraction of metagenomic DNA plays a crucial role in the study of microbial communities (Schloss and Handelsman, 2005).

2.8.1 Isolation of metagenomic DNA

The isolation of metagenomic DNA is done primarily with an objective to extract DNA from wide variety of microorganisms that can represent an authentic microbial population. The extraction of good quality DNA from sample without shearing is the secondary objective and the isolation of contaminant-free DNA which can later hamper the steps like PCR, restriction and ligation is the tertiary objective of metagenomic DNA isolation (Schmeisser *et al*., 2007). As the soil microbial population is composed of microorganisms like bacteria, archaea and protists, the susceptibility to the lysing procedures may vary (Kauffmann *et al*., 2004). Bertrand *et al.* (2005) reported that the soil microbial cells that are in physiologically dormant stage can also influence the quantity of DNA isolated from the soil.

Isolation of metagenomic DNA can be done by using direct as well as indirect extraction procedure. The direct extraction approach involves the direct lysis of the cells where as the indirect method requires extraction of the cells before lysis to obtain the DNA. It was found that more quantity of DNA was obtained from direct method hence; the direct method is preferred over indirect method (Schmeisser *et al*., 2007). The cells are lysed either physically or chemically or in combination to isolate DNA from the sample. The physical lysis method involves grinding of the sample using liquid nitrogen, thermal shock, ultrasonication, etc. The chemical lysis procedure involves the use of detergents like SDS (sodium dodecyl sulfate) which can destruct the cytoplasmic membrane of the microbial cell. EDTA (as chelating agent) and various Tris buffers are also used (Robe *et al*., 2003). The extracted DNA is then purified to eliminate the

contaminants. Siddhapura *et al*., (2010) developed an improved method for extracting metagenomic DNA from saline soil to obtain intact unsheared DNA flexible to further molecular biological applications. Among the soft lysis, harsh lysis and combination of both, the soft lysis method was found simple and efficient for rapid isolation of PCR amplifiable total genomic DNA.

Even though the manual method of metagenomic DNA extraction is efficient and cost effective, currently, most of the metagenomic studies rely on wide range of commercially available kits. This is due to their short hands-on time and adaptability to robotic platforms when conducting high-throughput studies. The sample homogenization, bacterial lysis and DNA purification techniques are important sources of technical variations, which can significantly distort the apparent composition, structure and diversity of the microbiota (Carrigg *et al*., 2007). These pitfalls can be managed by using the DNA isolation kits. The application of metagenomic DNA isolation kits for metagenome isolation provides simple, rapid isolation of inhibitor-free, PCR quality DNA from the samples.

Qiagen DNeasy Power Soil kit, HiPurA soil DNA isolation kit (Himedia), Sigma's GenElute™ Soil DNA Isolation Kit, Norgen´s Soil DNA Isolation Plus Kit, FastDNA Spin Kit for Soil and NucleoSpin® soil DNA isolation kit (Macherey-Nagel) are some of the several soil DNA extraction kits available commercially in the markets.

2.8.2 Purification of isolated metagenomic DNA

As the soil is a complex material, the extracted metagenomic DNA will be always associated with contaminants like humic acid which can hinder further steps like PCR, restriction, etc and hence its removal is inevitable (Tebbe and Vahjen, 1993). Humic acid undergoes oxidation to form quinone, that binds covalently with DNA and cause its denaturation (Young *et al*., 1993). Hence, organic solvent extraction using phenol, chloroform and iso-propanol is employed for lysing the cells during the isolation of DNA (Steffan *et al*., 1998). Sharma *et al*. (2007) introduced a purification technique to extract

good quality DNA by suspending Q- Sepharose beads in 10mM potassium phosphate buffer.

2.8.3 Next Generation Sequencing (NGS) and bioinformatics analysis

After the purification of isolated metagenomic DNA, high throughput sequencing technologies are employed to study the microbial diversity. The Next Generation Sequencing technologies are adopted and found to be much efficient for this purpose (Shendure and Ji, 2008). Higher sensitivity to detect low-frequency variants, faster turnaround time for high sample volumes, comprehensive genomic coverage, high throughput and lower cost of NGS over Sanger sequencing makes it comparatively reliable for the analysis of complex microbial communities. Many sequencing platforms employing NGS have been developed including pyrosequencing, Ion Torrent technology, Illumina/Solexa platform, and SOLiD (Sequencing by Oligonucleotide Ligation and Detection). Among the three NGS systems described before, the Illumina HiSeq 2000 features the biggest output and lowest reagent cost (Huse *et al.,* 2007). The short reads obtained from the Illumina platform is a result of deep coverage sequencing, but concurrently making the read based analysis bit difficult (Scholz *et al*., 2012).

The metagenomic analysis aims at regenerating all the genomes existing in an environmental sample. Two methods followed to obtain that are i) assemblage of contigs followed by taxonomic classification and functional assignments, ii) reconstruction of functional and taxonomic elements of the metagenome (Scholz *et al*., 2012). The online metagenome annotation tools IMG-M and MG-RAST are usually used for the metagenomic genome annotation of prokaryotic cells (Meyer *et al*., 2008). MG-RAST is an open source server used most commonly for genome annotation is used for comparative genomics (Overbeek *et al*., 2005). This server renders access to extensive amount of data including phylogenetic and metabolic reconstructions, metagenomes, etc. to which the data in the hand can be compared (Meyer *et al*., 2008).

2.9 Metagenomic studies carried out in Kerala Agricultural University

Several experiments were done at Kerala Agricultural University using metagenomic approach. Metagenomic analysis was employed to assess the bacterial diversity showed diverse groups of bacteria including Proteobacteria, Cyanobacteria, Acidobacteria, Chloroflexi and unidentified bacteria (Sah *et al.*, 2014). The bacteria belonged to saline tolerant, acidophilic, nitrogen fixing, sulphur reducing, sulphur oxidizing, strict anaerobes and pathogenic groups. A similar analysis done in long-term fertilized paddy field soil has shown an increased population of beneficial bacteria like N fixers in the plots with long-term application of organic fertilizer (Ashwini, 2016). The dominant phyla across all the samples were Actinobacteria, Acidobacteria, Proteobacteria, Firmicutes and Bacteroidetes. Verrucomicrobia, Chlorobi, Cyanobacteria, Chloroflexi and Spirochaetes were detected in all the samples with low abundance of OTUs. Among the dominant phyla, proportion of Actinobacteria (57.95%) was more abundant in plots receiving integrated regimes and low (25.80%) in plots receiving inorganic inputs alone. Conversely, Acidobacteria and Proteobacteria were more abundant in soils receiving inorganic inputs (INF) (Acidobacteria 14.31% and Proteobacteria 14.55%).

Diversity and phylogenetic relationship of indigenous bacteria in cowdung was assessed through metagenomics (Girija *et al*., 2013). Abundance of IAA and siderophore producers, N-fixers, phosphate solubilizers as well as efficient degraders of complex organic matter were detected in cowdung samples using metagenomics, thus justifying the inevitable role of cowdung in agriculture and waste management. Three predominant phyla included Firmicutes, Bacteriodetes and Proteobacteria. Metagenomic analysis was also employed to unveil the culturable and unculturable endophytic bacteria associated with *Phalaenopsis* roots (Girija *et al*., 2018). Metagenomic analysis in the rhizosphere of arecanut palms affected with yellowing revealed that the bacterial diversity in the rhizosphere of apparently healthy palms was found to be higher than that of the yellowing affected palms, which indicated that the increased population of identified bacterial taxa might have a significant role in maintaining the health of arecanut palms and reducing the

yellowing symptoms by mediating nutrient recycling and plant growth promotion (Mohan, 2019).

2.10 Future trends in metagenomics

Apart from gene annotations and phylogenetic reconstructions, bioprospecting with the aid of metagenomics can be considered as of utmost importance, as the soil serves as an important source of metabolite producing microbes (Daniel, 2005). The functional and metabolic ability of the microbial communities in the soil can be studied by the manipulation of ribonucleic acid (RNA) and hence metatranscriptomics proves to be another efficient technology that can be used to study the difference between expressed genes and non-expressed genes (Sorek and Cossart, 2010). In the year 2006, Wilmes and Bond proposed the term ‘metaproteomics’, for the extraction and analysis of total protein content from the environmental sample. The metagenomic analyses are commonly applied in the field of health, medicine, industries, food and agriculture. Apart from metagenomic study, metatranscriptomics and metaproteomics has expanded the boundary to explore the functional dynamics of the soil microflora (Simon and Daniel, 2011).

Materials and Methods

3. MATERIALS AND METHODS

The study on "Metagenomic analysis of bacterial diversity in the rice rhizosphere of Kole lands of Thrissur" was carried out during the period 2019-2021 at the Department of Agricultural Microbiology and Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Vellanikkara. The materials used and the methodologies employed in this laboratory oriented study are presented below.

3.1 MATERIALS

3.1.1. Glassware and plasticware

The glassware used for the experiments were supplied by Borosil, Merck and the plasticwares by Tarson India Ltd. and Eppendorf.

3.1.2. Chemicals used

The chemicals used for the study were obtained from agencies like Hi-media, Merck, SRL and Nice and the molecular biology reagents and buffers were procured from Sigma-Aldrich and GeNei.

3.1.3. Equipment items used

The equipment items available at the Department of Agricultural Microbiology, Centre for Plant Biotechnology and Molecular Biology and Radio-Tracer laboratory were utilized for the study. Equitron SLEFA and NatSteel horizontal autoclaves were used for the sterilization of glassware, microbiological media, water blanks, etc. Isolation, purification and screening of microbes were carried out in the laminar air flow chamber. GeNei OS-250 incubator cum shaker was used for the incubation of microbial culture and the centrifugation was carried out using SPINWIN MC-02 and Eppendorf 5804R centrifuges. The microscopic cells were examined using a compound binocular

microscope (Leica ICC50). The Eutech pH tutor was used to estimate the pH of microbial culture media, buffers and reagents. The total Nitrogen from soil samples were analyzed using KELPLUS VA DSL and DISTYL EM VA.

3.2. METHODOLOGY

3.2.1. Collection of rhizosphere soil samples

A total of six rice rhizosphere soil samples were collected, two each from three different locations of Kole lands of Thrissur (Puzhakkal, Mullassery and Cherpu) (Plate 1). The soil samples were collected by uprooting the plant along with the rhizosphere soil at maximum tillering stage (at a depth of around 15-20 cm) and brought to the laboratory for microbial analysis and metagenomic DNA isolation. Approximately, 500g soil was collected from each rhizosphere and the Global Positioning System (GPS) coordinates of the sample collected locations has been enlisted in the Table 1 and locations depicted in Plate 2.

Table 1. Coordinates and altitudes of the area selected for sample collection

Sample		Location	Latitude	Longitude
Puzhakkal (Pzk)	PzkR1	Adat	N 10.533195°	E 76.165156°
	PzkR2	Adat	N 10.540389°	E 76.161634°
Mullassery (Mls)	MlsR1	Elavathur	N 10.543431°	E 76.108386°
	MlsR2	Elavathur	N 10.544012°	E 76.109456°
Cherpu (Chr)	ChrR1	Cheruchenam	N 10.431664°	E 76.192674°
	ChrR2	Cheruchenam	N 10.430542°	E 76.191976°

3.2.2. Enumeration of rhizosphere microflora

Enumeration of the culturable microorganisms from the rice rhizosphere soil samples were carried out by serial dilution and plating method on appropriate media. The enumeration of culturable microflora was done on 10 different media (Table 2) in order

Plate 1. Locations of rhizosphere sample collection

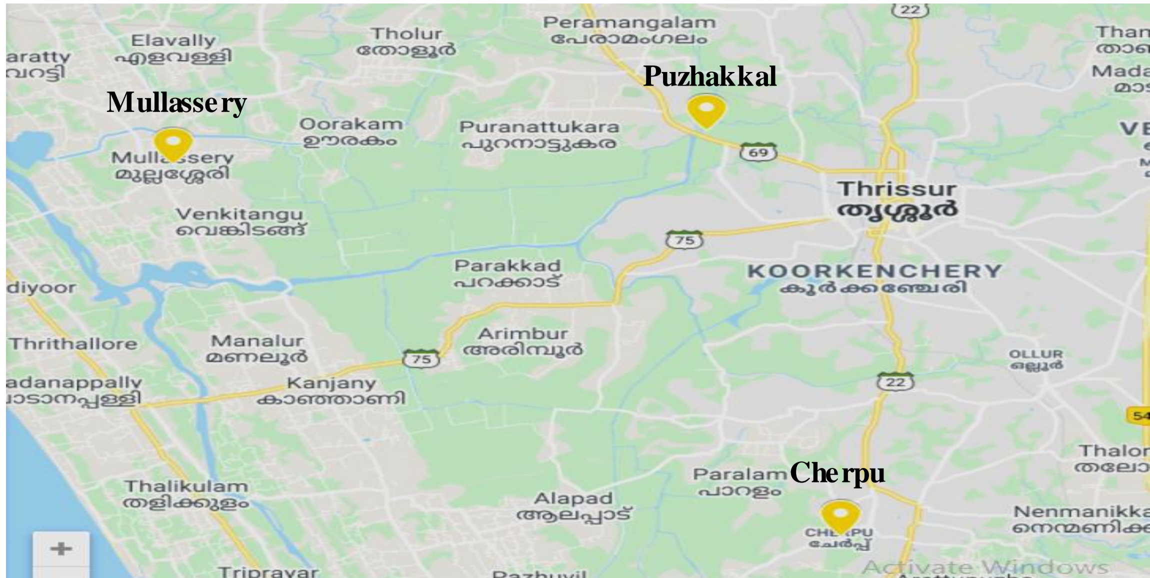


Plate 2. Map of sample collected locations

to differentiate the different functional groups present in the soil samples. The enumeration of bacteria was carried out on Luria Bertani agar and Nutrient agar whereas, the enumeration of fungi was done on Martin's rose Bengal agar and potato dextrose agar. Kenknight Munaier's agar and sodium caseinate agar were used for the enumeration of actinomycetes. The enumeration of nitrogen fixers was carried out on Jensen's nitrogen free agar and Ashby's agar and that of phosphorous solubilizers was carried out on Pikovskaya's agar. Alexandrov agar and zinc oxide agar medium were used for the enumeration of potassium and zinc solubilizers, respectively. The composition of media are given in Annexure V. The dilutions were later standardized after conducting experiments using respective medium.

- Ten gram of the collected soil sample was transferred to 90 mL sterile distilled water in a 250 mL conical flask under aseptic condition and the contents were mixed by shaking for ten minutes.

- One mL of the aliquot was taken using sterile pipette and transferred to 9 mL sterile distilled water blank.

- The suspension was shaken for a minute for homogenization and likewise further dilutions were carried out upto 10^{-6} for the isolation of specific groups of microorganisms. The dilutions were standardized for each medium and those dilutions which yielded 20-200 colonies per plate were selected for furthur experimentation.

- Twenty mL of the molten agar media was poured into sterile petriplates and allowed to solidify.

- 0.1 mL aliquot of the desired dilution was pipetted out and transferred aseptically on to the center of the surface of respective agar plate. The sample was spread over the surface of agar using the sterile L-shaped glass spreader by carefully rotating the Petri plate underneath at the same time.

- The plate was then kept for incubation at room temperature in an inverted position.
- The number of colonies observed in the respective media was recorded and population expressed as the number of colony forming units per gram of soil (cfu g^{-1}) for each category of soil sample. Alterations were made in dilutions to obtain optimum number of colony forming units.

Table 2. Media used for the isolation of culturable microflora by serial dilution and plating method

Organism targeted	Media used
Bacteria	Nutrient agar
Bacteria	Luria Bertani agar
Fungi	Martin's rose Bengal agar
Fungi	Potato dextrose agar
Actinomycetes	Kenknight and Munaier's Agar
Actinomycetes	Actinomycete isolation medium
N- Fixers	Jensen's nitrogen free agar
N-Fixers	Ashby's agar
Phosphate solubilizers	Pikovskaya's agar
Potassium solubilizers	Alexandrov Agar
Zinc solubilizers	Mineral salt agar amended with 0.1% ZnO

3.2.3. Purification and maintenance of isolates

A total of twenty four predominant bacterial isolates were purified by quadrant streak plate method and the purified isolates were preserved in agar slants at a low temperature of 4°C for further studies.

3.3. Characterization of purified isolates

3.3.1. Cultural characterization

The morphological and cultural characters of the purified isolates were observed. The size, shape, colour and elevation of the colonies were recorded.

3.3.3.1. Colony and cell morphology

The purified bacterial isolates were observed microscopically for the morphological characters such that cell size, shape, etc and the colony morphology was studied by culturing on agar plates.

3.3.3.2. Gram staining

The Gram staining technique was employed for differentiating the purified isolates into gram positive and gram negative.

- A loopful of purified inoculum was aseptically transferred onto a sterile glass slide with one or two drops of water to make a thin smear.
- Then the suspension was heat fixed to affix the bacteria to the slide.
- The smear was flooded with crystal violet (primary stain) for one minute and washed away.
- Grams iodine solution was added to the smear and kept for one minute and rinsed away with running water.
- The smear was decolourized using 95 per cent ethanol and rinsed off with water after 5 seconds.

- The decolourized smear was then counterstained with safranin for one minute and washed off.

The smears were observed under microscope for the visualization of colour (purple/red), shape and arrangement of cells. Red colour indicated that the bacterium was Gram negative and purple colour was indicative of Gram positive bacteria.

3.4. *In vitro* screening of bacterial isolates for Plant Growth Promoting (PGP) traits

The purified bacterial isolates were screened qualitatively for Plant Growth Promoting (PGP) activities including the production of ammonia and indole acetic acid and phosphate solubilization under *in vitro* conditions. The media composition used for the experiments are shown in Annexure V.

3.4.1 Screening for IAA production

The purified bacterial isolates were screened for IAA production by inoculating in sterile Luria-Bertani broth supplemented with tryptophan (one mg ml^{-1}) and the tubes were incubated in dark for 7 days. After incubation, the cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant was collected and to this, four ml of Salkowski reagent was added. The IAA production was indicated by the development of pink colour (Ahmad *et al.*, 2008).

3.4.2 Screening for phosphate solubilization activity

The screening of bacterial isolates for phosphate solubilization was carried out on Pikovskaya's agar. The cultures grown on Pikovskaya's broth were spot inoculated on Pikovskaya's agar plates. These plates were incubated for 7 days at 30^{0}C. The phosphate solubilization efficiency was evaluated in terms of per cent phosphate solubilization, as indicated below (Panhwar *et al.*, 2012).

$$\text{Solubilization efficiency (\%SE)} = \frac{\text{Solubilization diameter}}{\text{Colony diameter}} \times 100$$

3.4.3 Screening for ammonia production

The bacterial isolates were screened for the production of ammonia by inoculating the isolates in four per cent peptone water and the tubes were incubated for 48 hours at 28°C. After incubation, 0.5 ml Nessler's reagent was added to the tubes. The production of ammonia was indicated by the development of brown to yellow colour (Cappuccino and Sherman, 1992).

3.5. Physico-chemical analysis of the rhizospheric soil samples

The soil samples were analysed for pH, EC and the nutrient concentrations. The methodologies adopted are provided in Table 3.

Table 3. Methodologies adopted for the physico-chemical and microbial biomass carbon analysis of the soil samples

Parameters	Method employed	Reference
Soil pH	Soil water suspension of 1:2.5 (read using pH meter)	Jackson, 1958
Electrical Conductivity	Soil water suspension of 1:2.5 (read using EC meter)	Jackson, 1958
Organic Carbon (%)	Walkley and Black wet digestion method	Walkley and Black, 1934
Total Nitrogen	Micro-kjeldahl method	Jackson, 1973
Available Phosphorous	Bray no.1 method (reduced molybdate blue colour method)	Bray and Kurtz, 1945
Available Potassium	Neutral ammonium acetate method (using flame photometry)	Jackson, 1958
Available Calcium and Magnesium	Atomic absorption spectrometry	Hesse, 1971
Available Sulphur	Turbidi-metrico method (Extraction using $CaCl_2$)	Massoumi and Cornfield, 1963
Available Iron, Manganese, Zinc and Copper	Atomic absorption sphectrometry (HCl extraction)	Sims and Johnson, 1991
Available Boron	Azomethine-H method (Hot water extraction)	Berger and Troug, 1939
Microbial Biomass C	Fumigation - extraction method	Vance *et al.*, 1987

3.6 Isolation of metagenomic DNA

The soil metagenomic DNA extraction was carried out in order to establish the metagenomic libraries of the six soil samples. The major limitations taken into account during the metagenomic DNA isolation were i) DNA extraction from wide range of microorganisms to procure a representative of the microbial population ii) shearing of the large molecular weight DNA during the isolation procedure iii) isolation of contaminant-free DNA, etc. (Schmeisser *et al.*, 2007). Also, the preferred extraction procedures should not be harsh as it can destroy lyze-sensitive DNA present in the sample (Robe *et al.*, 2003).

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

- One gram of soil sample was taken in a sterile 30 mL centrifuge tube. 10 mL of extraction buffer was added into the tube and incubated at 37^0C for 10-12 hours with continuous shaking (150 rpm).

- The samples were re-extracted using one mL of the same extraction buffer and centrifuged at 5000 rpm for 10 minutes.

- The supernatants were collected and mixed with four mL lysis buffer and kept for incubation at 65^0C for 2 hours with intermittent shaking at every 15 minutes interval. Then the suspension was centrifuged at 10,000 rpm at 4^0C for 10 minutes.

- The top aqueous layer formed was extracted by adding equal volume of chloroform: isoamyl alcohol (24:1) followed by centrifugation at 10,000 rpm for 20 minutes at 4^0C.

- The top aqueous layer obtained was again collected and centrifuged at 10,000 rpm for 10 minutes at 4^0C with equal amount of chloroform: isoamyl alcohol (24:1).

- Then the suspension was treated with 1/10 volume of 7.5M potassium acetate and the DNA was precipitated with 2 volumes of ice cold 100 % ethanol followed by centrifugation at 10,000 rpm for 10 minutes.

- The DNA precipitated was then air dried and suspended in 50µL sterile distilled water.

3.6.2 Direct method - Short procedure (Siddhapura *et al*., 2010)

- Twenty milligram of soil sample was taken in a 1.5 mL sterile centrifuge tube. 400 µL of extraction buffer was added to the tube and vortexed for 10-15 minutes.

- The suspension was kept for incubation at room temperature for 1 hour and is centrifuged at 12,000 rpm for 5 minutes.

- The supernatant was collected in a fresh centrifuge tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to it. Later, the suspension was centrifuged at 10,000 rpm for 20 minutes at 4^0C and the supernatant was collected.

- The DNA was precipitated by adding equal volume of ice cold isopropanol followed by 15 minutes incubation.

- Then the DNA pellet was collected by centrifuging the suspension at 13,000 for 5 minutes and discarding the supernatant.

- The pellets were washed twice by adding 70 per cent ethanol (600µL) followed by centrifugation at 10,000 rpm for 5 minutes.

- Then the precipitated DNA was air dried and suspended in 25µL sterile distilled water.

3.6.3 Extraction of metagenomic DNA by using DNeasy® PowerSoil® Kit

The DNeasy PowerSoil kit by QIAGEN was used to extract good quality metagenomic DNA from the soil samples (containing high humic acid content). The metagenomic DNA extraction was done by lysing the samples mechanically in a bead beating tube and chemically by treating with the solutions provided. Then the DNA was collected in a MB spin column provided with the kit and washed with ethanol (wash solution). The DNA was finally obtained by eluting the DNA from the column using elution buffer.

3.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to analyze the quality of extracted metagenomic DNA. The components used for the preparation are given in Annexure IV.

- The electrophoresis of the isolated metagenomic DNA was done with 2 per cent agarose gel.
- The gel was prepared by dissolving 2g of agarose in 100mL 1X TAE buffer followed by heating for proper homogenization.
- Once the solution was cooled, 3 µL of ethidium bromide is added.
- Then the mixture was poured into the casting tray and a comb was placed on it.
- The gel was allowed to set for 45 minutes. Then the comb was taken carefully without damaging the wells.
- The agarose gel was then gently placed in the gel tank in such a way that the wells are placed near the cathode.

- 1X TAE buffer was poured into the gel tank till it covers the gel. Then the DNA (5µL) is loaded along with loading dye (2µL) into the respective wells.

- The molecular marker (ladder) was also loaded in one lane. Then the power plugs are connected and a constant electric potential of 70V was applied till the dye reaches three-fourth portion of the gel from the top end.

- Later, the agarose gel was taken out and visualized for the presence of DNA bands under transilluminator.

3.8 Quantitative analysis of metagenomic DNA

3.8.1 Nanodrop quantification

The concentration and purity of the metagenomic DNA samples were assessed using nanodrop spectrophotometer (NanoDrop Technologies., USA).

- The optical surfaces of the instrument were cleaned with distilled water and a dry lab wipe.

- The blank value was set using sterile distilled water or elution buffer in the case of DNA isolated with the commercial kit.

- Then the samples were loaded on the optical surface and the absorbance were recorded at 260nm and 280 nm.

- The purity of the samples were assessed by calculating the 260/280 ratio and 260/230 ratio.

- The quantity of the DNA samples were measured using the relationship,

$$1 \text{ OD at } 260\text{nm} = 50\ \mu g\ \mu L^{-1} \text{ of DNA}$$

$$\text{Hence, } OD_{260} \times 50 \text{ gives the concentration of DNA in } \mu g\ \mu L^{-1}$$

3.8.2 Qubit Assay

The quantity of the samples were also assessed using Qubit double stranded DNA (dsDNA) high sensitivity assay kit (Life Technologies), which uses fluorescent dyes that specifically binds the double stranded DNA. After caliberating the instrument with standards, the samples were read and the concentration of the samples was recorded.

3.9 Metagenomic DNA sequencing

The metagenomic DNA extracted from the six soil samples were analyzed for the qualitative and quantitative standards recommended for sequencing. The samples were sequenced at a private scientific facility, AgriGenome Lab Pvt Ltd., Kochi.

3.9.1 16S rRNA metagenomic sequencing library preparation using Next Generation Illumina MiSeq™

The amplicon gene library was prepared by amplifying the V3 and V4 hypervariable regions of 16 S rRNA gene using specific primers. The amplicons obtained were then sequenced and subjected to subsequent classification of microorganisms.

3.9.1.1 Amplicon PCR

The amplicon PCR of metagenomic DNA was done using specific primers designed for the V3 and V4 for regions of 16S rRNA. The master mix comprised of 5 µL of 5X Phusion HF reaction buffer, 0.5 µL of 40mM dNTP, 2µL each of forward and reverse primers (10 pmol/ µL), 0.2 µL of F-540 special Phusion HS DNA polymerase (2U/ µL), 5 ng of input DNA and sterile distilled water to make up the volume to 25 µL.

The PCR reactions were set at a preset programme of initial denaturation for 30 seconds at 98^0C. The denaturation temperature was set at 98^0C for 10 seconds for 30 cycles followed by annealing at 55^0C for 30 seconds. The primer extension was set at 72^0C for 30 seconds and final extension at 72^0C for 5 minutes followed by a hold at 4^0C.

The amplicons obtained were then quantified using Qubit fluorometer with the Qubit dsDNA HS assay kit (Invitrogen, USA).

3.9.1.2 PCR clean-up

The PCR clean-up was done using AMPure XP beads to eliminate the free primers as well as primer dimers from the product in order to obtain pure V3 and V4 regions of 16S rRNA. The reagents used for PCR clean-up was 52.5 µL Tris pH 8.5 (10mM), 20 µL AMPure XP beads and 400 µL freshly prepared 80 per cent ethanol for each sample. The purified PCR products were stored at -20^{0}C.

3.9.1.3 Index PCR

The index PCR was done with an objective to attach the dual indices and Illumina sequencing adapters to the cleaned-up PCR amplicons. The index PCR master mix was comprised of 10 µL 5X Phusion HF reaction buffer, 1 µL of 40mM dNTP, 2 µL each of forward and reverse primers (10 pmol/ µL), 0.4 µL of F-540 special Phusion HS DNA polymerase (2U/ µL) and 10 µL of PCR I amplicons (minimum 5ng) and water to make up the volume to 50 µL.

The PCR reaction was set as initial denaturation at 98^{0}C for 30 seconds followed by denaturation at 98^{0}C for 10 seconds repeated for 15 cycles, annealing at 55^{0}C for 30 seconds, primer extension at 72^{0}C for 30 seconds, final extension at 72^{0}C for 5 minutes and finally hold at 4^{0}C.

3.9.1.4 PCR clean-up 2

The product obtained from index PCR was cleaned-up before the quantification using AMPure XP beads to remove the free primers and primer dimers. The reagents used for clean-up were 25.5 µL 10mM Tris pH 8.5, 56 µL AMPure XP beads and 400 µL freshly prepared 80% ethanol. Standard protocol was followed and the purified PCR product obtained were stored at -20^{0}C.

3.9.2 Library quantification, normalization and pooling

The quantification of library was done by fluorometric quantification method using dsDNA binding dyes recommended by Illumina. The final concentrated library was diluted to 4 nM with 10mM Tris pH 8.5. Five µL aliquot of diluted DNA from each samples were mixed to pool the libraries with unique indices. More than 100,000 reads per sample was considered to be sufficient for the complete survey of bacterial population of the metagenomic sample.

3.9.3 Library denaturing and MiSeq sample loading

For cluster generation and sequencing, the pooled libraries were denatured using sodium hydroxide (NaOH), followed by dilution with hybridization buffer and then heat denaturation before Illumina MiSeq sequencing. A minimum of 5% PhiX must be included in each run to attain an internal control of low diversity libraries. Finally, the denatured library was immediately loaded into the Illumina MiSeq reagent catridge for sequencing. The sequences obtained were in FastQ format, which was further used for *in silico* analysis.

3.10 NGS data analysis

The total raw sequence reads obtained were subjected to quality control analysis for the parameters like base composition, base quality distribution, base distribution, GC distribution etc. The unwanted sequences from the orginal paired-end data were trimmed off and a consensus V3 and V4 sequences were constructed using multiple sequence alignment program Clustal Omega (http://www.ebi.ac.uk/Tools/ msa/clustalo/).

The V3 and V4 sequences obtained were quality filtered using spacer filter, conserved region filter, mismatch filter, etc. to remove too short sequences, many ambiguous base pairs and chimeras. The singletons produced due to sequencing errors were eliminated to keep away the errors in assessment of operational taxonomic units

(OTUs). The chimeras that may be misinterpreted as novel sequence were detected using the *de novo* program UCHIME in VSEARCH sequence analysis tool.

The bioinformatics pipeline QIIME (Quantitative insights into Microbial Ecology) was used to cluster the sequences into operational taxonomic units based on the sequence similarity. The chimera-free full-length 16S rDNA sequences were obtained using the PyNAST (Python Nearest Alignment Space Termination) alignment program by identifying the representative sequences from the operational taxononomic units followed by alignment against the template sequence (De Santis *et al.*, 2006).

The taxonomic classification was carried out using an open source web application server MG RAST (Metagenomic Rapid Annotation using Subsystems Technology). The raw sequence data fed in fasta format were automatically processed by the pipeline to obtain the taxonomic distribution data by comparing the sequences against RDP database. The taxonomic distribution data obtained can be represented as graphs and charts.

The offline tool MEGAN (MEtaGenome Analyzer) was used to obtain phylogenetic trees and statistical data of the metagenome at various taxonomic levels. The BIOM files which provides information about the number of reads and OTUs corresponding to each taxa were fed into MEGAN as input. MEGAN collects the reads and compares it with the sequence databases and allocates a taxon ID based on the NCBI taxonomy which provides sufficient information for the graphical and statistical analysis.

Alpha diversity metrics summarize the structure of an ecological community with respect to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups), or both. The microbial diversity within the samples was assessed using Shannon, Chao1 and Observed species metrics and the metric calculation was performed using QIIME software. A rarefaction curve can be used to determine whether a sample has been sequenced to an extent sufficient to represent its true

diversity. Rarefaction curves plots the number of individuals on the x-axis against the number of species on the y-axis.

The sequence data obtained from the Illumina platform was then submitted in Sequence Read Archive (SRA) of GenBank database and the accession number was obtained.

Results

4. RESULTS

The study entitled 'Metagenomic analysis of bacterial diversity in the rice rhizosphere of Kole lands of Thrissur' emphasized on the diversity analysis of bacterial community in the rice rhizosphere ecosystem of Kole lands of Thrissur. The results obtained from the investigation are presented in this chapter.

4.1 Collection of rice rhizosphere soil samples

A total of six rice rhizosphere soil samples were collected, *ie.,* two samples from the three different locations of Kole lands of Thrissur *viz.* Puzhakkal (Pzk), Mullassery (Mls) and Cherpu (Chr) (Plate 1). Soil samples were collected by uprooting the plant along with the rhizosphere soil at maximum tillering stage, as detailed under Materials and Methods, for microbial analysis and metagenomic DNA isolation.

4.2 Physico-chemical properties of rice rhizosphere soil samples collected from Thrissur Kole lands

The rhizosphere soil samples collected from the rice fields of Thrissur Kole lands were analysed for various physico-chemical properties. The parameters considered for the study included pH, electrical conductivity, microbial biomass carbon, % organic carbon content and the soil nutrient content. The primary nutrients analysed were total nitrogen, available phosphorous and available potassium. The secondary nutrients analysed included available calcium, magnesium and sulphur and the micronutrients analysed were iron, manganese, copper, zinc and boron (Table 4).

4.2.1 Electrical conductivity and pH

The electrical conductivity and pH of all the soil samples were analyzed. The electrical conductivity of all the soil samples was found to be normal (Table 4). The lowest value of 0.18 dSm^{-1} was recorded in sample from Chr and the highest value in the sample Pzk (0.27 dSm^{-1}).

All the samples recorded low pH, which indicated that the soils are strongly acidic nature. The sample Chr recorded the lowest pH of 4.29 (extremely acidic), followed by Mls and Pzk with a pH of 4.44 and 4.59 respectively.

4.2.2 Organic Carbon content

All the samples were tested for organic carbon content and the results showed that all the samples possess high level of organic carbon, as per the critical limits of Tandon (2005). The sample Pzk recorded 1.93 per cent, Mls and Chr recorded values of 1.23 per cent and 1.12 per cent organic carbon respectively.

4.2.3 Total nitrogen

Total nitrogen content of all the samples were analysed using micro-Kjeldahl method and the highest value was found in the sample Pzk (0.50 per cent) and the lowest was found in the sample Chr (0.13 per cent). All the soils were high in total nitrogen when compared to the critical limit of 0.004 per cent given by Tandon (2005).

4.2.4 Available phosphorous

Analysis of rhizosphere soil samples for available phosphorous showed that two samples were deficient in available phosphorous (Pzk, with 6.73 Kg ha^{-1} and Mls, with 8.3 Kg ha^{-1}). The sample Chr recorded sufficient level of available P (10.13 Kg ha^{-1}), when compared to the critical limits of 10 Kg ha^{-1} reported by Tandon (2005).

4.2.5 Available potassium

The available potassium was found to be low in all the samples tested, when compared to the critical value of 120 to 280 Kg ha^{-1}, proposed by Tandon (2005). Lowest value was recorded by the sample Chr (59.47 Kg ha^{-1}). The available K content of the other two samples Pzk and Mls were found to be 69.02 and 90.89 Kg ha^{-1} respectively.

4.2.6 Available calcium

The rhizosphere soil samples were tested for available calcium and the results did not show much variation among the samples. The sample Chr recorded sufficient level of available calcium content with a value of 307.53 mg Kg^{-1} and the remaining samples were deficit in available calcium content. The sample Mls recorded the least value of 150.58 mg Kg^{-1}.

4.2.7 Available magnesium

All the samples analysed were deficient in available magnesium, in comparison to the critical limits proposed with a highest value recorded for the sample Pzk (60 mg Kg^{-1}) and the least amount for the sample Chr (39.29 mg Kg^{-1}).

4.2.8 Available sulphur

All the soil samples tested possessed sufficient levels of sulphur, among that the sample Pzk recorded the highest value of 60.60 mg Kg^{-1} and the least value was observed for sample Chr (25.27 mg Kg^{-1}).

4.2.9 Micronutrients

4.2.9.1 Iron

The iron content was found to be high in all the samples as per the critical limits proposed by Tandon (2005). The sample Pzk recorded a highest value of 583.88 mg $Kg^{-1}$and the least value was recorded by the sample Chr with an iron content of 263.19 mg Kg^{-1} (Table 4).

4.2.9.2 Manganese

The manganese content was found sufficient in all the samples. The highest value of manganese content was recorded by the sample Chr with a value of 9.69 mg Kg^{-1} and lowest by the sample Pzk with 3.62 mg Kg^{-1}.

4.2.9.3 Zinc

The zinc content was sufficient in all the samples tested and among the samples, lowest concentration of zinc was found in the sample Mls (2.39 mg Kg^{-1}) and the highest value was shown by the sample Chr (3.49 mg Kg^{-1}).

4.2.9.4 Copper

All the samples were found to contain sufficient content of copper. The sample Chr recorded the highest value of 3.42 mg Kg^{-1} among the samples and the sample Pzk recorded the least value of 2.87 mg Kg^{-1}.

4.2.9.5 Boron

All the samples (Pzk and Mls) were found to be deficient in boron content, when compared to the critical limits proposed by Tandon (2005).

Table 4. Physico-chemical parameters of rice rhizosphere soil samples

Samples	pH	EC (dS m^{-1})	OC (%)	Total N (%)	P (Kg ha^{-1})	K (Kg ha^{-1})	Ca (mg Kg^{-1})	Mg (mg Kg^{-1})	S (mg Kg^{-1})	Fe (mg Kg^{-1})	Mn (mg Kg^{-1})	Zn (mg Kg^{-1})	Cu (mg Kg^{-1})	B (mg Kg^{-1})
Pzk	4.59	0.27	1.93	0.50	6.73	69.02	292.07	60.00	60.60	583.88	3.62	2.87	2.87	0.02
Mls	4.44	0.23	1.23	0.33	8.30	90.89	150.48	39.29	54.62	466.06	6.72	2.39	2.99	0.02
Chr	4.29	0.18	1.12	0.13	10.13	59.47	307.53	41.72	25.27	263.19	9.69	3.49	3.42	0.05
Critical values for rice crop (Tandon, 2005)	4.6	1.0	0.5	0.004	10	115	300	120	10	4.5	2.5	0.6	0.2	0.5

4.3 Biological Properties of the soil

The rice rhizosphere soil samples collected from three different locations of Kole lands were analysed for biological properties including microbial biomass carbon, population of microorganisms like bacteria, fungi, actinobacteria, nitrogen fixers and solubilizers of phosphate, potassium and zinc. The results are detailed in Table 5.

4.3.1 Microbial biomass carbon

All the samples were analyzed for microbial biomass carbon and the highest value was recorded by the sample Pzk (684.11 $\mu g\ g^{-1}$) and lowest in the sample Chr (258.52 $\mu g\ g^{-1}$). The values of microbial biomass carbon are detailed in Table 5.

4.3.2 Enumeration of culturable microbial population of the soil samples

The population of bacteria, fungi, actinomycetes, nitrogen fixers, phosphate solubilizers, potassium solubilizers and zinc solubilizers were estimated using selective media and suitable dilutions. The population of the above mentioned microorganisms from all the six rhizosphere samples is illustrated in Table 5.

4.3.3 Bacteria

The bacterial population of all the samples was enumerated and among the samples, the highest bacterial population was observed in the sample Pzk with 25.25 x 10^5 cfu g^{-1} and the lowest was found in the sample Chr with a population of 13.00 x 10^5 cfu g^{-1}.

4.3.4 Fungi

Fungal population was enumerated on PDA and RBA. The sample Pzk recorded the highest population of 17.85 x 10^3cfu g^{-1}. The population of fungi was lowest in the sample Mls (9.60 x 10^3cfu g^{-1}).

4.3.5 Actinobacteria

The population of actinobacteria in rhizosphere soil samples was enumerated on Kenknight's and actinomycete isolation medium. The sample Pzk recorded higher population of 4.70 x 10^3cfu g^{-1} and the sample Chr recorded the lowest actinobacterial population (1.70 x 10^3cfu g^{-1}).

4.3.6 Nitrogen fixers

The enumeration of nitrogen fixers were carried out on Jensen's and Ashby's agar. The sample Mls recorded the highest population of 11.35 x 10^4 cfu g^{-1} and the least population was observed in the sample Chr (5.80 x 10^4 cfu g^{-1}).

4.3.7 Phosphate solubilizers

The enumeration for phosphate solubilizing bacteria and fungi were carried out in Pikovskaya's agar. The sample Pzk recorded the highest population of 14.55 x 10^3 cfu/g. The population of phosphate solubilizers was found to be less in the sample Chr (4.25 x 10^3cfu g^{-1}).

4.3.8 Potassium solubilizers

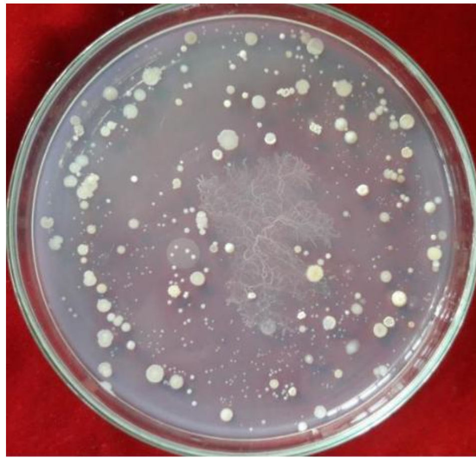
The population of potassium solubilizing bacteria and fungi were enumerated on Alexandrov agar and among the three samples, the highest population was recorded by the sample Mls (2.70 x 10^3cfu g^{-1}). The population was found to be least in the sample Chr with a value of 1.53 x 10^3cfu g^{-1}.

4.3.9 Zinc solubilizers

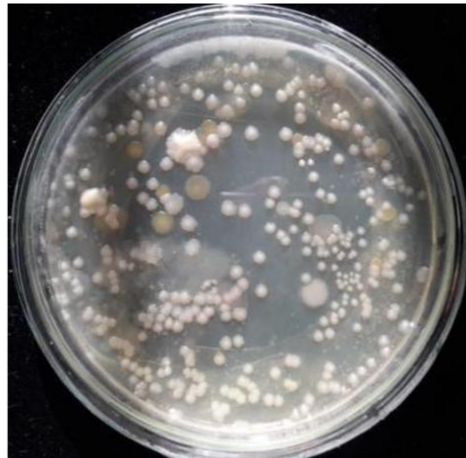
The zinc solubilizing microbial population was found to be comparatively low in all the samples. The sample Pzk recorded a population of 0.15 x 10^3cfu g^{-1}, which was found to be the highest among all the samples. No zinc solubilizers could be detected in the sample Chr.

Table 5. Microbial biomass carbon and the culturable microbial diversity in the rice rhizosphere samples

Samples	Microbial biomass carbon ($\mu g\ g^{-1}$)	Microbial population in cfu g^{-1} soil						
		Bacteria x 10^5	Fungi x 10^3	Actinomycetes x 10^3	N-fixers x 10^4	P-solubilizers x 10^3	K-solubilizers x 10^3	Zn- solubilizers x 10^3
Puzhakkal (Pzk)	684.11	25.25	17.85	4.70	9.55	14.55	3.40	0.15
Mullassery (Mls)	562.60	21.05	9.60	2.25	11.35	8.15	2.70	0.05
Cherpu (Chr)	258.52	13.00	9.90	1.70	5.80	4.25	1.53	0.00

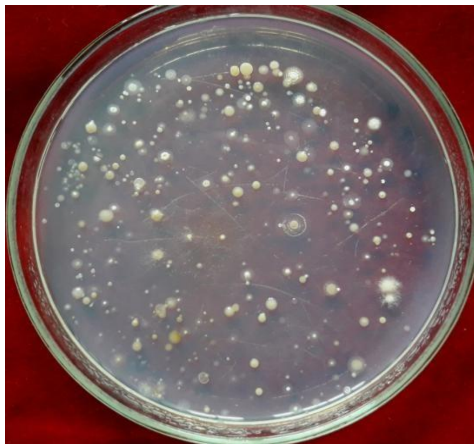


Mls-R1

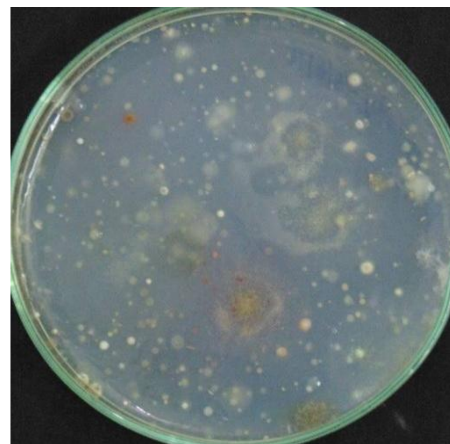


Pzk-R1

Plate 3.a. Culturable bacterial diversity enumerated using serial dilution plating



Pzk-R2



Mls-R2

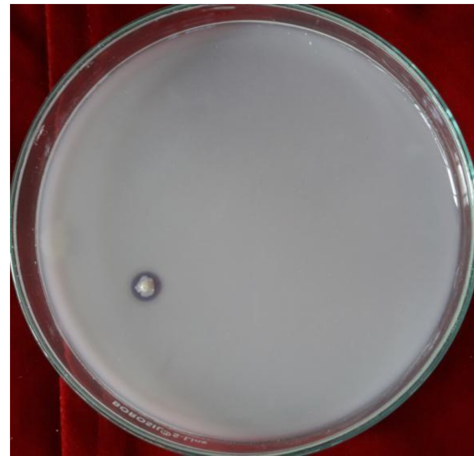
Plate 3.b. Culturable diversity of actinomycetes using serial dilution plating

Pzk-R2

Pzk-R1

Plate 3.c. Culturable diversity of fungi using serial dilution plating

Zinc solubilizers (Mls-R2)



Potassium solubilizer (Chr-R1)

Plate 3. Diversity of culturable microflora in the rice rhizosphere of Kole lands of Thrissur

4.4 Purification and maintenance of bacterial isolates

A total of 24 predominant bacterial isolates (eight from each of the three locations) comprising of nitrogen fixers, phosphate solubilizers, IAA producers, etc. were purified and maintained as agar slants at $4^{0}C$ for further characterization and screening for plant growth promoting (PGP) traits.

4.5 Cultural and morphological characterization

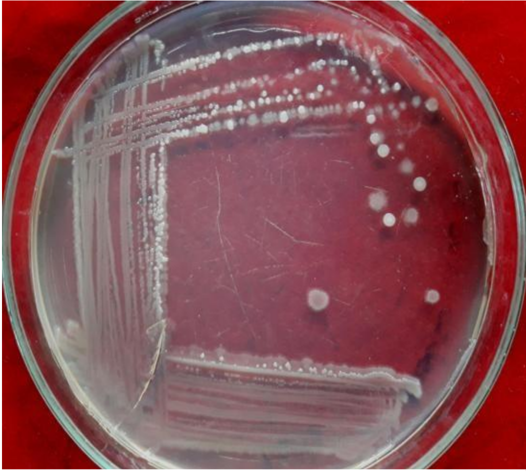
All the twenty four predominant bacterial isolates were observed for the cultural and morphological characters such as colony colour, form, margin, elevation. The colour of the colonies were yellow, white, creamy white, muddy white and were mostly circular, pin point, flat, raised at the centre. The cell shape and cell arrangements were also observed. Most of the isolates produced smooth colonies. Gram's reaction was positive for all the isolates except the bacteria Mls-BC4 obtained from the sample Mls.

4.6 *In vitro* screening of bacterial isolates for Plant Growth Promoting (PGP) activities

The bacterial isolates were screened for the production of indole acetic acid (IAA), nitrogen fixation, phosphate solubilization and ammonia production (Plate 6). The results are detailed in Table 6. Ten isolates exhibiting phosphate solubilization were used for calculating the phosphate solubilization efficiency (Table 7). Thirteen bacterial isolates were found to show IAA production which was ranked based on the intensity of colour produced. Eight bacterial isolates exhibited ammonia production.

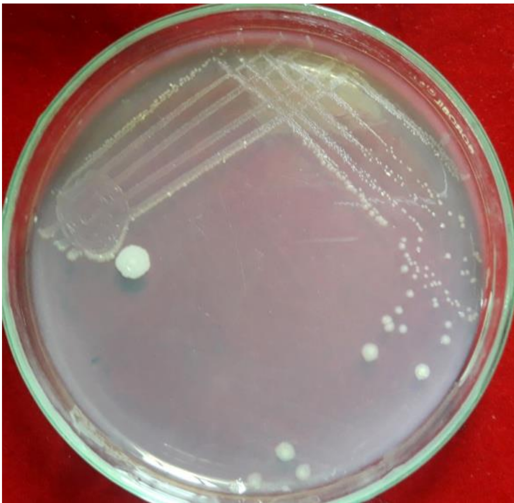
4.6.1. Screening for indole acetic acid production

The ability of bacterial isolates to produce IAA was studied with tryptophan as supplement. Among the 24 selected isolates, only 13 bacterial isolates were found to produce IAA. The colour intensity varied from light pink to blood red colour among the IAA producing samples and was found to be highest in the sample Chr-BC7.

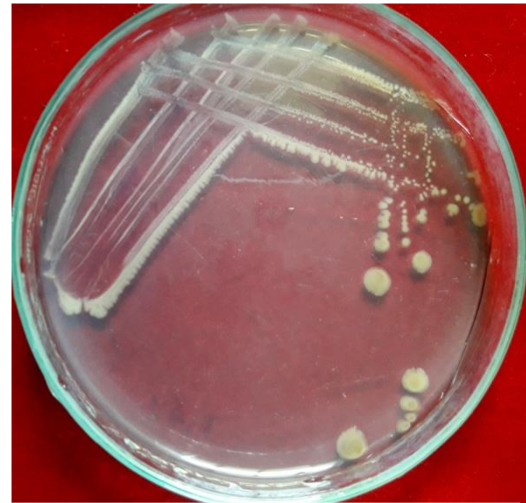


Pzk-BC5

Mls-BC1

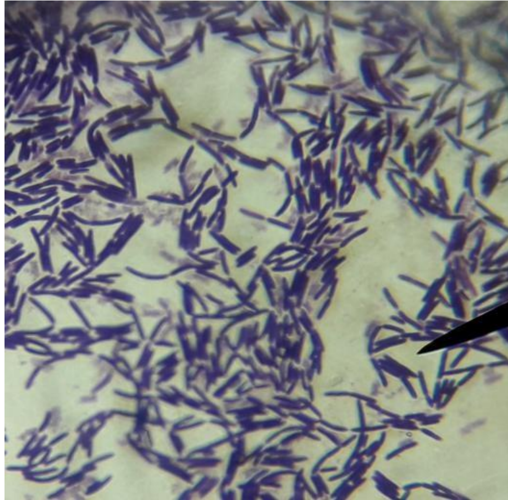


Mls-BC6

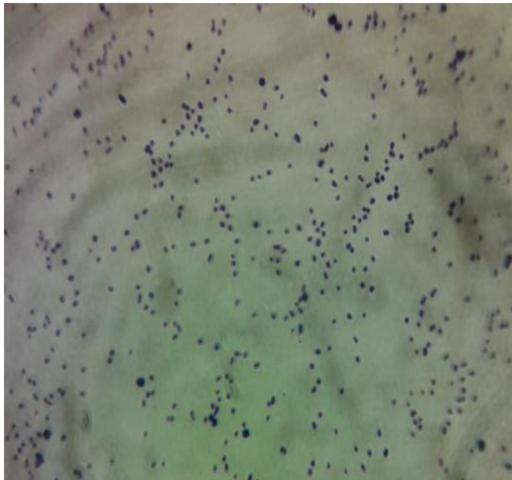


Chr-BC5

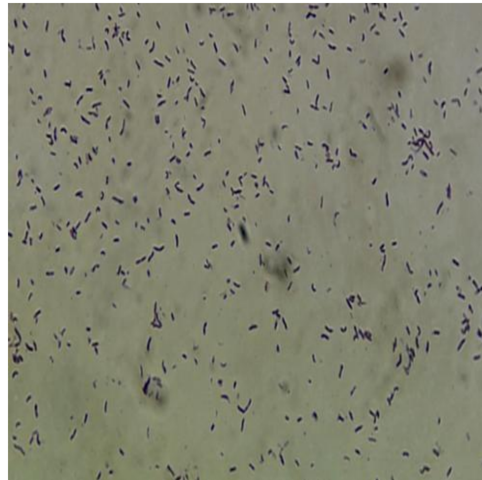
Plate 4. Purified predominant bacterial isolates



Pzk-BC5



Mls-BC7



Chr-BC8

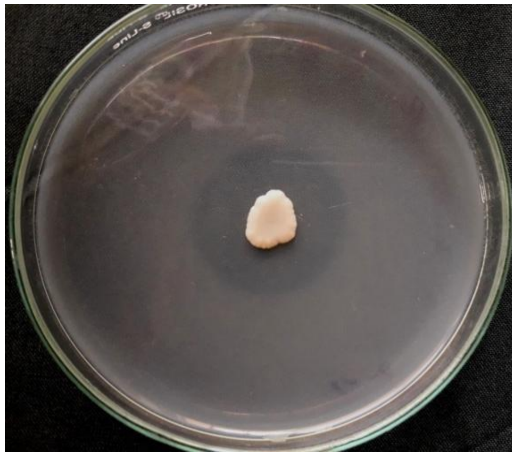
Plate 5. Microscopic images of predominant bacteria isolated under 100X magnification

Table 6. Screening for Plant Growth Promoting (PGP) activities of the predominant bacterial isolates

Bacterial isolates	N-fixation	Phosphate solubilization	IAA production	Ammonia production
Pzk-BC1	-	+	+	-
Pzk-BC2	-	+	+	+
Pzk-BC3	+	-	-	+
Pzk-BC4	-	-	-	-
Pzk-BC5	-	-	+	-
Pzk-BC6	+	-	-	-
Pzk-BC7	+	-	+	+
Pzk-BC8	-	-	-	-
Mls-BC1	-	+	++	-
Mls-BC2	-	+	+	-
Mls-BC3	+	+	+++	+
Mls-BC4	-	-	-	-
Mls-BC5	-	-	+++	-
Mls-BC6	-	+	-	-
Mls-BC7	-	-	+++++	-
Mls-BC8	+	-	+++	+
Chr-BC1	+	-	-	-
Chr-BC2	+	-	+	++
Chr-BC3	-	+	-	-
Chr-BC4	+	-	++	+
Chr-BC5	+	-	-	-
Chr-BC6	-	+	+	-
Chr-BC7	-	+	-	-
Chr-BC8	+	+	-	++

BC: Predominant bacterial isolate **N-fixation/ P-solubilization - absence; + presence**

IAA/ ammonia production – absence; + low; ++moderate; +++good; ++++excellent



P- solubilizer

N- fixer

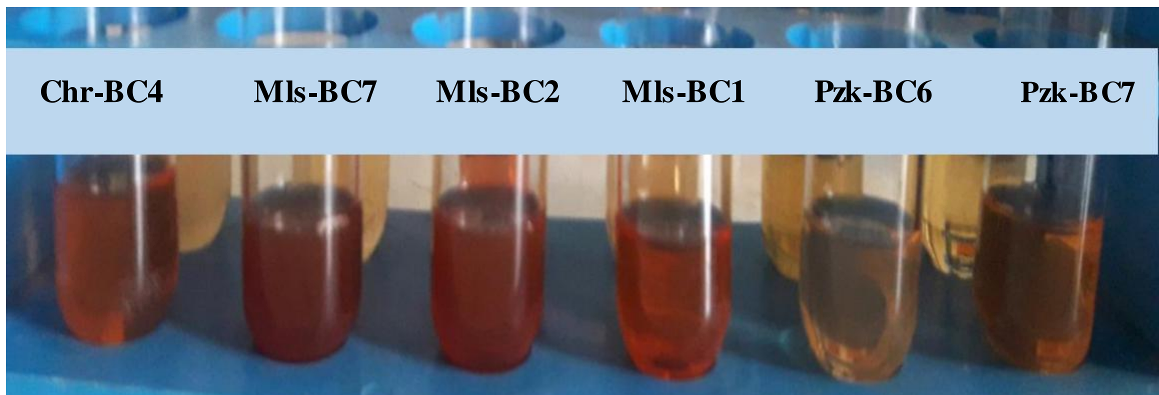


Plate 6.a. Screening of predominant bacterial isolates for IAA production

Plate 6. PGP characterization of the predominant bacterial isolates

4.6.2. Screening for phosphate solubilization

All the 24 bacterial isolates were screened for phosphate solubilization in Pikovskaya's agar. Ten isolates exhibited production of clear zone around the colony, which was indicative of phosphate solubilization. The solubilization efficiency is given in Table 8. The maximum solubilization efficiency was displayed by the isolate Chr-BC6.

Table 8. Phosphate solubilization efficiency by the selected bacterial isolates

Isolate name	Solubilization efficiency in Pikovskaya's agar in 7 days (%)
Pzk-BC1	34.00
Pzk-BC2	32.85
Mls-BC1	25.00
Mls-BC2	55.00
Mls-BC3	30.00
Mls-BC6	17.77
Chr-BC3	22.00
Chr-BC6	61.50
Chr-BC7	28.00
Chr-BC8	46.66

4.7 Quality and quantity of the isolated metagenomic DNA

The quantity of the isolated metagenomic DNA was assessed using NanoDrop spectrophotometer and Qubit fluorometer. The metagenomic DNA concentration and the ratio of absorbance 260/280 nm were estimated and the values are shown in the Table 8. The quality of the V3-V4 amplicons obtained from the extracted metagenomic DNA was analyzed by electrophoresis on two per cent agarose gel (Plate 8). The V3-V4 amplicons of 16S rDNA were used for the further downstream processes.

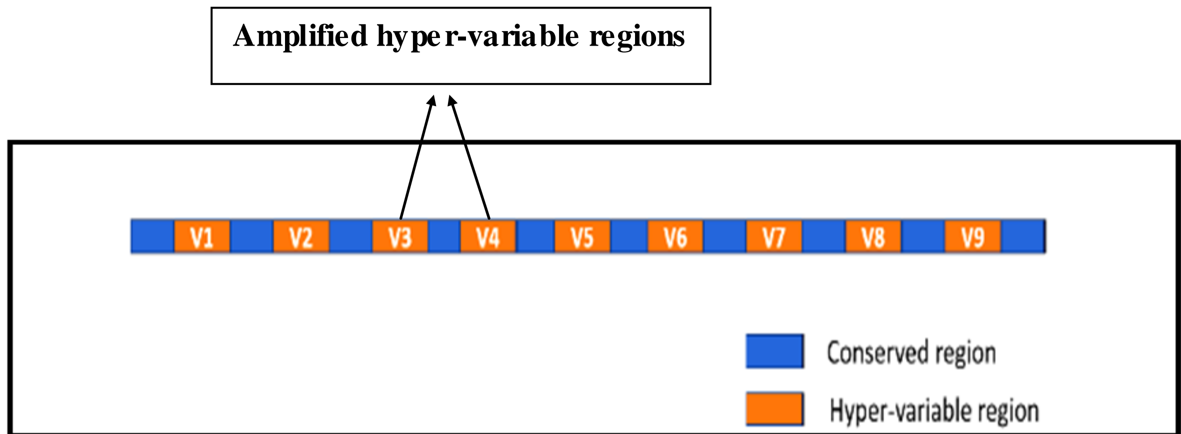
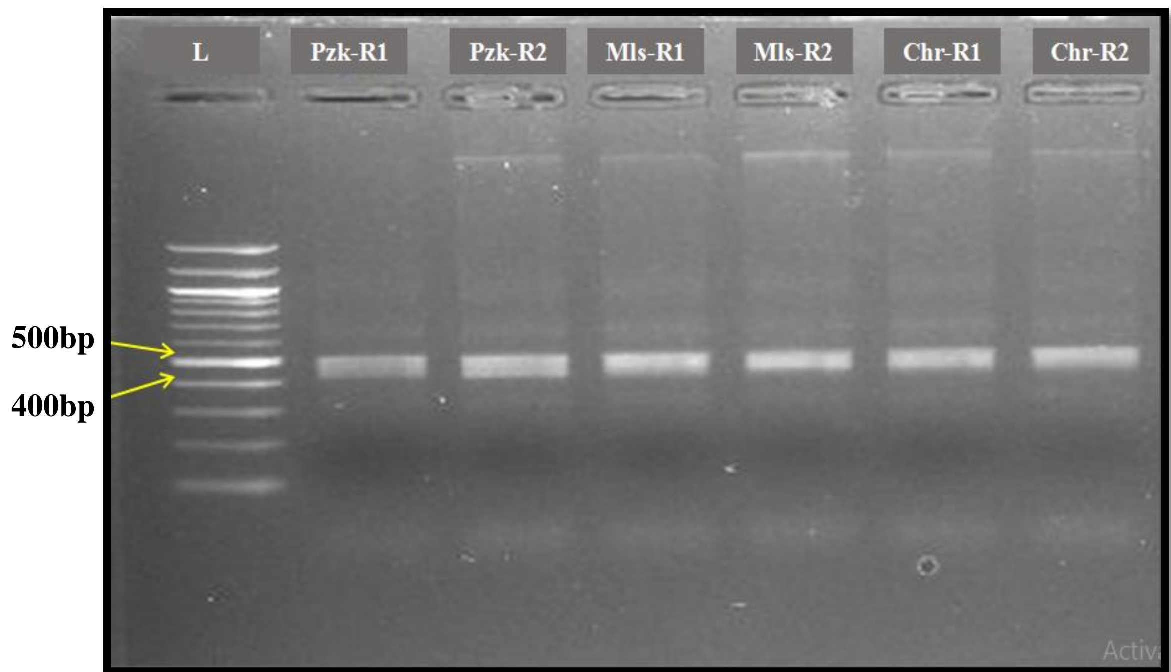


Plate 7. V3 and V4 regions of 16S rRNA gene



L- NEB 100bp ladder

Plate 8. V3-V4 amplicons of metagenomic DNA on 2% agarose gel

Table 8. Qualitative and quantitative parameters of the isolated metagenomic DNA

Sl. No.	Sample	Qubit concentration (ng/µl)	NanoDrop concentration (ng/µl)	A 260/280	Total elute concentration (ng)
1	Puzhakkal 1 (PzkR1)	7.70	20	1.67	18.42
2	Puzhakkal 2 (PzkR2)	13.8	38	1.75	31.8
3	Mullassery 1 (MlsR1)	9.15	26	1.73	39.9
4	Mullassery 2 (MlsR2)	14.9	42	1.71	38.4
5	Cherpu 1 (ChrR1)	10.9	32	1.67	30.0
6	Cherpu 2 (ChrR2)	11.3	32	1.71	35.4

4.8 Quality checking of Fastq sequences

The raw reads obtained from Illumina sequencing platform after demultiplexing was subjected to FastQC program to check the quality of the reads with default parameters. The base quality (Phred score; Q), base composition, GC content, ambiguous bases (other than A,T,G,C) and adapter dimers were thoroughly checked prior to the Bioinformatics analysis.

4.9 Base quality Score distribution

The base quality distributions of all samples were found to be greater than 80 per cent with a phred score value above 30 (Table 9). The phred score indicates the mean read quality and the value above 30 suggests an error probability of around 0.001. The schematic representation displays the sequencing cycle in the X-axis and the percentage of total reads in the Y-axis (Plate 9).

Table 9. Raw read summary: Read quantity and quality

Sl. No.	Sample	Read orientation	Mean read quality (Phred score)	Number of reads	% GC	% Q < 10	% Q 10-20	% Q 20-30	% Q > 30	Number of bases (MB)	Mean read length (bp)
1	Pzk-R1	R1	34.73	94,730	56.43	0.09	8.08	4.19	87.64	23.68	250.00
		R2	30.70	94,730	56.59	0.99	19.55	8.91	70.54	23.68	250.00
2	Pzk-R2	R1	34.73	67,397	57.14	0.09	7.74	4.29	87.88	16.85	250.00
		R2	30.52	67,397	57.36	1.03	19.63	9.46	69.88	16.85	250.00
3	Mls-R1	R1	34.15	59,645	56.14	0.08	10.07	4.84	85.01	14.91	250.00
		R2	30.00	59,645	56.66	0.95	22.18	9.59	67.28	14.91	250.00
4	Mls-R2	R1	34.76	83,615	57.28	0.09	7.86	4.05	88.01	20.90	250.00
		R2	30.54	83,615	57.16	1.00	19.94	9.14	69.91	20.90	250.00
5	Chr-R1	R1	34.56	66,925	56.94	0.09	8.63	4.38	86.91	16.73	250.00
		R2	30.11	66,925	57.26	1.00	21.44	9.68	67.88	16.73	250.00
6	Chr-R2	R1	35.21	39,870	56.18	0.09	6.59	3.63	89.68	9.97	250.00
		R2	30.94	39,870	56.28	1.03	18.52	8.76	71.69	9.97	250.00

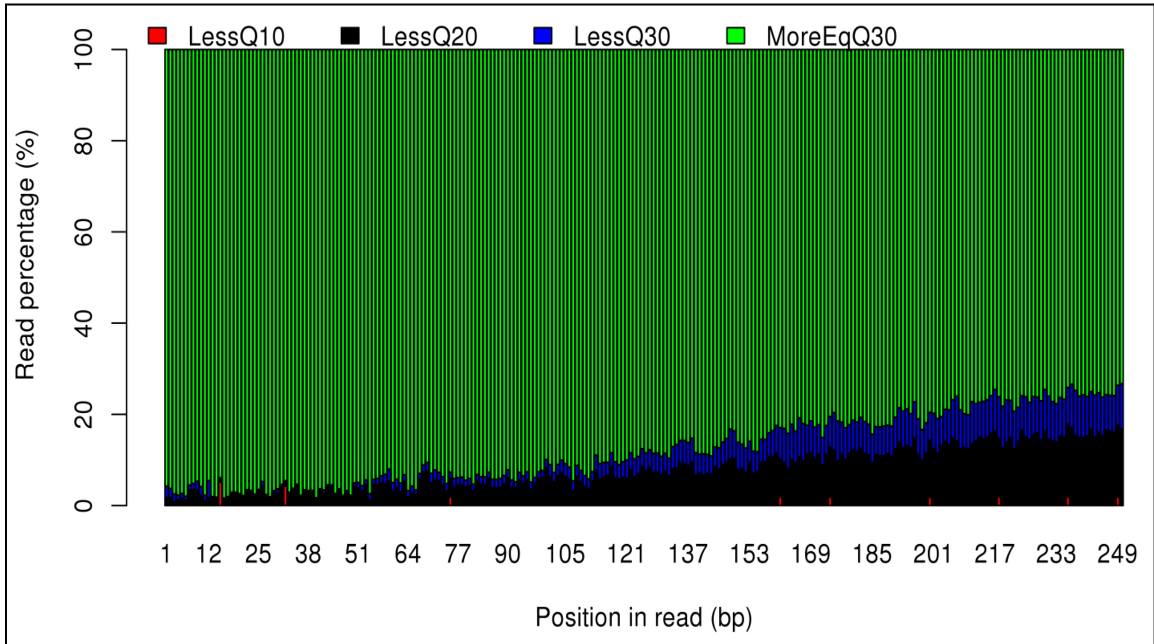


Fig. a. Read 1

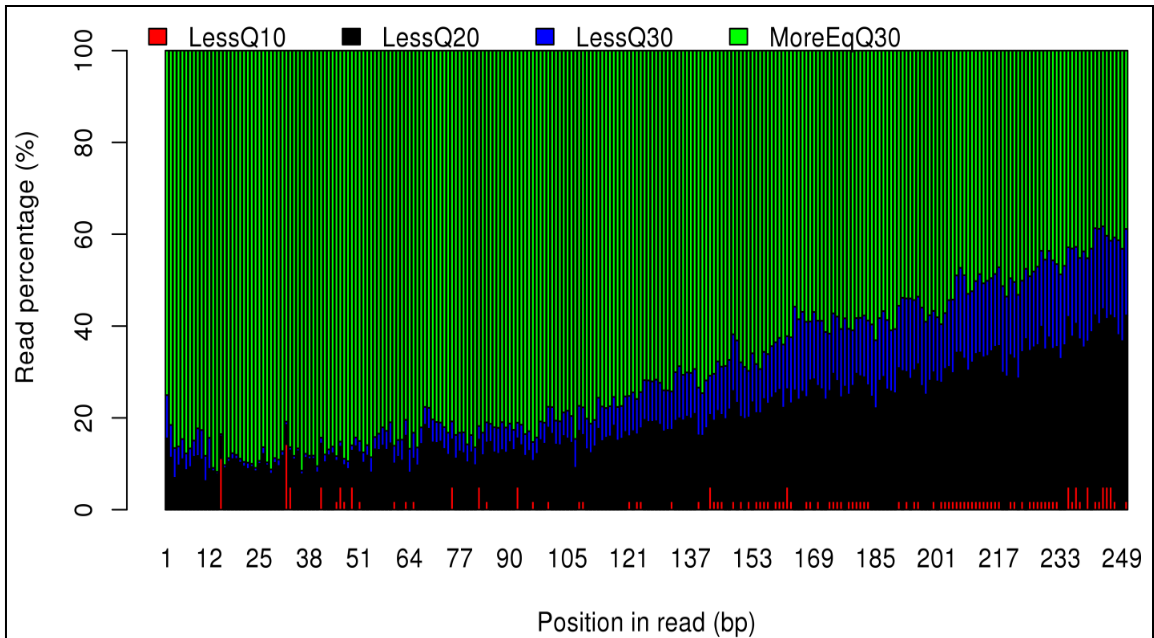


Fig. b. Read 2

Plate 9.a. Base quality distribution of sample Pzk

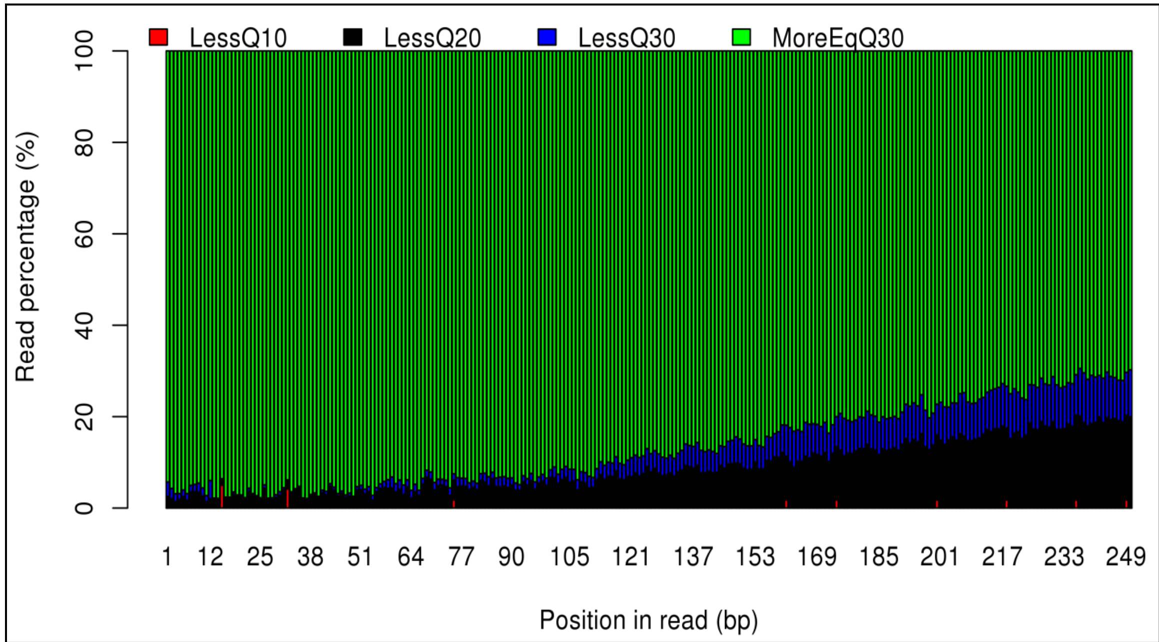


Fig. c. Read 1

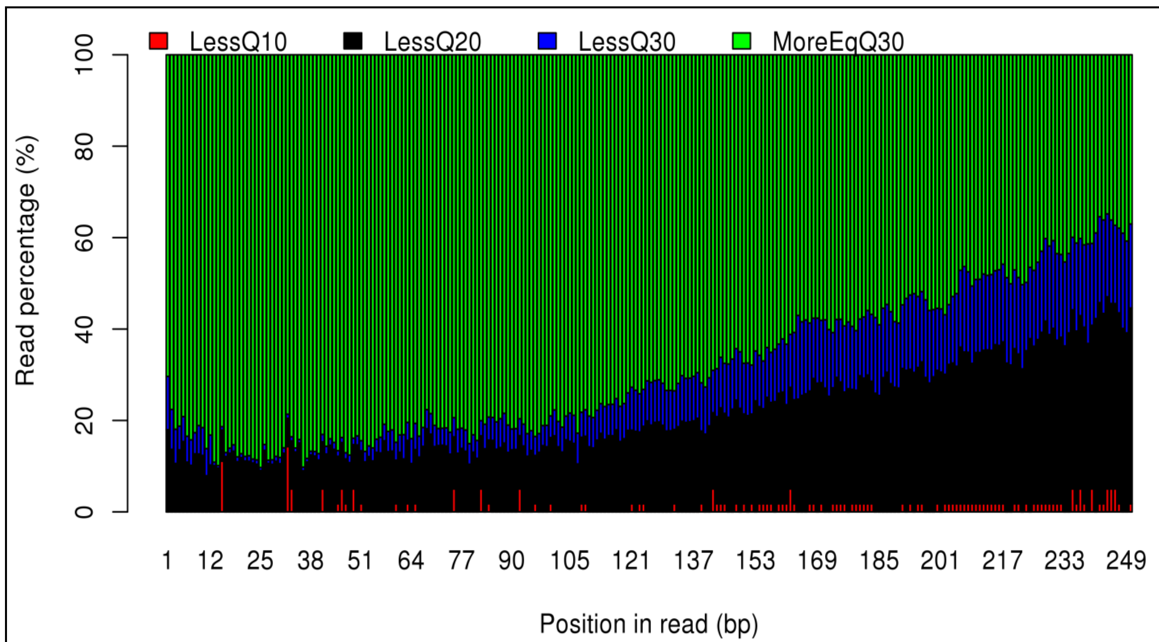


Fig. d. Read 2

Plate 9.b. Base quality distribution of sample MIs

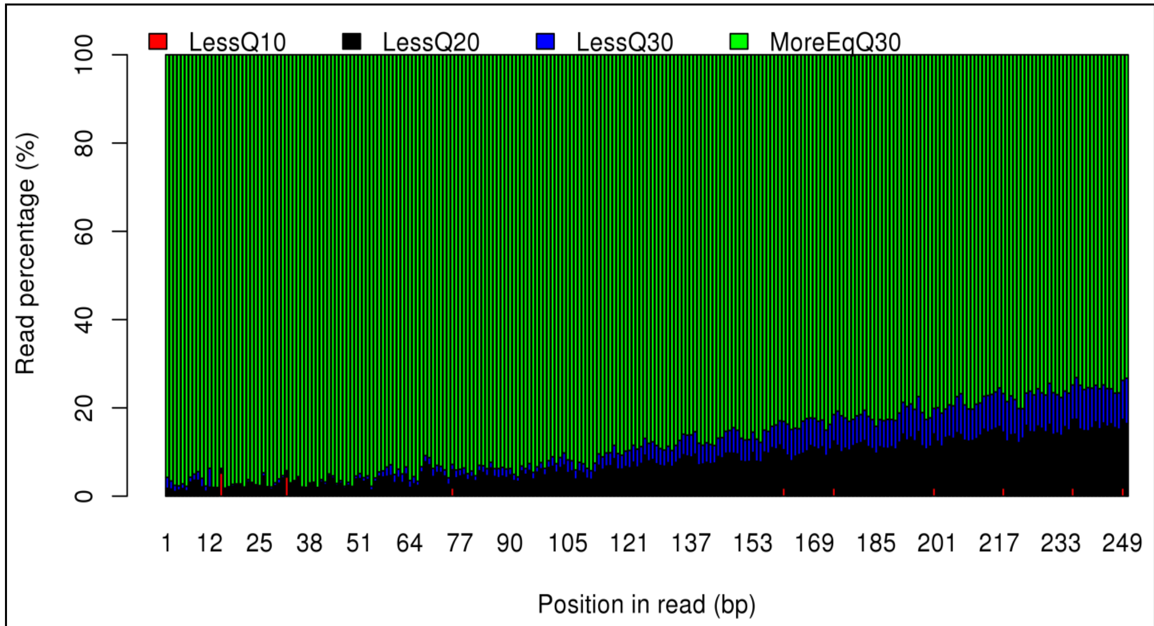


Fig. e. Read 1

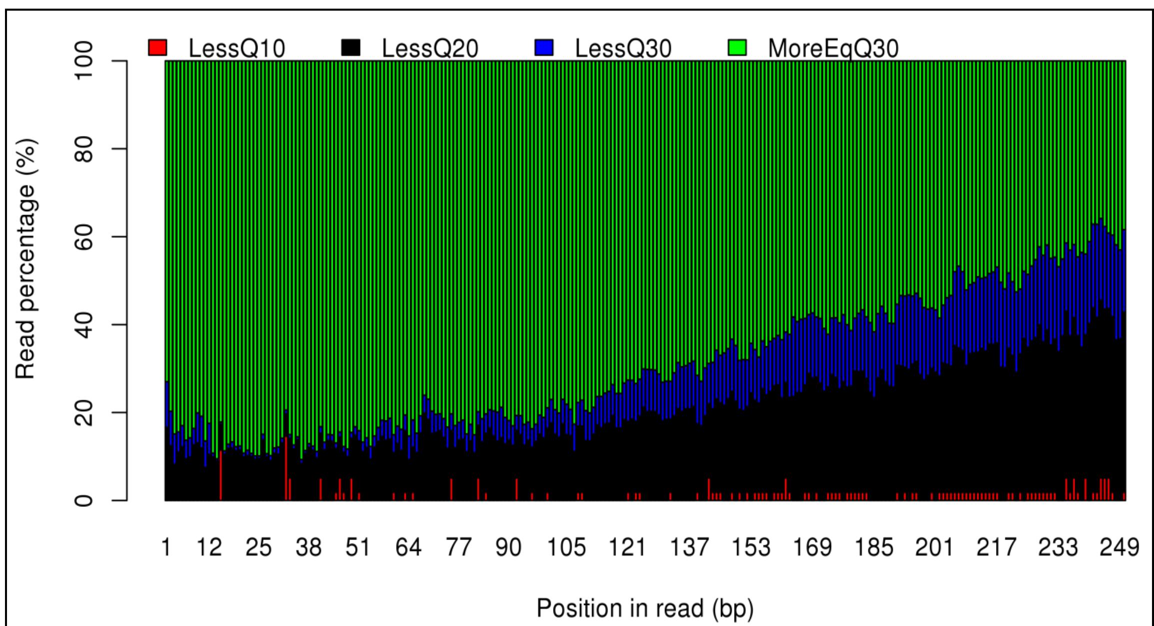


Fig. f. Read 2

Plate 9.c. Base quality distribution of sample Chr

Plate 9. Base quality distribution of all the samples

4.10 Base composition

Each sample was analysed for composition of nucleotides in the sequence read and the results are given in the Table 10. The content of T was the minimum among all the bases (20.91 per cent) in the sample Mls. Maximum content was noticed in the base C (28.89 per cent) in the same sample Mls. The graphical representation of base composition displays each base in specific colour and the X and Y-axis of the graph indicates the sequencing cycles and the nucleotide percentage, respectively (Plate 10).

Table 10. Base composition of the 16S rDNA amplicons of the isolated metagenomic DNA samples

Sample	Base composition (%)			
	A	C	G	T
Pzk	21.55	28.89	27.92	21.09
Mls	21.68	28.75	28.13	20.91
Chr	21.50	28.85	27.92	21.18

4.11 GC distribution

All the reads of samples were analyzed for average GC composition. The graphical representation with average GC content (%) in X-axis and percentage of total reads in Y-axis shows that all the reads of samples have an average GC content in the range of 30-60 per cent.

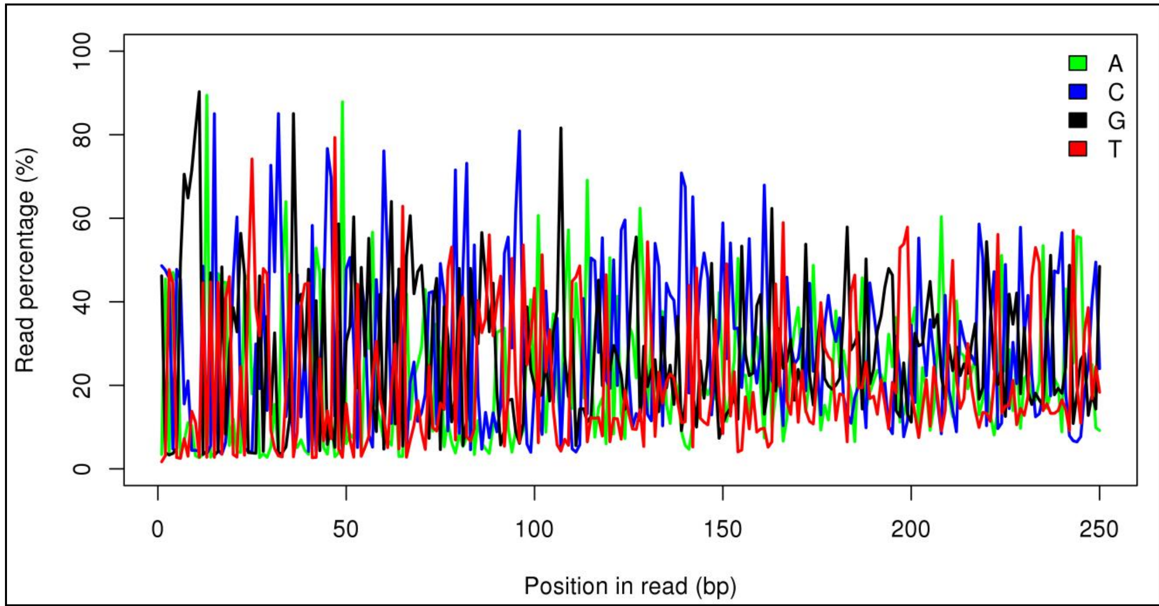


Fig. a. Read1

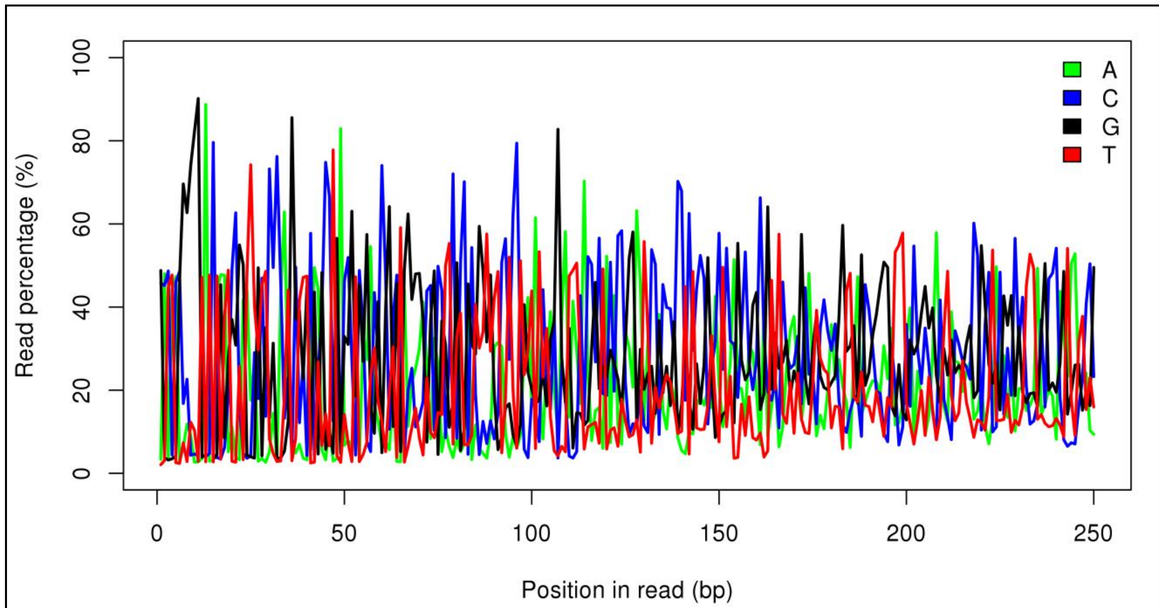


Fig. b. Read 2

Plate 10.a. Base composition of sample Pzk

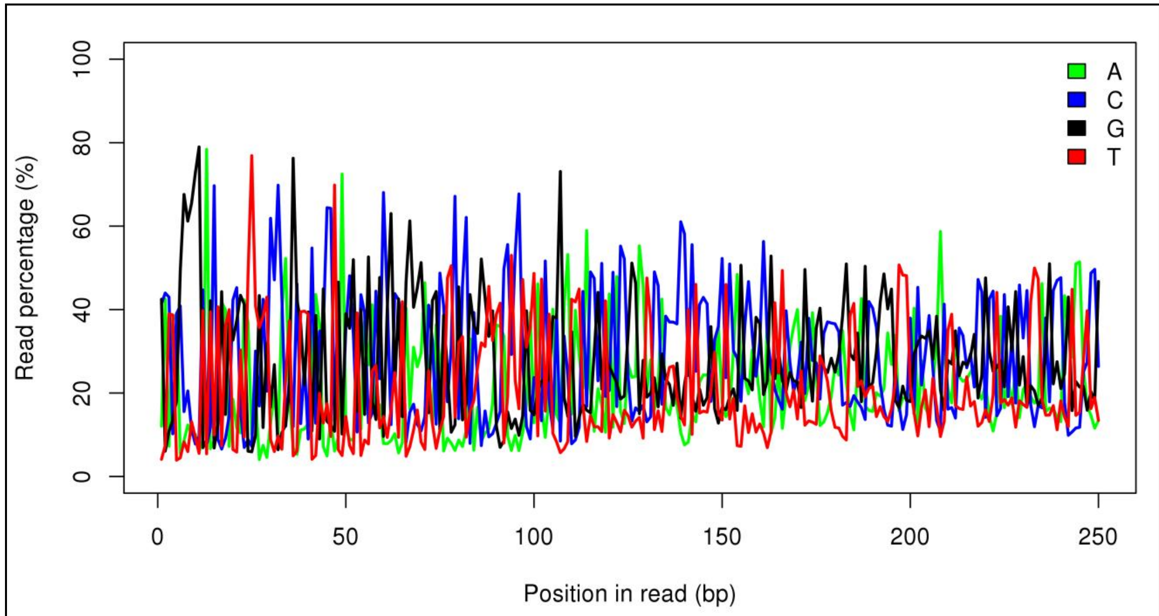


Fig. c. Read 1

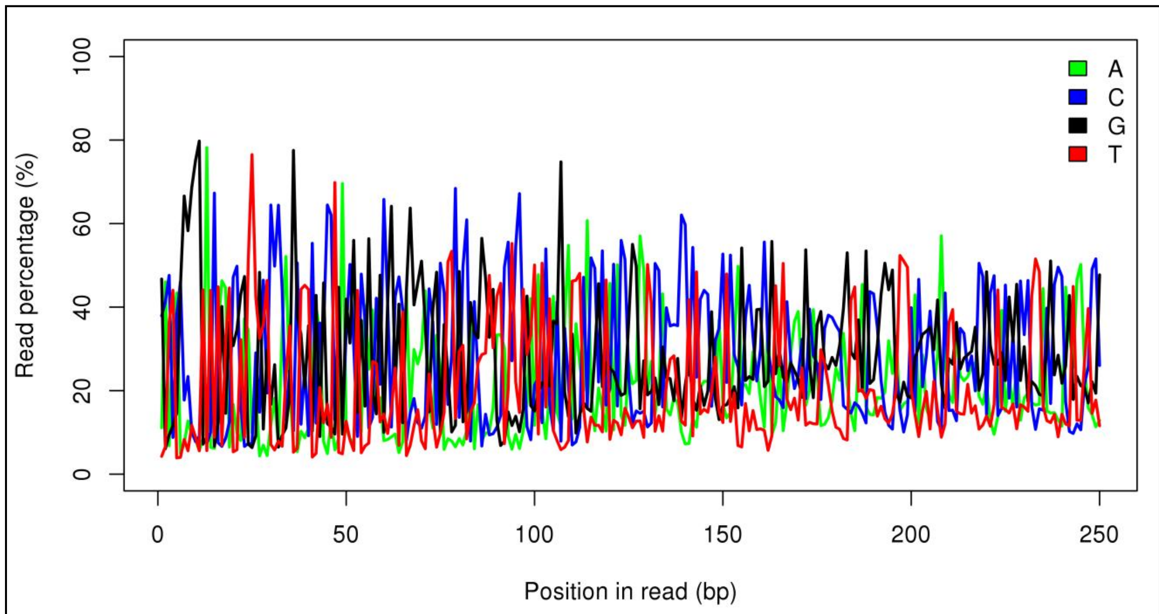


Fig. d. Read 2

Plate 10.b. Base composition of sample Mls

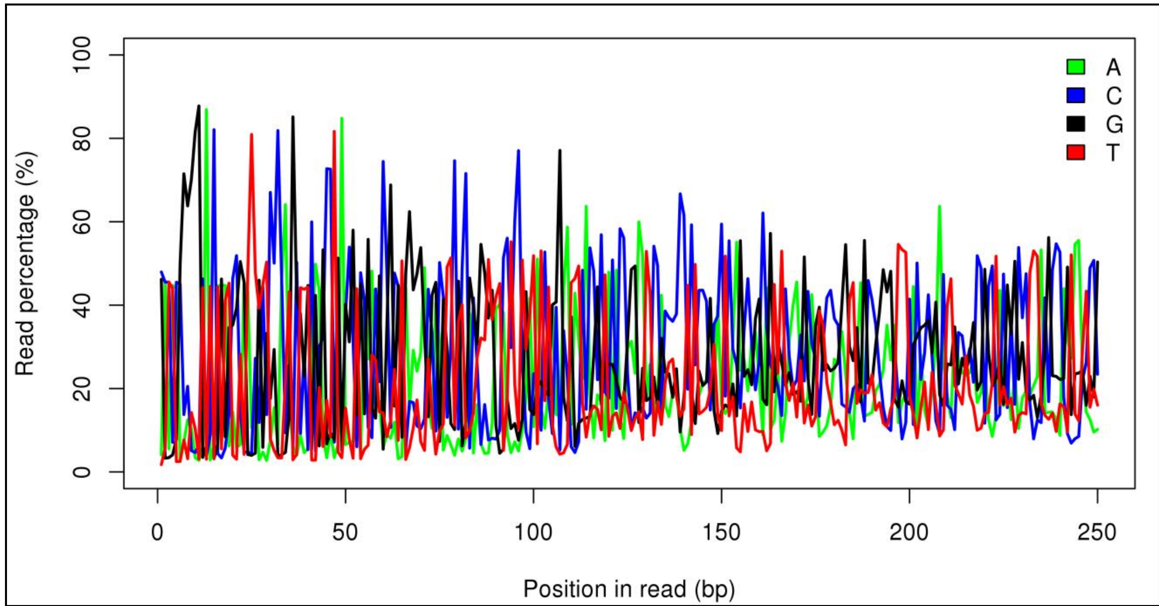


Fig. e. Read 1

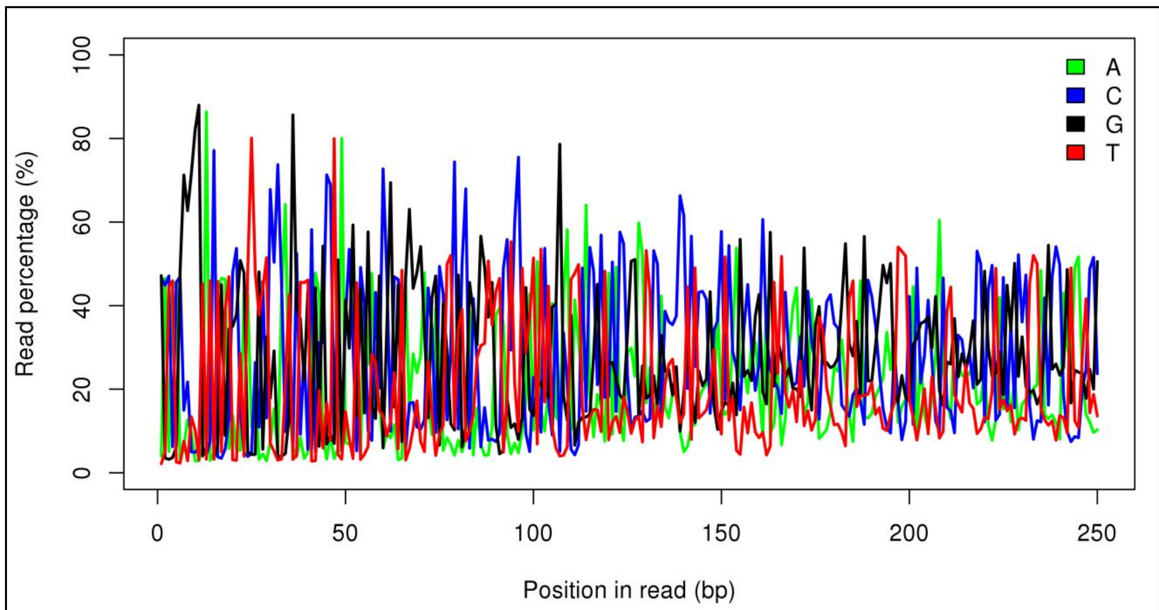


Fig. f. Read 2

Plate 10.c. Base composition of sample Chr

Plate 10. Base composition of all the samples

4.12 Identification of V3 and V4 regions of the paired end sequences

The consensus V3-V4 region was obtained after separating them from Illumina paired-end sequences followed by trimming of sequencing primers. The forward V3 specific primer and reverse V4 specific primers were trimmed using In-house PERL script. The properly paired end reads with Phred score quality (Q>20) were considered for V3-V4 consensus generation. The high quality paired-end reads were pair-wisely allowed to merge using FLASH program to get the V3-V4 amplicon consensus FASTA sequences. An average contig length of 350 to 450bp with zero mismatches was obtained from all samples. A summary of trimmed consensus reads is shown in Table 11.

Table 11. Trimmed and consensus read summary

Sample name	Total paired-end reads	Reads passed with primers	Total consensus sequences
Pzk	162127	162127	105768
Mls	143260	143260	72380
Chr	106795	106795	65224

4.13 Pre-processing of reads: Chimera filter

The chimeric sequences were removed from the consensus reads using the de-novo chimera removal method UCHIME implemented in the tool VSEARCH. The reads thus obtained were used for the analysis of taxonomical classification. A detailed table of chimera filter based on individual sample is given in Table 12.

Table 12. Pre-processed reads obtained after chimera filter

Sample Name	Consensus Sequences	Chimeric Sequences	Pre-processed Consensus Sequences
Pzk	105768	23550	82218
Mls	72380	22793	49587
Chr	65224	18664	46560

4.14 Taxonomic classification and relative abundance of the OTUs

The Operational Taxonomic Units (OTU) picking and taxonomy classification were performed using the pre-processed consensus V3-V4 sequences. Pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity using Uclust program available in QIIME software. A total of 35417 OTUs were identified from 178365 reads. From 35417 total OTUs, 24212 OTUs with less than 2 reads were removed and 11205 OTUs were selected for further analysis (Table 13).

Table 13. Summary of OTUs

Total Pre-processed Consensus	178365
Total OTUs Picked	35417
Total singleton OTUs	24212
Total OTUs after singleton removal	11205

QIIME1 program was used for the entire downstream analysis. The representative sequences from each clustered OTUs were picked and aligned against SILVA core set of sequences using PyNAST program. Further, taxonomy classification was performed using RDP classifier by mapping each representative sequence against SILVA OTUs database. The sequence that does not have any alignment against taxonomic database is categorized as Unknown. The taxonomical classification was carried out and the top ten taxa were represented graphically using this method.

4.15 Bacterial diversity analyzed using QIIME program

The sequences obtained after filtering process were analyzed for bacterial diversity using the tools QIIME, PyNAST and RDP classifier. The analysis carried out using the program QIIME yielded a wide range of bacterial taxa which indicated the community structure of the representative samples. Proteobacteria (27%) and Chloroflexi

(24%) were found to be the most predominant bacterial phyla followed by Acidobacteria (21%). Actinobacteria (10%) and Nitrospira (7%) were found to be the other major bacterial phyla obtained from the samples. In the sample Pzk, Proteobacteria constituted the major proportion of bacterial phyla followed by Chloroflexi and Acidobacteria. In the sample Mls, phylum Chloroflexi constituted the majority of bacterial population followed by Acidobacteria and Proteobacteria respectively. The major proportion of bacterial phyla in the sample Chr was contributed by Chloroflexi followed by Proteobacteria and Acidobacteria respectively. The bacterial population belonging to unknown categories was also obtained at a notable proportion. The samples also yielded novel categories of bacterial phyla at lower proportions.

4.17 Bacterial diversity analysed using MEGAN6

A BIOM file which comprises the supplementary concise detail of the bacterial diversity was used as an input file for the MEGAN6 program. The bacterial taxonomic compositions of the three samples (Pzk, Mls and Chr) were obtained from MEGAN6 using NCBI taxonomy. Comparative analysis was carried out between the samples for bacterial diversity at different taxonomic levels using graphical representations and phylogenetic trees. The comparison between the samples and among the phyla showed an increased proportion of Proteobacteria in the sample Pzk and an increased population of Chloroflexi in the sample Mls. The bacterial population of the sample Chr was observed to be less when compared with Pzk and Mls. The archaeal diversity of the samples was also analyzed and the phyla Euryarchaeota and Crenarchaeota were found to be dominant in all the samples.

4.16 Bacterial diversity analyzed using MG-RAST pipeline

The bacterial diversity of the samples was also analyzed using MG-RAST pipeline. The fastq sequences obtained were used as an input file for MG-RAST and taxonomic analysis was performed. The pictorial representations using krona aids in easy comparison and the population indices were calculated by using the tab separated files in

csv format. The diversity of bacterial population at different taxonomic level was studied and a considerably increased population of delta-proteobacteria was found in the sample Pzk.

The bacterial and archaeal diversity at phylum level was assessed to discover the most abundant phyla in each sample. The sample Pzk showed higher bacterial and archaeal population than the samples Mls and Chr. Considering the archaeal population, the Euryarchaeota was found to be the most predominant archaeal phylum in sample Pzk and the population was far higher than that of Proteobacteria. The phylum level archaeal and bacterial distribution has been listed in Table 14.

Table 14. Phylum-level bacterial and archaeal diversity of the rhizosphere samples

Domain	Phylum	No. of reads assigned		
		Pzk	Mls	Chr
Bacteria	Acidobacteria	8484	8776	5996
	Caldiserica	14	0	0
	Calditrichaeota	7	2	0
	Deferribacteres	4	6	2
	Elusimicrobia	20	26	38
	Bacteroidetes	1720	1775	1789
	Fibrobacteres	19	23	16
	Gemmatimonadetes	645	483	609
	Fusobacteria	0	7	2
	Nitrospinae	2	0	3
	Nitrospirae	2776	1500	2689
	Proteobacteria	10909	5824	7294
	Chlamydiae	18	8	6
	Kiritimatiellaeota	7	4	2
	Lentisphaerae	0	0	2
	Planctomycetes	331	305	326
	Verrucomicrobia	319	739	1358
	Spirochaetes	658	361	163
	Actinobacteria	4010	3138	1241
	Armatimonadetes	57	5	11
	Chloroflexi	9888	10412	7473
	Firmicutes	533	531	267
	Tenericutes	0	0	2
Archaea	Euryarchaeota	18901	1906	2854
	Crenarchaeota	9218	4038	4620
	Thaumarchaeota	1545	1509	289

4.18 Diversity of Archaebacteria in the rice rhizosphere samples

Three phyla constituting 13 genera were observed from the rhizosphere samples indicating the wide array of archaeal population in the samples. Crenarchaeota was found to be the most predominant archaeal phylum in samples Mls and Chr followed by Euryarchaeota and Thaumarchaeota. The sample Pzk showed exceptionally higher archaeal population with predominant phylum Euryarchaeota which was even higher than the bacterial population in the sample. The genus-level diversity of Archaebacteria is shown in Table 15.

4.18.1 Archaeal diversity in the sample Pzk

In sample Pzk, the archaeal population was found higher than the bacterial population. The archaeal phylum Euryarchaeota was observed to be most abundant with population belonging to 5 classes followed by Crenarchaeota. The archaeal population of Methanomicrobiaceae was found to be the most dominant family of Euryarchaeota. The phylum Crenarchaeota was found to harbor the largest number of unclassified archaeal population. *Nitrososphaera* turned out to be the most abundant genus of the phylum Thaumarchaeota.

4.18.2 Archaeal diversity in the sample Mls

The phylum Crenarchaeota was observed to be the most dominant archaeal phylum followed by Euryarchaeota and Thaumarchaeota. Phylum Euryarchaeota was mainly composed of genus *Methanocella* while the genus *Nitrososphaera* was found to be the only genus of phylum Thaumarchaeota. The phylum Crenarchaeota accommodated a wide diversity of unclassified archaeal population.

4.18.3 Archaeal diversity in the sample Chr

The phylum Crenarchaeota constituted the major portion of archaeal population followed by Euryarchaeota. The members belonging to Thaumarchaeota was found to be least in Chr with only one genus *Nitrososphaera*. The phylum Crenarchaeota was found to harbor wide array of unclassified genera.

Table 15. Genus-level diversity of archaeal population in the rice rhizosphere samples

Phylum	**Class**	**Order**	**Family**	**Genus**
Genus-level archaeal assemblage in the sample Pzk				
Euryarchaeota	Thermoplasmata	Methanomassilicoccales	Methanomassilicoccaceae	Unclassified (213)
	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium* (222) *Methanobrevibacter* (1)
	Halobacteria	Halobacteriales	Unclassified	Unclassified (1)
	Methanomicrobia	Methanocellales	Methanocellaceae	*Methanocella* (290)
		Methanomicrobiales	Methanomicrobiaceae	Unclassified (1290)
			Methanoregulaceae	*Methanolinea* (498)
				Methanoregula (129)
			Methanospirillaceae	*Methanospirillum* (1)
		Methanosarcinales	Methanosarcinaceae	*Methanosarcina* (125)
	Thermococci	Unclassified	Unclassified	Unclassified (4)
Crenarchaeota	Unclassified	Unclassified	Unclassified	Unclassified (9218)
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera* (1131)
Genus-level archaeal assemblage in the sample Mls				
Euryarchaeota	Thermoplasmata	Methanomassilicoccales	Methanomassilicoccaceae	*Unclassified (16)*
	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium (57)*
	Methanomicrobia	Methanocellales	Methanocellaceae	*Methanocella (333)*
		Methanomicrobiales	Methanomicrobiaceae	*Unclassified (28)*
			Methanoregulaceae	*Methanolinea (21)*
				Methanoregula (17)

			Methanospirillaceae	*Methanospirillum (2)*
		Methanosarcinales	Methanosarcinaceae	*Methanosarcina (62)*
Crenarchaeota	Unclassified	Unclassified	Unclassified	*Unclassified (4038)*
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera (900)*
Genus-level archaeal assemblage in the sample Chr				
Euryarchaeota	Thermoplasmata	Methanomassilicoccales	Methanomassilicoccaceae	*Unclassified (119)*
	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium (130)* *Methanobravibacter (1)*
	Halobacteria	Halobacteriales	Unclassified	*Unclassified (1)*
	Methanomicrobia	Methanocellales	Methanocellaceae	*Methanocella (208)*
		Methanomicrobiales	Methanomicrobiaceae	*Unclassified (61)*
			Methanoregulaceae	*Methanolinea (182)*
				Methanoregula (20)
			Methanospirillaceae	*Methanospirillum (4)*
		Methanosarcinales	Methanosarcinaceae	*Methanosarcina (47)*
Crenarchaeota	Unclassified	Unclassified	Unclassified	*Unclassified (4620)*
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera (46)*

4.19 Bacterial diversity in the sample Pzk

The phylum Proteobacteria was found to be the most abundant in the sample Pzk with a total of 10909 reads, followed by Chloroflexi and Acidobacteria. Bacterial population belonging to 20 phyla was observed in the sample indicating the community structure of the bacteria present in the sample (Plate 11). Actinobacteria, Nitrospirae and Bacteroidetes were found to be the other major bacterial phyla. Majority of the Proteobacteria belonged to class Deltaproteobacteria with genus *Desulfobacca* as the dominant one followed by *Geobacter* and *Sulfuricurvum* among the known genera. The genus *Thioalkalispira* of class Gammaproteobacteria and *Psuedolabrys* of class Alphaproteobacteria were found to be the other major genera of Proteobacteria. The genus *Anaerolinea* and *Ktedonobacter* contributed to the major portion of phylum Chloroflexi while, the genus *Thermoanaerobaculum* occupied the major portion of phylum Acidobacteria. Unclassified genera belongs to the order Frankiales was found to be predominant in phylum Actinobacteria. The proportion of unclassified bacteria was found to be very high in the class Deltaproteobacteria of phylum Proteobacteria. Bacterial population belonging to novel phyla was also observed in notable amount from the sample Pzk. The genus-level taxonomic assemblage of bacterial diversity from the sample Pzk has been given in the Table 16.

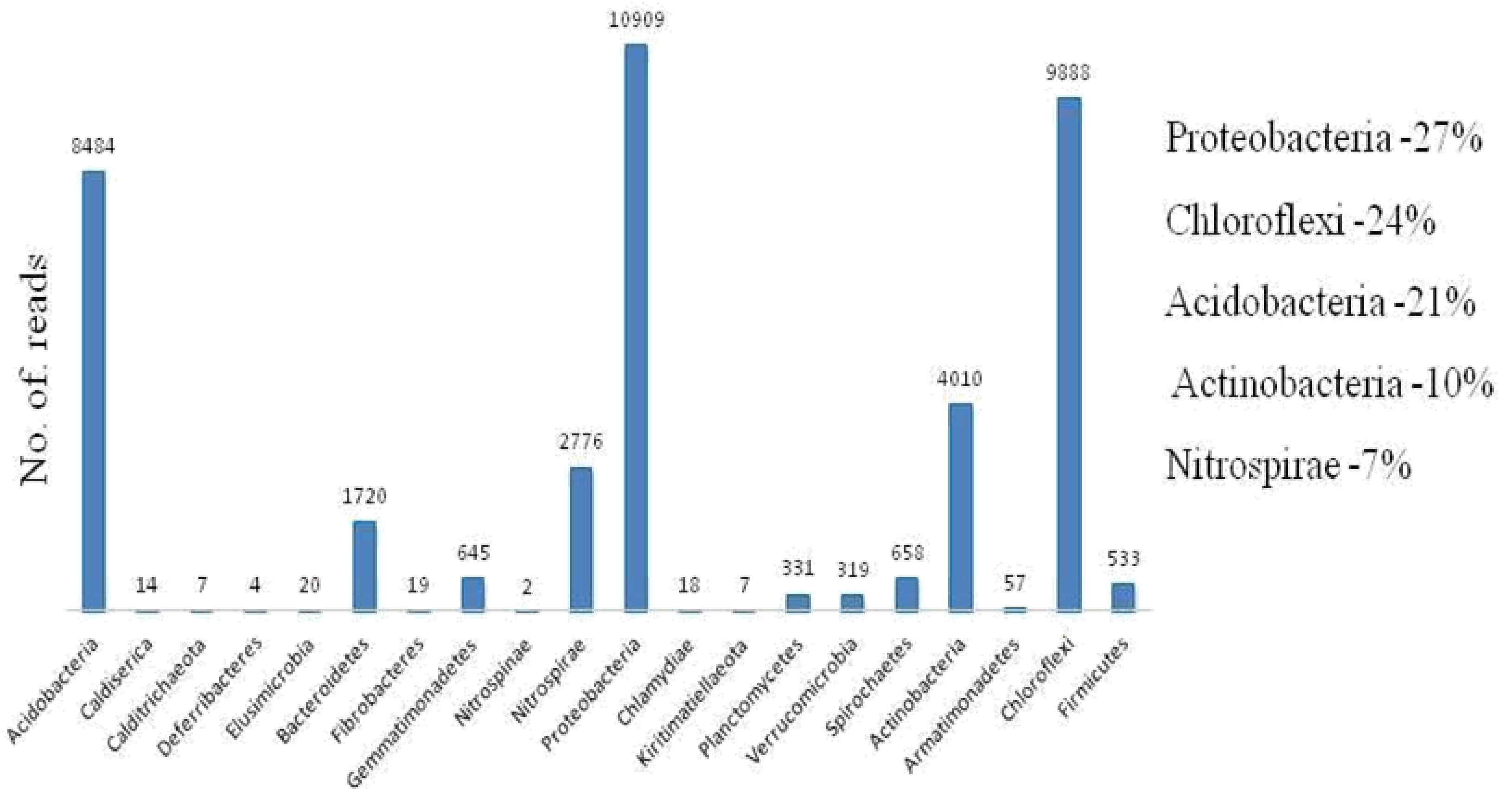


Plate 11. Phylum-level bacterial diversity of sample Pzk

Table 16. Genus-level taxonomic assemblage of bacterial diversity from the sample Pzk

Phylum	**Class**	**Order**	**Family**	**Genus**
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Acidipila* (2)
	Holophagae	Holophagales	Holophagaceae	*Geothrix* (13)
	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	*Thermoanaerobaculum* (280)
Caldiserica	Caldisericia	Caldisericales	Unclassified	Unclassified (14)
Calditrichaeota	Calditrichae	Calditrichales	Calditrichaceae	Unclassified (7)
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	*Mucispirillum* (4)
Elusimicrobia	Endomicrobia	Endomicrobiales	Endomicrobiaceae	Unclassified (20)
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroids* (24)
			Barnesiellaceae	Unclassified (2)
			Lentimicrobiaceae	Unclassified (41)
			Muribaculaceae	Unclassified (6)
			Porphyromonoadaceae	*Odoribacter* (10)
				Parabacteroids (6)
			Rikenellaceae	*Alistipes* (16)
				Rikenella (1)
Fibrobacteres	Chitinivibrionia	Unclassified	Unclassified	Unclassified (3)
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas* (168)
				Gemmatirosa (118)
Nitrospinae	Unclassified	Unclassified	Unclassified	Unclassified (2)
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium* (11)

Contd.

		Micropepsales	Micropepsaceae	Unclassified (28)
		Rhizobiales	Bradyrhizobiaceae	*Rhodoblastus* (14)
			Hyphomicrobiaceae	*Hyphomicrobium* (4)
			Hyphomicrobiaceae	*Pedomicrobium* (3)
			Rhizobiaceae	Unclassified (6)
			Rhodobiaceae	*Bauldia* (7)
			Xanthobacteraceae	*Psuedolabrys* (151)
		Rhodobacterales	Rhodobacteraceae	*Amaricoccus* (2)
				Rhodobacter (10)
		Rhodospirillales	Acetobacteraceae	*Acidiphilium* (20)
				Rhodopila (2)
				Roseomonas (22)
			Geminicoccaceae	Unclassified (16)
			Rhodospirillaceae	*Inquilinus* (2)
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium* (2)
				Polymorphobacter (2)
				Sphingomonas (2)
	Deltaproteobacteria	Unclassified	Unclassified	*Deferrisoma* (2)
		Desulfarculales	Desulfarculaceae	Unclassified (238)
		Desulfobacterales	Desulfobacteraceae	*Desulfonema* (1)
			Desulfobulbaceae	*Desulfobulbus* (5)
				Desulfurivibrio (3)
		Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio* (15)
		Desulfuromonadales	Geobacteraceae	*Geobacter* (115)

Contd.

		Myxococcales	Archangiaceae	Unclassified (146)
			Kofleriaceae	*Haliangium* (58)
			Phaselicystidaceae	*Phaselicystis* (18)
			Polyangiaceae	*Pajaroellobacter* (40)
				Sorangium (4)
			Sandaracinaceae	Unclassified (3)
		Syntrophobacterales	Syntrophaceae	*Desulfobacca* (419)
				Desulfomonile (62)
				Smithella (21)
				Syntrophus (56)
			Syntrophobacteraceae	*Syntrophobacter* (66)
			Syntrophorhabdaceae	*Syntrophorhabdus* (88)
		Campylobacterales	Campylobacteraceae	*Sulfurospirillum* (1)
			Helicobacteraceae	*Helicobacter* (24)
				Sulfuricurvum (180)
				Sulfurimonas (16)
	Gammaproteobacteria	Acidiferrobacterales	Acidiferrobacteraceae	*Sulfurifustis* (61)
		Chromatiales	Thioalkalispiraceae	*Thioalkalispira* (277)
		Legionellales	Coxiellaceae	*Aquicella* (2)
				Coxiella (28)
			Legionellaceae	*Legionella* (2)
		Methylococcales	Methylococcaceae	*Methylobacter* (1)
				Methylocaldum (12)
				Methylomonas (7)

Contd.

		Nevskiales	Steroidobacteraceae	Unclassified (4)
		Oceanospirillales	Halomonadaceae	*Salinicola* (4)
		Xanthomonadales	Rhodanobacteraceae	*Dyella* (3)
				Luteibacter (1)
				Rhodanobacter (1)
			Xanthomonadaceae	*Stenotrophomonas* (2)
Chlamydiae	Chlamydiae	Chlamydiales	Unclassified	Unclassified (18)
Kiritimatiellaeota	Kiritimatiellae	Kiritimatiellales	Kiritimatiellaceae	*Kiritimatiella* (7)
Lentisphaerae	Oligosphaeria	Oligosphaerales	Unclassified	Unclassified (0)
Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	Unclassified (5)
		Tepidisphaerales	Unclassified	Unclassified (88)
	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Pirellula* (2)
			Isosphaeraceae	*Aquisphaera* (20)
				Singulisphaera (5)
			Unclassified	Unclassified (1)
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	*Akkermansia* (8)
Spirochaetes	Spirochaetia	Spirochaetales	Leptospiraceae	Unclassified (32)
			Spirochaetaceae	*Treponema* (1)
Actinobacteria	Actinobacteria	Catenulisporales	Actinospicaceae	*Actinospica* (17)
			Catenulisporaceae	*Catenulispora* (4)
		Corynebacteriales	Mycobacteriaceae	*Mycobacterium* (76)
			Nocardiaceae	*Nocardia* (4)
		Frankiales	Unclassified	Unclassified (2146)
		Kineosporiales	Kineosporiaceae	*Kineosporia* (9)

Contd.

		Micrococcales	Cellulomonadaceae	*Cellulomonas* (4)
			Intrasporangiaceae	Unclassified (4)
			Microbacteriaceae	Unclassified (12)
		Actinomycetales	Micromonosporaceae	*Luedemannella* (1)
				Micromonospora (1)
			Nocardioidaceae	*Actinopolymorpha* (2)
				Nocardioides (4)
			Streptomycetaceae	*Streptomyces* (38)
		Streptosporangiales	Streptosporangiaceae	*Microbispora* (2)
				Streptosporangium (2)
Armatimonadetes	Fimbriimonadia	Fimbriimonadales	Fimbriimonadaceae	Unclassified (2)
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	*Anaerolinea* (223)
				Bellilinea (1)
				Leptolinea (59)
				Longilinea (2)
	Chloroflexia	Chloroflexales	Roseiflexaceae	Unclassified (2)
	Dehalococcoidia	Dehalococcoidales	Dehalococcoidaceae	Unclassified (2)
	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	*Ktedonobacter* (115)
Firmicutes	Clostridia	Christensenellales	Christensenellaceae	Unclassified (14)
		Clostridiales	Clostridiaceae	*Fonticella* (11)
				Oxobactor (3)
			Gracilibacteraceae	Unclassified (2)
			Heliobacteriaceae	Unclassified (38)
			Lachnospiraceae	*Blautia* (4)

Contd.

			Peptococcaceae	*Desulfosporosinus* (8)
				Peletomaculum (3)
				Thermincola (6)
			Peptostreptococcaceae	*Paeniclostridium* (2)
				Romboutsia (3)
			Ruminococcaceae	*Caproiciproducens* (2)
			Syntrophomonadaceae	*Syntrophomonas* (4)
			Sporomusaceae	*Anaerospora* (2)
				Pelosinus (2)
				Sporomusa (2)
	Bacilli	Bacillales	Alicyclobacillaceae	*Alicyclobacillus* (5)
				Tumebacillus (7)
			Bacillaceae	*Bacillus* (25)
			Paenibacillaceae	*Paenibacillus* (1)
			Planococcaceae	Unclassified (1)
		Lactobacillales	Aerococcaceae	*Vagococcus* (1)
				Streptococcus (2)

4.20 Bacterial diversity in the sample Mls

Analysis of the sample Mls for bacterial diversity at phylum-level revealed that Chloroflexi was the most abundant bacterial phylum followed by Acidobacteria and Proteobacteria. *Anaerolinea* and *Ktedonobacter* were found to be the predominant genera of phylum Chloroflexi. The genus *Thermoanaerobaculum* possessed major proportion of the phylum Acidobacteria. The third largest phylum being Proteobacteria, was largely composed of class Deltaproteobacteria followed by Alphaproteobacteria and Gammaproteobacteria. *Sulfuricurvum*, *Syntrophobacter* and *Haliangium* were observed to be the dominant genera of class Deltaproteobacteria.

Actinobacteria, Bacteroidetes and Nitrospirae were the other major phyla observed in the sample Mls. Bacterial population belonging to the order Frankiales were found to be abundant in the phylum Actinobacteria with majority of unclassified genera. The major proportion of Bacteroidetes was contributed by unclassified genera and *Acetobacteroides* among the known genera. A large proportion of unclassified genera and population belonging to novel phyla were also observed in the sample Mls. The genus-level taxonomic assemblage of bacterial diversity in the sample Mls is provided in Table 17.

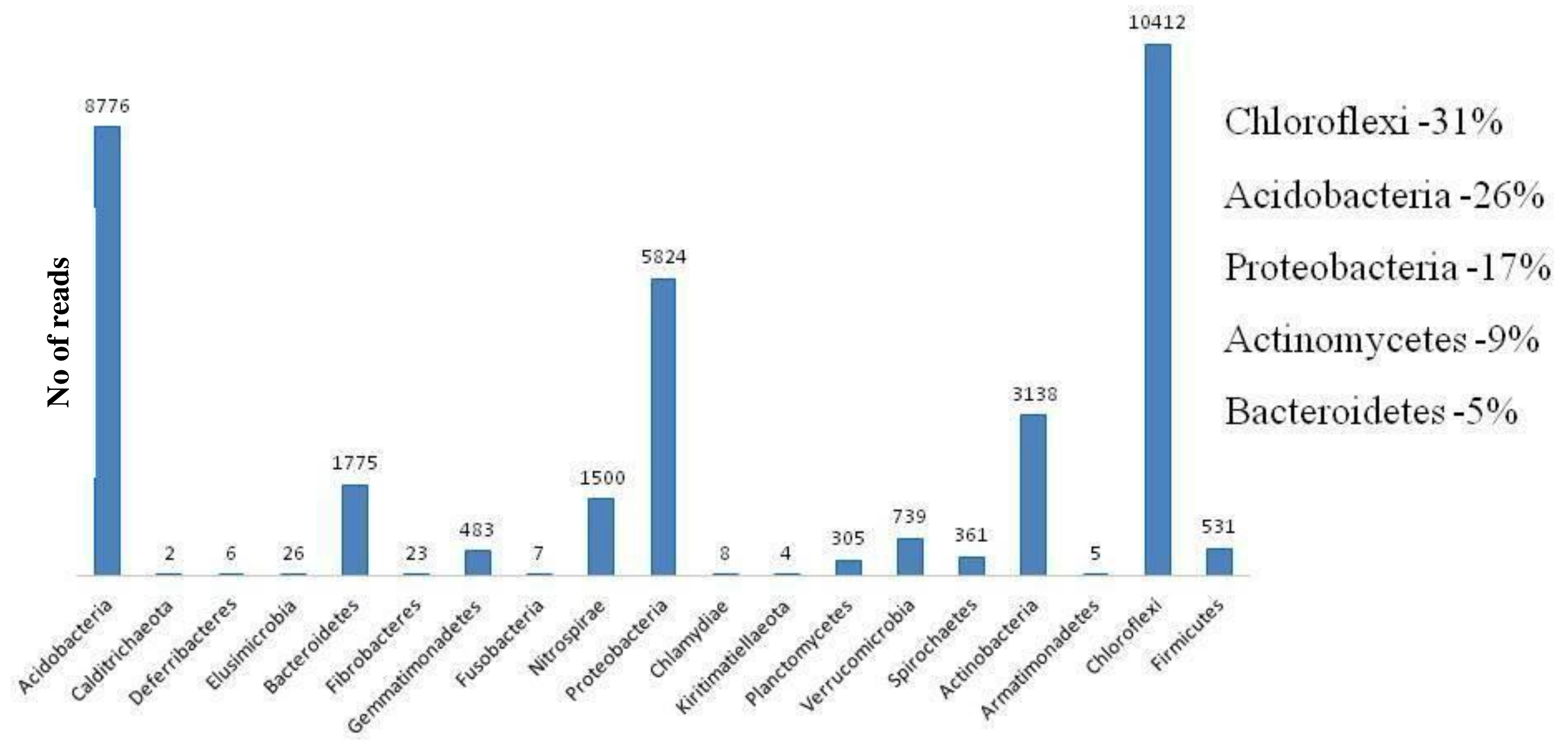


Plate 12. Phylum-level bacterial diversity of sample MIs

Table 17. Genus-level taxonomic assemblage of bacterial diversity from the sample Mls

Phylum	Class	Order	Family	*Genus*
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Acidipila* (9)
				Chloracidobacterium (2)
	Holophagae	Holophagales	Holophagaceae	*Geothrix* (11)
				Holophaga (2)
	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	*Thermoanaerobaculum* (154)
Calditrichaeota	Calditrichae	Calditrichales	Calditrichaceae	Unclassified (2)
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	*Mucispirillum* (6)
Elusimicrobia	Endomicrobia	Endomicrobiales	Endomicrobiaceae	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroids* (30)
			Lentimicrobiaceae	Unclassified (76)
			Muribaculaceae	Unclassified (15)
			Porphyromonoadaceae	*Odoribacter* (3)
				Parabacteroids (3)
			Paludibacteraceae	*Paludibacter* (21)
			Rikenellaceae	*Acetobacteroides* (62)
				Alistipes (6)
				Rikenella (1)
Fibrobacteres	Fibrobacteria	Fibrobacterales	Fibrobacteraceae	*Fibrobacter* (2)
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas* (107)
				Gemmatirosa (20)

Contd.

Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Thermodesulfovibrio* (2)
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium* (22)
		Micropepsales	Micropepsaceae	Unclassified (95)
		Rhizobiales	Bradyrhizobiaceae	*Rhodoblastus* (16)
			Hyphomicrobiaceae	*Pedomicrobium* (2)
			Rhizobiaceae	Unclassified (5)
			Rhodobiaceae	*Bauldia* (27)
				Parvibaculum (2)
			Xanthobacteraceae	*Psuedolabrys* (32)
		Rhodobacterales	Rhodobacteraceae	*Rhodobacter* (6)
		Rhodospirillales	Acetobacteraceae	*Acidiphilium* (13)
				Acidocella (2)
				Rhodopila (7)
				Rhodovastum (1)
				Roseomonas (7)
			Rhodospirillaceae	*Azospirillum* (2)
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium* (2)
				Sphingomonas (5)
	Deltaproteobacteria	Unclassified	Unclassified	*Deferrisoma* (5)
		Desulfarculales	Desulfarculaceae	Unclassified (178)
		Desulfobacterales	Desulfobacteraceae	*Desulfobacterium* (2)
			Desulfobulbaceae	*Desulfobulbus* (9)
				Desulfurivibrio (2)
		Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio* (25)

Contd.

		Desulfuromonadales	Geobacteraceae	*Geobacter* (79)
		Myxococcales	Archangiaceae	Unclassified (94)
			Kofleriaceae	*Haliangium* (168)
			Phaselicystidaceae	*Phaselicystis* (22)
			Polyangiaceae	*Pajaroellobacter* (48)
			Sandaracinaceae	Unclassified (5)
		Syntrophobacterales	Syntrophaceae	*Desulfobacca* (156)
				Desulfomonile (65)
				Smithella (4)
				Syntrophus (17)
			Syntrophobacteraceae	*Syntrophobacter* (171)
			Syntrophorhabdaceae	*Syntrophorhabdus* (83)
		Campylobacterales	Campylobacteraceae	*Sulfurospirillum* (9)
			Helicobacteraceae	*Helicobacter* (45)
				Sulfuricurvum (613)
				Sulfurimonas (30)
	Gammaproteobacteria	Acidiferrobacterales	Acidiferrobacteraceae	*Sulfurifustis* (13)
		Chromatiales	Halothiobacillaceae	*Halothiobacillus* (2)
			Thioalkalispiraceae	*Thioalkalispira* (51)
		Enterobacterales	Enterobacteriaceae	*Enterobacter* (1)
		Legionellales	Coxiellaceae	*Aquicella* (9)
				Coxiella (3)
		Methylococcales	Methylococcaceae	*Methylocaldum* (1)
				Methylogaea (2)

Contd.

				Methylomonas (11)
		Nevskiales	Steroidobacteraceae	Unclassified (19)
		Psuedomonadales	Moraxellaceae	*Acinetobacter* (2)
			Pseudomonadaceae	*Pseudomonas* (4)
		Salinisphaerales	Unclassified	Unclassified (4)
		Xanthomonadales	Rhodanobacteraceae	*Dokdonella* (2)
				Dyella (24)
				Luteibacter (4)
				Rhodanobacter (40)
Chlamydiae	Chlamydiae	Chlamydiales	Unclassified	Unclassified (6)
Kiritimatiellaeota	Kiritimatiellae	Kiritimatiellales	Kiritimatiellaceae	*Kiritimatiella (4)*
Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	Unclassified (4)
		Tepidisphaerales	Unclassified	Unclassified (167)
	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Gemmata* (3)
			Isosphaeraceae	*Aquisphaera* (1)
				Singulisphaera (5)
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	*Akkermansia* (6)
			Rubritaleaceae	Unclassified (1)
			Verrucomicrobiaceae	*Prosthecobacter* (2)
Spirochaetes	Spirochaetia	Spirochaetales	Leptospiraceae	Unclassified (5)
			Spirochaetaceae	*Treponema* (7)
Actinobacteria	Actinobacteria	Catenulisporales	Actinospicaceae	*Actinospica* (42)
			Catenulisporaceae	*Catenulispora* (2)
		Corynebacteriales	Corynebacteriaceae	Unclassified (2)

Contd.

			Mycobacteriaceae	*Mycobacterium* (149)
			Nocardiaceae	*Nocardia* (13)
		Frankiales	Unclassified	Unclassified (1551)
		Kineosporiales	Kineosporiaceae	*Kineosporia* (38)
		Micrococcales	Intrasporangiaceae	Unclassified (2)
			Microbacteriaceae	Unclassified (2)
		Actinomycetales	Micromonosporaceae	*Micromonospora* (16)
			Nocardioidaceae	*Nocardioides* (10)
			Pseudonocardiaceae	*Saccharopolyspora* (2)
			Streptomycetaceae	*Streptomyces* (27)
		Actinomycetales	Thermomonosporaceae	*Actinomadura* (4)
Armatimonadetes	Fimbriimonadia	Fimbriimonadales	Fimbriimonadaceae	Unclassified (1)
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	*Anaerolinea* (605)
				Bellilinea (2)
				Leptolinea (90)
	Chloroflexia	Chloroflexales	Chloroflexaceae	Unclassified (4)
			Roseiflexaceae	Unclassified (4)
	Dehalococcoidia	Dehalococcoidales	Dehalococcoidaceae	Unclassified (1)
	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	*Ktedonobacter* (185)
Firmicutes	Clostridia	Christensenellales	Christensenellaceae	Unclassified (32)
		Clostridiales	Clostridiaceae	*Fonticella* (5)
				Oxobactor (1)
			Clostridiales Family XII. Incertae Sedis	*Anaerovorax* (1)

Contd.

			Gracilibacteraceae	Unclassified (6)
			Heliobacteriaceae	Unclassified (70)
			Lachnospiraceae	*Blautia* (8)
				Roseburia (2)
			Peptococcaceae	*Desulfitobacterium* (3)
				Desulfosporosinus (2)
				Peletomaculum (5)
				Thermincola (6)
			Peptostreptococcaceae	*Romboutsia* (2)
			Sporomusaceae	*Pelosinus* (2)
				Sporomusa (4)
	Bacilli	Bacillales	Alicyclobacillaceae	*Alicyclobacillus* (6)
				Tumebacillus (6)
			Bacillaceae	*Bacillus* (16)
			Paenibacillaceae	*Oxalophagus* (2)
				Paenibacillus (6)
		Lactobacillales	Aerococcaceae	*Aerococcus* (10)
				Vagococcus (1)

4.21 Bacterial diversity in the sample Chr

A dominance of phylum Chloroflexi was recorded in the sample Chr, followed by Proteobacteria and Acidobacteria. Nitrospirae and Bacteroidetes were the other major phyla observed in the sample followed by Verrucomicrobia and Actinobacteria. The genera *Ktedonobacter* and *Anaerolinea* constituted the major proportion of phylum Chloroflexi. The class Deltaproteobacteria dominated the phylum proteobacteria with *Desulfobacca* and *Geobacter* as the dominant genera. A large proportion of unclassified genera were found in the phylum Proteobacteria. The phylum Acidobacteria was majorly composed of *Thermoanaerobaculum* while the phylum Nitrospirae was dominated by the genus *Nitrospira*. The genus *Akkermansia* dominated the phylum Verrucomicrobia while unclassified genera belonging to Lentimicrobiaceae family constituted the major proportion of phylum Bacteroidetes. Population belonging to novel phyla was also observed from the sample Chr. The genus-level assemblage of bacterial diversity has been shown in Table 18.

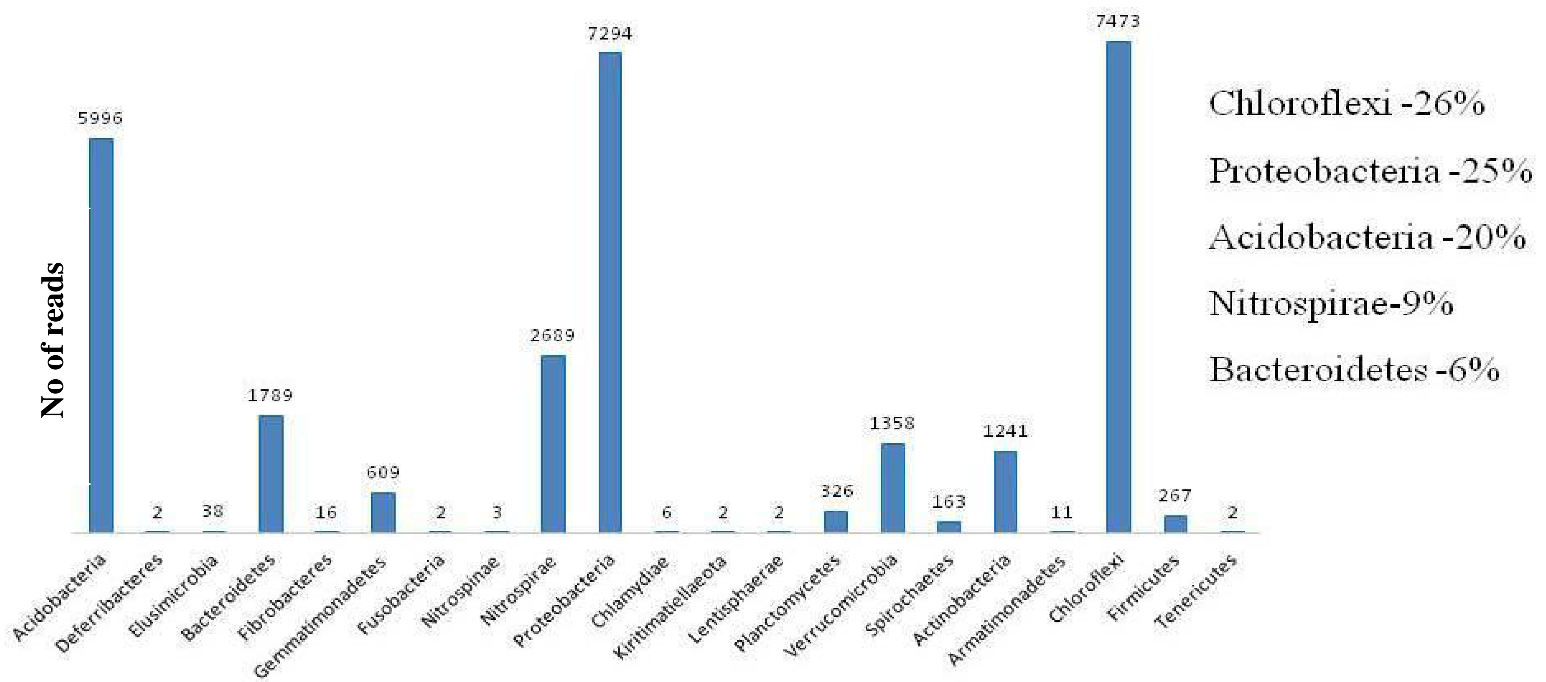


Plate 13. Phylum-level bacterial diversity of sample Chr

Table 18. Genus-level taxonomic assemblage of bacterial diversity from the sample Chr

Phylum	Class	Order	Family	Genus
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Acidipila* (1)
	Holophagae	Holophagales	Holophagaceae	*Geothrix* (12)
	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	*Thermoanaerobaculum* (152)
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	*Mucispirillum* (2)
Elusimicrobia	Endomicrobia	Endomicrobiales	Endomicrobiaceae	Unclassified (8)
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroids* (30)
			Lentimicrobiaceae	Unclassified (169)
			Muribaculaceae	Unclassified (1)
			Porphyromonoadaceae	*Odoribacter* (13)
			Paludibacteraceae	*Paludibacter* (4)
			Rikenellaceae	*Acetobacteroides* (8)
Fibrobacteres	Chitinivibrionia	Unclassified	Unclassified	Unclassified (1)
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas* (76)
				Gemmatirosa (23)
Fusobacteria	Fusobacteria	Fusobacterales	Leptotrichiaceae	*Oceanivirga* (2)
Nitrospinae	Unclassified	Unclassified	Unclassified	Unclassified (3)
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira* (59)
				Thermodesulfovibrio (2)
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium* (19)

Contd.

		Micropepsales	Micropepsaceae	Unclassified (22)
		Rhizobiales	Bradyrhizobiaceae	*Rhodoblastus* (4)
			Hyphomicrobiaceae	*Hyphomicrobium* (2)
			Hyphomicrobiaceae	*Pedomicrobium* (1)
			Rhodobiaceae	*Bauldia* (6)
			Xanthobacteraceae	*Psuedolabrys* (97)
		Rhodospirillales	Acetobacteraceae	*Acidiphilium* (4)
				Rhodopila (2)
				Rhodovastum (1)
				Roseomonas (9)
			Rhodospirillaceae	*Inquilinus* (2)
				Reyranella (22)
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium* (3)
				Sphingomonas (2)
	Deltaproteobacteria	Unclassified	Unclassified	*Deferrisoma* (2)
		Desulfarculales	Desulfarculaceae	Unclassified (151)
		Desulfobacterales	Desulfobacteraceae	*Desulfonema* (1)
			Desulfobulbaceae	*Desulfurivibrio* (7)
		Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio* (8)
		Desulfuromonadales	Geobacteraceae	*Geobacter* (215)
		Myxococcales	Archangiaceae	Unclassified (132)
			Kofleriaceae	*Haliangium* (128)
			Phaselicystidaceae	*Phaselicystis* (16)
			Polyangiaceae	*Pajaroellobacter* (94)

Contd.

			Sandaracinaceae	Unclassified (5)
		Syntrophobacterales	Syntrophaceae	*Desulfobacca* (221)
				Desulfomonile (33)
				Smithella (3)
				Syntrophus (30)
			Syntrophobacteraceae	*Syntrophobacter* (137)
			Syntrophorhabdaceae	*Syntrophorhabdus* (136)
		Campylobacterales	Helicobacteraceae	*Helicobacter* (44)
				Sulfuricurvum (16)
				Sulfurimonas (1)
	Gammaproteobacteria	Acidiferrobacterales	Acidiferrobacteraceae	*Sulfurifustis* (76)
		Chromatiales	Chromatiaceae	Unclassified (6)
			Thioalkalispiraceae	*Thioalkalispira* (130)
		Enterobacterales	Enterobacteriaceae	*Enterobacter* (1)
		Legionellales	Coxiellaceae	*Coxiella* (12)
		Methylococcales	Methylococcaceae	*Methylobacter* (1)
				Methylocaldum (2)
				Methylomonas (2)
		Nevskiales	Steroidobacteraceae	Unclassified (39)
		Oceanospirillales	Halomonadaceae	*Halomonas* (2)
		Pasteurellales	Pasteurellaceae	Unclassified (2)
		Psuedomonadales	Moraxellaceae	*Acinetobacter* (4)
		Xanthomonadales	Rhodanobacteraceae	*Dyella* (4)
				Rhodanobacter (16)

Contd.

			Xanthomonadaceae	*Stenotrophomonas* (2)
Chlamydiae	Chlamydiae	Chlamydiales	Unclassified	Unclassified (6)
Kiritimatiellaeota	Kiritimatiellae	Kiritimatiellales	Kiritimatiellaceae	*Kiritimatiella* (2)
Lentisphaerae	Oligosphaeria	Oligosphaerales	Unclassified	Unclassified (2)
Planctomycetes	Phycisphaerae	Tepidisphaerales	Unclassified	Unclassified(135)
	Planctomycetacia	Planctomycetales	Isosphaeraceae	*Aquisphaera* (6)
				Singulisphaera (3)
			Unclassified	Unclassified (12)
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	*Akkermansia* (7)
			Rubritaleaceae	Unclassified (1)
Spirochaetes	Spirochaetia	Brevinematales	Brevinemataceae	*Brevinema* (2)
		Spirochaetales	Leptospiraceae	Unclassified (16)
			Spirochaetaceae	*Treponema* (1)
Actinobacteria	Actinobacteria	Catenulisporales	Actinospicaceae	*Actinospica* (11)
			Catenulisporaceae	*Catenulispora* (2)
		Corynebacteriales	Mycobacteriaceae	*Mycobacterium* (36)
			Nocardiaceae	*Nocardia* (4)
		Frankiales	Unclassified	Unclassified (605)
		Kineosporiales	Kineosporiaceae	*Kineosporia* (6)
		Micrococcales	Microbacteriaceae	Unclassified (1)
		Actinomycetales	Micromonosporaceae	*Luedemannella* (3)
			Nocardioidaceae	*Nocardioides* (10)
			Streptomycetaceae	*Streptomyces* (13)
Armatimonadetes	Chthonomonadetes	Chthonomonadales	Unclassified	Unclassified (3)

Contd.

	Fimbriimonadia	Fimbriimonadales	Fimbriimonadaceae	Unclassified (3)
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	*Anaerolinea* (224)
				Bellilinea (1)
				Leptolinea (36)
	Chloroflexia	Chloroflexales	Roseiflexaceae	Unclassified (18)
	Dehalococcoidia	Dehalococcoidales	Dehalococcoidaceae	Unclassified (1)
	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	*Ktedonobacter* (445)
Firmicutes	Clostridia	Christensenellales	Christensenellaceae	Unclassified (3)
		Clostridiales	Clostridiales Family XII. Incertae Sedis	*Fusibacter* (2)
				Anaerovorax (1)
			Gracilibacteraceae	Unclassified (2)
			Heliobacteriaceae	Unclassified (24)
			Lachnospiraceae	*Blautia* (8)
			Peptococcaceae	*Desulfitobacterium* (2)
				Desulfosporosinus (5)
				Peletomaculum (2)
				Thermincola (2)
			Peptostreptococcaceae	Romboutsia (1)
			Syntrophomonadaceae	Syntrophomonas (2)
			Sporomusaceae	Sporomusa (1)
	Bacilli	Bacillales	Alicyclobacillaceae	Alicyclobacillus (2)
				Tumebacillus (13)
			Bacillaceae	Bacillus (33)
			Planococcaceae	Unclassified (1)

Contd.

		Lactobacillales	Aerococcaceae	Aerococcus (8)
Tenericutes	Mollicutes	Unclassified	Unclassified	Unclassified (2)

4.24 Comparison of bacterial diversity among all the samples

The prokaryotic diversity of all the samples was analyzed with the help of various tools like QIIME, MG-RAST and MEGAN. The highest prokaryotic population was recorded in the sample Puzhakkal (Pzk) followed by Mullassery (Mls), while the lowest population was recorded by the sample Cherpu (Chr). The highest population of Proteobacteria was recorded in the sample Pzk and Chloroflexi in the sample Mls. Sample Chr recorded a comparatively higher bacterial population of phyla Nitrospirae and Bacteroidetes. In sample Pzk, the bacterial population belonging to class Acidobacteria occupied the major proportion followed by Deltaproteobacteria and Gammaproteobacteria. Acidobacteria and Ktedonobacteria were found to be the predominant classes in samples Mls and Chr. The Ktenonobacterales and Acidobacterales were the found to be the dominant orders in all the samples. The comparison of prominent genera among the samples were carried out and *Desulfobacca* was found to be the dominant genera followed by *Thermoanaerobaculum* and *Thioalkalispira*. The predominant genera found in the sample Mls was *Sulfuricurvum* followed by *Anaerolinea* and *Syntrophobacter*. In the sample Chr, the genus *Ktedonobacter* was observed to be abundant followed by *Anaerolinea* and *Desulfobacca*. *Anaerolinea* was found higher in Mls than Chr and *Desulfobacca* was found to be higher in Pzk than Chr. Unknown prokaryotic population was found abundant at species level.

The microbial diversity within the samples was analyzed by calculating Shannon, Chao 1 and observed species metrics. The metric calculation was performed using QIIME software. The Chao1 metric estimates the species richness while Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. The observed species metric is the count of unique OTUs identified in the sample. The rarefaction curve showed higher species richness in the sample Pzk and a higher species diversity in the sample Mls (Plate 15).

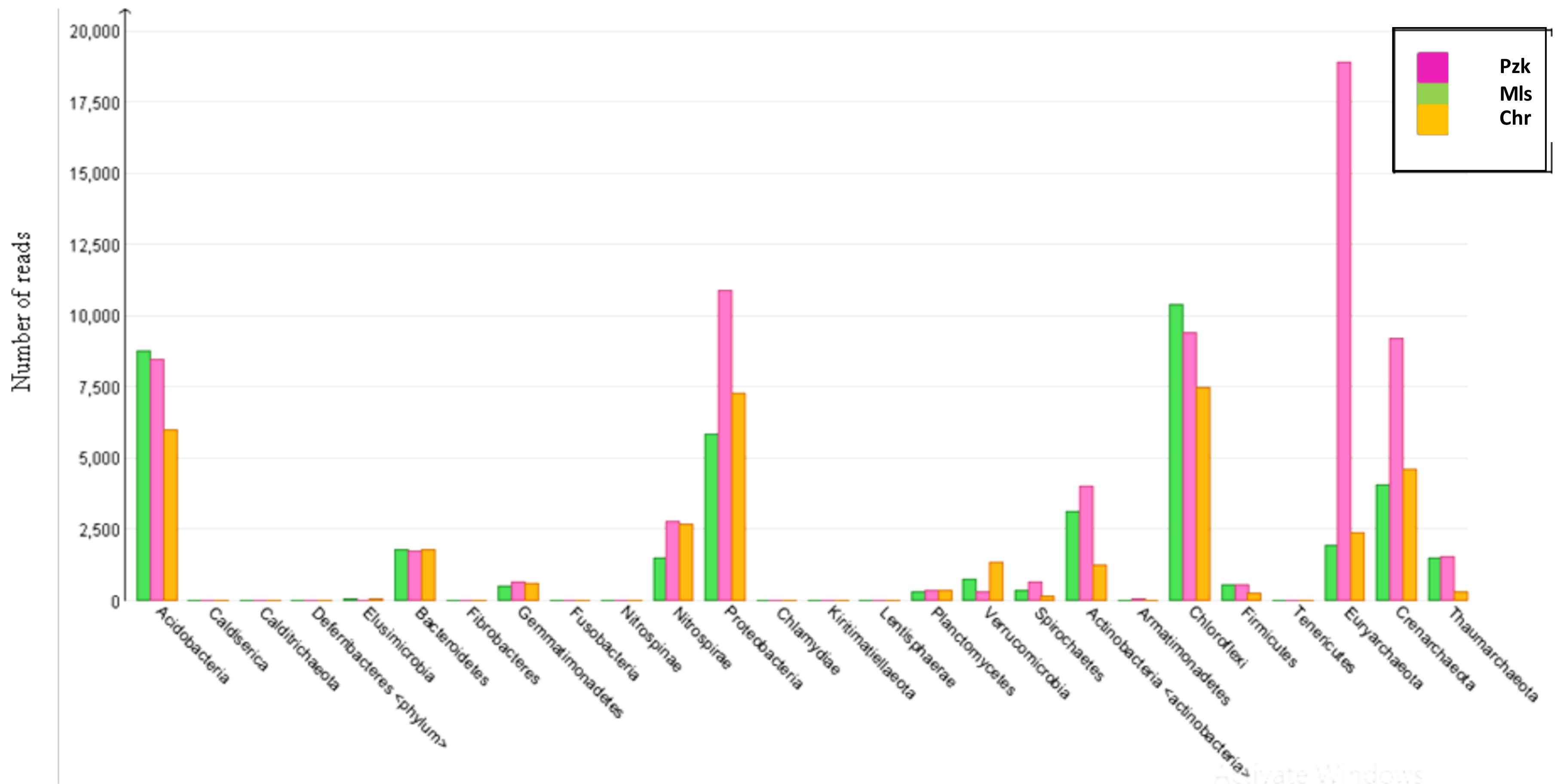


Plate 14. Bacterial and archaeal diversity at phylum-level using MEGAN

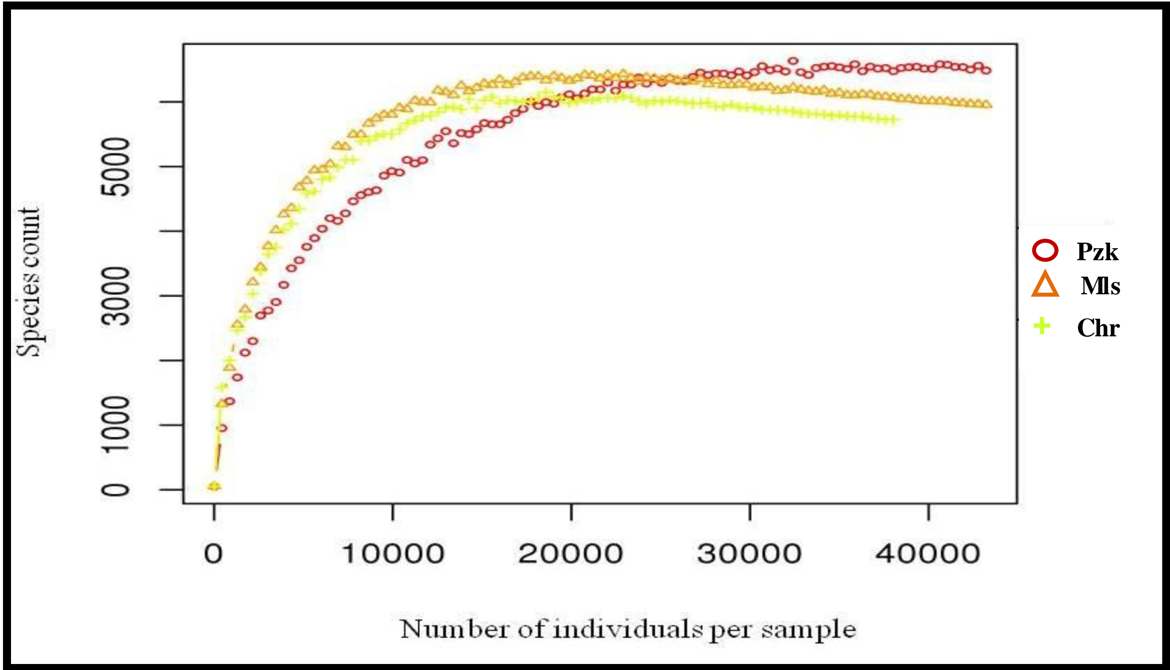


Plate 15.a. Chao 1 rarefaction curve for all the samples

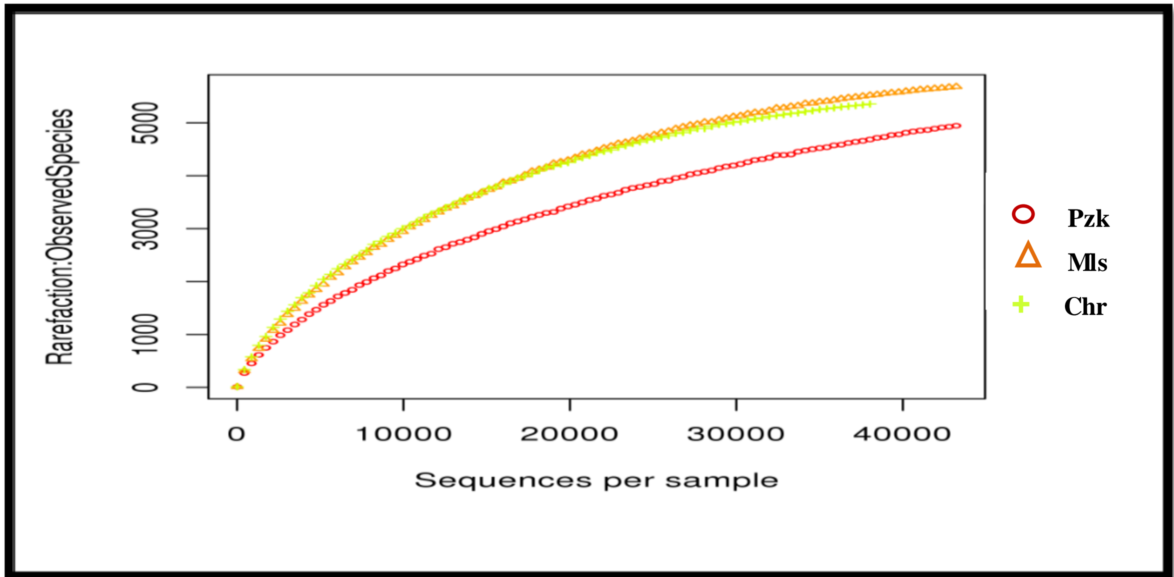


Plate 15.b. Observed species rarefaction curve for all the samples

Plate 15. Rarefaction analysis using QIIME

4.25 Submission of sequences in NCBI database

The metagenomic sequences obtained were submitted in the Sequence Read Archive (SRA) for the accessibility by the public domain. The sequences of the samples Pzk, Mls and Chr were submitted in the SRA portal under the bioaccession numbers SAMN17776076, SAMN17776077 and SAMN17776078 respectively.

Discussion

5. DISCUSSION

Agriculture plays an important role in overall development and social transformation of the country. Rice production has been contributing a significant part of national economy. According to recent statistics, India is the second largest producer and largest exporter of rice in the world (Statista, 2021). Rice cultivation contributes a major portion of overall food production in Kerala and rice is considered to be the staple food of the State. According to 2017-18 agricultural statistics data of Kerala, the area under paddy cultivation was 1.94 lakh ha, of which 1.89 lakh ha was wetland paddy. The overall rice production of the same year was 5.21 lakh tonnes which showed a decline of 11.7 per cent compared to 2008-09 production (Envis, 2021).

Kole lands of Kerala are a complex ecological system and are known for the higher productivity of rice. Kole lands play a vital role in ensuring food security and ecological balance of the state. Kole lands account for about 20 per cent of rice production in Kerala with a productivity of 5.0 to 6.0 tonnes per hectare (Johnkutty and Venugopal, 1993). The name '*kole*' originated from a Malayalam word which means bumper yield or high returns, if flood waters do not damage the crop and refers to the peculiar type of paddy cultivation carried out from December to May. The importance of the ecosystem of this area is the reason for its inclusion as a Ramsar site, and it is being considered as a separate high value biodiversity area. This area is a part of the Vembanad-Kole Wetland System, which is the largest brackish, humid tropical wetland ecosystem in the Southwest coast of India. The Vembanad-Kole system is fed by 10 rivers, which originate from the Western Ghats and flow into the Arabian sea.

The Kole lands remain submerged under flood water for about six months in a year and this seasonal alteration gives it both terrestrial and water related properties which determine the ecosystem structure. It is reported that about 233.74 mm^3 water is contained in the Kole lands.

Several studies have been undertaken in Kole lands for exploring the diversity of birds, fishes, flora, butterflies, etc. However, no systematic studies have been taken up on microbial diversity. Soil microbes are the key elements of soil nutrient cycling. The microbial community aids plants in nutrient uptake and also acts as antagonists to a wide range of phytopathogens (Matilla and Krell, 2018). The recalcitrant forms of soil nutrients are metabolized by soil microbes to release these elements for plant nutrition (Richardson *et al.*, 2009). Such nutrient transformations by the microbes are the main grounds for plant growth and sometimes it can act as a rate-limiting step in the ecological productivity (Schimel and Bennett, 2004). The plant growth enhancing microbes play a vital role in plant stress management by improving nutrient access, production of secondary metabolites, plant hormones and also provide induced and acquired systemic resistance against biotic stress (Narusaka *et al*., 1999).

The traditional methods of culturing microbes limit the analysis to those which grow under *in vitro* conditions (Rondon *et al*., 2000). However, 99 per cent of microbes present in the environment are unculturable (Sekiguchi, 2006). To overcome this limitation, different DNA-based molecular methods have been developed. In general, methods based on 16S rRNA gene analysis provide vast information about the archaeal and bacterial diversities in extreme environments. The recovery and analysis of 16S rDNA directly from the environmental DNA provides an insight of microbial populations without the need to culture them (Ghazanfar and Azim, 2009).

In this context, the present investigation is taken up with the objective of assessing the diversity of bacterial community in the rice rhizosphere of Kole lands of Thrissur, using the culture independent metagenomic approach. The results obtained from the study have been discussed in this chapter.

The Thrissur Kole lands are spread over eight blocks. Of these, three blocks were selected for the study, namely Puzhakkal (Pzk), Mullassery (Mls) and Cherpu (Chr) and rhizosphere soil samples from the active root zone of rice were collected from one padasekharam representing each block. The padasekharams were located in Adat,

Elavathur and Cheruchenam, representing Pzk, Mls and Chr blocks respectively. Puzhakkal block is reported to have sandy soil whereas the other two locations have clayey soil. Sampling was carried out at maximum tillering stage of the crop, as the population and diversity of rhizosphere microorganisms have been reported to be maximum at this stage, in rice (Hussain *et al.,* 2012).

The six soil samples collected from three locations were subjected to physico-chemical and biological characterization. It has been reported that soil physical as well as chemical properties affect soil bacterial as well as fungal community structures (Sui *et al*., 2021). The physico-chemical parameters characterized included pH, electrical conductivity, organic C, total N, available P, K, Ca, Mg, S and micronutrients viz. Fe, Mn, Cu, Zn and B. Remarkably low pH was observed in all the samples and the lowest pH of 4.29 was recorded in Chr sample (Table 4). Earlier reports also indicated that the rice soils of Thrissur Kole lands are generally acidic due to the underlying peat horizon and the pH ranged from 4.9 to 6.1 (Thomas, 2003). Vineetha *et al*. (2016) reported that the soil pH in Kole lands tends to increase when soils become more reduced due to water saturation because of the consumption of free protons with reduction processes. The electrical conductivity was normal and did not show much variation among the samples.

The organic carbon was found to be higher in the sample Pzk than the samples Mls and Chr. Soil acts as a major source and utilizer of organic C in the environment and thereby creates the most dynamic platform for the growth and proliferation of plants and microorganisms (Tate, 1995). The decomposition process carried out by the soil microbes along with themselves add up the organic content of soil (Schmidt *et al*., 2011). The soil microbial carbon use efficiency (CUE) is considered as an important characteristic of soil microbial community metabolism (Sinsabaugh *et al.*, 2013). A previous study conducted by van Hees *et al.* (2005) reported that the CUE of soil microbial communities is closely related with the decomposition of complex compounds and respiration rate of unit carbon assimilation. A study by Wang *et al*. (2015) suggested that the long-term paddy

cultivation stabilizes the soil organic C and enhances the soil microbial activity in saline marshy soil.

Soil organic matter is known to modify the physico-chemical reactions which influence the nutrient availability in the soil. The macro and micronutrients composition in the rhizosphere soils did not show much variation among the samples. All the samples were found to be deficient in major nutrients except for S while, the samples were found to be sufficient in all the micronutrients except B. A negative correlation was found between Ca and Fe which indicates the influence of iron in calcium uptake. In acidic soils, the Ca uptake was found to be inhibited by the precipitation of Fe in the plant root tips (Rorison, 1973).

The rhizosphere samples were also analyzed for their biological properties viz. culturable microbial population and microbial biomass C. Microbial biomass C estimation was carried out using Fumigation-extraction method proposed by Vance *et al*. (1987). The microbial biomass C measures the mass of the microorganisms present in the soil and it provides more accurate values than the direct count method (Schnurer *et al*., 1985). This is also considered as an indicator of soil quality. In the present investigation, a notable difference in microbial biomass carbon among the samples was not observed. The microbial biomass carbon value was found higher in the sample Pzk and lowest in the sample Chr. An earlier study conducted by Neale *et al*. (1997) suggested that the acidic soils with less pH supported the acid-tolerant bacteria but failed to support the acid-intolerant ones, which in fact reduced the microbial biomass C. Previous reports suggest that the microbial biomass C would only show trends in long-term and in short-term it may be equivalent to any other chemical indicators present in the soil. However, the microbial biomass C changes more rapidly than the other soil chemical indicators viz. soil organic C (Hargreaves *et al*., 2003).

The population of bacteria, fungi, actinomycetes, nitrogen fixers, phosphate solubilizers, potassium solubilizers and zinc solubilizers was estimated in all the rhizosphere soil samples, using selective media. Among the different groups of

microorganisms, population of bacteria was highest (in the range of 10^6 cfu g^{-1}). This is generally true for any soil. The culturable microbial population was found higher in the sample Pzk and lower in the sample Chr. These soils also recorded an organic C content of 1.93 % and 1.12 % for Pzk and Chr respectively, which indicates a positive correlation between the microbial biomass C and microbial population. Total N content was also higher for Pzk (0.5%) and lower for Chr (0.13 %) and this could also have contributed to the difference in population of culturable microflora.

The fungal population was comparatively less in the samples (to the tune of 10^3 cfu g^{-1} soil) and this could be attributed to the low levels of oxygen under flooded conditions in the wetland ecosystem. Predominant fungal genus was *Trichoderma* spp. The culturable population of actinomycetes did not show remarkable variations among the samples and was found to be slightly higher in the sample Pzk, as compared to the other locations.

The population of nitrogen fixers were found to be higher in the sample Mls followed by Pzk. Chr recorded lowest population of N-fixers and this soil also recorded the minimum content of total N in soil. It has been reported that the presence of mineral N in soil inhibits N-fixation by bacteria (El-Sharkawi, 2012). However, in the present investigation, the content of available N was not estimated in the soil.

Phosphate- solubilizing populations were observed to be higher in the sample Pzk (14.55 x 10^3 cfu g-1) and lowest in the sample Chr (4.25 x 10^3 cfu g^{-1}). This could be because of the lower content of available P in Pzk (6.73 Kg ha^{-1}), as compared to the higher content of P in Chr (10.13 Kg ha^{-1}). It has already been reported that phosphate solubilizers proliferate and solubilize insoluble P, in P deficient soils, improving the available P content (Liang *et al*., 2020). The populations of potassium and zinc solubilizers were found less in all the samples and the sample Chr failed to show the presence of Zn solubilizers. All the three soil samples were sufficient in Zn levels and this could be the reason for the low population of Zn solubilizers. A similar study by

Saravanan *et al*. (2007) reported few zinc solubilizing bacteria such as *Bacillus* and *Thiobacillus*.

Microbial biomass C and microbial population are the two factors that determine the soil microflora. A slight increase in the microbial population was observed in sample Pzk, which corresponded to higher microbial biomass C in Pzk. Previous reports have suggested that the presence of Fe hydroxides inhibit the biodegradation of organic matter. In the present investigation, the content of iron was found to be high in all the samples. A study done by Jones (1998) suggested that the microorganisms have the capacity to utilize the metal-organic complexes and the non-complex forms of organic acids for their metabolism.

The plant rhizosphere microorganisms play a crucial role in the maintenance of plant health. The plant rhizosphere microflora is engineered as a result of selective pressures exerted by the abiotic factors as well as biological processes taking place in the soil (da Rocha *et al*., 2009). The exudates secreted from the plant roots are found responsible for chemotaxis and the shaping of rhizosphere microflora (Chaparro *et al*. 2014). The rice root exudates were found to be composed of carbohydrates, amino acids and other organic acids (Aulakh *et al*., 2001). The quality and quantity of root exudates depends on the plant species, its developmental stage and environmental conditions (Gransee and Wittenmayer, 2000). Since the root exudates heavily influence the rhizosphere microflora, a variation in the release of root exudates with soil conditions and cropping pattern can result a similar shift in the population dynamics of soil microbes.

The microbial enumeration was followed by the purification of 24 predominant bacterial isolates. These isolates were then screened for PGPR traits viz. production of IAA, phosphate solubilization and ammonia production (Table 6). In the present investigation, 14 bacterial isolates were found capable to synthesize IAA and phosphate solubilization was displayed by ten isolates. Eight bacterial isolates exhibited ammonia production. The PGPR performs multiple functions like nutrient cycling, suppression of antagonistic microbes, seedling growth enhancement, etc. (Barea *et al*., 2005). Many

bacteria are known to solubilize phosphate in soil complexes, rendering phosphorus available to plants. However, the degree of solubilization varies with microorganisms, which is an important trait for plant growth promotion by the microorganism for increasing crop yield (Rodriguez *et al*., 2006). The plant growth promoters produced by the rhizosphere microflora influence the plant growth and development via induction of some important physiological responses at various developmental stages of plant (Solano *et al*., 2008). A study by Marcela *et al*. (2018) revealed that the rhizobacteria *Pseudomonas fluorescens* BRM 32111 and *Burkholderia pyrrocinia* BRM-32113 are found to impart allelochemical stress tolerance to upland rice plants. An investigation by Mukherjee and Sen (2017) found that inoculation of maize with *Acetobacter* in the presence of half of the recommended dose of NPK induced the parameters to match those of the recommended dose of the NPK.

As the soil acts as a large reservoir of soil microbes and 99 per cent of them are uncultivable, the enumeration of all the bacterial population using serial dilution plate method is not possible. Hence, in the present study, a culture-independent approach has been adopted to unravel the total bacterial diversity from the rice rhizosphere of Kole lands of Thrissur. The culture-independent tool called metagenomics based on 16S rRNA gene was employed for this. Handelsman (2004) introduced the term metagenomics to deeply understand the unculturable microorganisms that remains unexplored and unidentified using 16S rDNA sequences. Metagenomics helps to eliminate the plate count anomaly and renders information about 95-99 per cent of the microbial population of the sample that remains uncultivable (Nichols, 2007). The study of microbial population using metagenomics can be used to construct a link between the phylogenetic information and the environmental functions (Riesenfeld *et al*., 2004).

Extraction of good quality metagenomic DNA is the major hassle in metagenomic studies. Basically, two methods are adopted for the extraction of metagenomic DNA manually: direct and indirect methods. In the former method, the cell are lysed and DNA is extracted directly from the soil while in the later, the cells are first isolated from the

soil and then the DNA is extracted. In the present investigation, the direct method of DNA extraction by Siddhapura *et al*. (2010) was initially adopted for the extraction of metagenomic DNA. However, the DNA obtained was highly contaminated with impurities like humic acid as the protocol lacked any clean-up or filtration step for the elimination of these impurities. As the rice rhizosphere soil samples were naturally rich in humus, the presence of humus in the DNA extracted could have inhibited the PCR step. Therefore DNeasy PowerSoil kit by QIAGEN was used to extract good quality metagenomic DNA from the soil samples. The metagenomic DNA extraction was done by lysing the samples mechanically in a bead beating tube and chemically by treating with the solutions provided. Then the DNA was collected in a MB spin column provided with the kit and washed with ethanol (wash solution). The DNA was finally obtained by eluting from the column using elution buffer. The metagenomic DNA thus obtained was of good quality, without PCR inhibitors as evidenced by an intact band on agarose gel and amplification of 16S rRNA gene.

The lysis method carried out using DNeasy PowerSoil kit included chemical lysis with lysing buffers in combination with mechanical lysis with ceramic beads. This combinational lysis was observed to be highly efficient in the extraction of DNA from Gram positive and archaeal cells that are difficult to lyse and from the other inaccessible cells residing in the soil pockets, without hampering DNA from Gram negative bacteria (Frostegard *et al*., 1999). The DNA extraction using bead beating method was found to yield high quantity of DNA from the samples. Harsh protocols are recommended for the isolation of samples which are abundant in Gram positive bacteria while gentle lysis protocol are recommended for the extraction of sample with abundant in Gram negative bacteria. Even though various protocols are available for metagenomic DNA extraction, commercial kits are found to be more efficient in the optimum lysis of cells as well as for the removal of PCR inhibitors and other contaminants (Keisam *et al*., 2016). In this study, DNeasy PowerSoil kit was used to evade the biases involved in the extraction of DNA using manual protocols. The study conducted by Ashwini *et al.* (2019) reported that the direct method of metagenomic DNA isolation failed to extract archaeal sequences,

but the use DNeasy PowerSoil kit in the current study rendered optimum lysis to acquire archaeal diversity from the rhizosphere soil samples.

In general, 16S rRNA gene sequences are used to reveal the community composition of any environmental samples. This gene which encodes for the 16S ribosomal RNA is 1500bp in size and is made up of nine variable regions distributed between the conserved regions. It was reported that the 16S rDNA was used as best molecular chronometer for cataloguing purposes as it has greater resolving power than other oligonucleotides (Woese, 1987). The bacteria were classified based on the differences in V3 and V4 hypervariable regions of 16S rDNA which are amplified using specific primers. The 16S rRNA gene sequences obtained from Illumina platform was reported to be an effective tool in diversity analysis and taxonomic assemblage of the environmental samples (Logares *et al*., 2014). Even though this method of classification is considered as an efficient one, only 17 per cent of the sequences are allotted to known bacterial genera. A previous study on phylum level bacterial diversity reported that the soil ecosystem consisted of about 32 bacterial phyla and ambiguity existed with the allocation of sequences to known and unknown taxa of Proteobacteria (Janssen, 2006). An investigation carried out by Claesson *et al*. (2010) identified that the hypervariable V3/V4 and V4/V5 combinations displayed high simulation coverage and accuracy than the other combinations. In the current study also, V3 and V4 hypervariable regions were exploited for the metagenomic analysis of bacterial diversity from the rice rhizosphere of Kole lands of Thrissur.

Metagenomics is a high throughput Next Generation Sequencing (NGS) technology, which generates an enormous amount of data which are then, analyzed using bioinformatics tools. This technology being relevant in the comprehensive study of bacterial diversity, has been exploited in various fields including microbial exploration from guts, intestinal tracts, rumen and soil. However, the management of large scale data generated from NGS platform and the requirement of complex computational algorithms for the analysis has always been a major pitfall of NGS technology (Scholz *et al*., 2012).

The major NGS platforms include Roche 454, Illumina and Ion Torrent. In this study, Illumina platform was employed for NGS sequencing. The Illumina platform involves amplification of V3-V4 hypervariable regions using specific primers, ligation of adapters followed by library quantification. Then the denatured library was exposed to MiSeq sequencing.

The Fastq sequences obtained from MiSeq sequencing were analyzed for their quality using parameters like base quality distribution, base composition and GC distribution. Then the sequences were trimmed and filtered to eliminate mismatches, singletons and chimeras to procure total number of Operational Taxonomic Units (OTUs). The bioinformatics pipeline QIIME (Quantitative insights into Microbial Ecology) developed by Knight and Caporaso labs was used to cluster the sequences into Operational Taxonomic Units based on sequence similarity. In general, Operational Taxonomic Unit is referred as a group of related organisms. In the context of metagenomics, an OTU is described as a cluster of 16S rDNA sequence variant with a minimum per cent of 97 identity threshold at genus level. An identity threshold of 98 or 99 per cent is considered as ideal for species level classification.

QIIME1 program was used for the downstream analysis. The representative sequences from each clustered OTUs were picked and aligned against SILVA core set of sequences using PyNAST program. Further, taxonomy classification was performed using RDP (Ribosomal Database Project) classifier by mapping each representative sequence against SILVA OTUs database. The sequence that does not have any alignment against taxonomic database is categorized as Unknown.

MG-RAST (Metagenomic Rapid Annotation using Subsystems Technology) developed by Argonne Laboratory was also employed for the taxonomic characterization of OTUs using RDP database. RDP, the rRNA sequence based database comprises the ribosomal information of archaeal, bacterial and fungal sequences (Maidak *et al.*, 1994). The taxonomic classification was also carried out using MEGAN (MEtaGenome Analyzer) and phylogenetic trees and statistical data were obtained. In the following

discussion, the prokaryotic diversity of individual sample and the comparison among the samples are described.

In the sample Pzk, the major proportion of the total reads was constituted by the domain bacteria. The bacterial phylum Proteobacteria was found to be most abundant followed by Chloroflexi and thirdly by Acidobacteria. Proteobacteria was found to be the most versatile, dominant and diverse phylum of the rhizosphere ecosystem (Sanguin *et al*., 2006). Bacterial population belonging to 20 phyla was observed from the sample indicating the population dynamics of the bacteria present in the sample. Actinobacteria and Bacteroidetes were found to be other major bacterial phyla. Majority of the Proteobacteria was found to be occupied by the class Deltaproteobacteria with genus *Desulfobacca* as the dominant one followed by *Geobacter* and *Sulfuricurvum* among the known genera. The genus *Geobacter* was found capable to oxidize organic compounds and metals, including Fe, radioactive metals and petroleum compounds which have greater environmental significance (Childers *et al*., 2002). The genus *Thioalkalispira* of class Gammaproteobacteria and *Psuedolabrys* of class Alphaproteobacteria was found to be the other major genera of Proteobacteria. The bacteria belonging to novel genera were found abundant in this sample. The genus *Anaerolinea* and *Ktedonobacter* contributed the major portion of phylum Chloroflexi and the *Thermoanaerobaculum* occupied the major portion of phylum Acidobacteria. Unclassified genera belongs to the order Frankiales was found to be predominant in phylum Actinobacteria. The proportion of unclassified bacteria was found to be very high in the class Deltaproteobacteria of phylum Proteobacteria. In the sample Pzk, majority of the genera was anaerobes and the population of PGPR bacteria was found comparatively very less. Unlike other samples, the archaeal population was found higher than the bacterial population in sample Pzk. The archaeal phylum Euryarchaeota was observed to be most abundant followed by Crenarchaeota. The phylum Crenarchaeota was found to harbor the most number of unclassified archaeal population. The genus *Nitrososphaera* was turned out to be the most abundant genera of the phylum Thaumarchaeota. *Nitrososphaera* is an ammonia oxidizing archaeon found in the soil (Tourna *et al*., 2011). A study conducted by Chen *et*

al., (2008) reported that the ammonia oxidizing archaea is dominant than the ammonia oxidizing bacteria in the rice rhizosphere ecosystem.

The bacterial diversity of sample Mls at phylum-level was analyzed and Chloroflexi was found to be the most abundant bacterial phylum followed by Acidobacteria and thirdly by Proteobacteria. Bacterial population belonging to 18 phyla was observed from this sample. *Anaerolinea* and *Ktedonobacter* were found to be the predominant genera of phylum Chloroflexi. The genus *Thermoanaerobaculum* possessed major proportion of the phylum Acidobacteria. The genus *Thermoanaerobaculum* was known to be the first cultivated genus of phylum Acidobacteria subdivision 23 (Blake *et al*., 2014). The third largest phylum being Proteobacteria, was largely composed of class Deltaproteobacteria followed by Alphaproteobacteria and Gammaproteobacteria. *Sulfuricurvum*, *Syntrophobacter* and *Haliangium* were observed to be the dominant genera of class Deltaproteobacteria. The genus *Syntrophobacter* is often used in the process of waste water treatment for the degradation of organic compounds from lactate and propionate (Plugge *et al*., 2012). The genus *Haliangium* belonging to the family Kofleriaceae is known to produce antifungal compounds haliangicins (Colegate *et al*., 2007). Actinobacteria, Bacteroidetes and Nitrospirae were the other major phyla observed in the sample Mls. Bacterial population belonging to the order Frankiales were found to be abundant in the phylum Actinobacteria with majority of unclassified genera. The major proportion of Bacteroidetes was contributed by unclassified genera and *Acetobacteroides* among the known genera. A large proportion of unclassified and novel genera were found in this sample. The genus *Nitrospira* of phylum Nitrospirae was found to have nitrate oxidizing property (Koch *et al*., 2015). The population of *Bacillus*, *Paenibacillus*, *Arthrobacter*, *Burkholderia*, etc. was also found in a notable amount. The phylum Crenarchaeota was observed to be the most dominant archaeal phylum followed by Euryarchaeota and Thaumarchaeota respectively. The phylum Euryarchaeota was majorly composed of genus *Methanocella* while the genus *Nitrososphaera* was found to be the only genera of phylum Thaumarchaeota. The phylum Crenarchaeota accommodated a wide diversity of unclassified archaeal population.

The phylum Chloroflexi was found dominant in the sample Chr followed by Proteobacteria and thirdly by Acidobacteria. Nitrospirae and Bacteroidetes were the other major phyla observed from the sample followed by Verrucomicrobia and Actinobacteria. A study by Campbell and Kirchman (2013) reported that the OTUs of phylum Verrucomicrobia are found highly sensitive to the changing soil conditions viz. temperature. The sample showed a slight higher diversity of bacterial population at phylum level but the population density was found comparatively lesser in Chr. The genera *Ktedonobacter* and *Anaerolinea* constituted the major proportion of phylum Chloroflexi. The class Deltaproteobacteria dominated the phylum proteobacteria with *Desulfobacca* and *Geobacter* as the dominant genera. A large proportion of unclassified genera were found in the phylum Proteobacteria. The phylum Acidobacteria was majorly composed of *Thermoanaerobaculum* while the phylum Nitrospirae was dominated by the genus *Nitrospira*. The genus *Akkermansia* was found abundant in the phylum Verrucomicrobia while unclassified genera belonging to Lentimicrobiaceae family constituted the major proportion of phylum Bacteroidetes. Lentimicrobiaceae contains slow-growing anaerobes that can ferment wide range of compounds to malate, acetate, formate, propionate and hydrogen (Sun *et al*., 2016). The phylum Crenarchaeota constituted the major portion of archaeal population followed by Euryarchaeota. The members belonging to Thaumarchaeota was found to be least in Chr with only one genus *Nitrososphaera*. The phylum Crenarchaeota was found to harbor wide array of unclassified genera.

A comparative analysis was done among all the samples and Pzk reported highest prokaryotic population followed by Mls, and the lowest population was recorded by the sample Chr. The phylum level diversity was found more in the sample Chr while the population diversity of known genera was found higher in the sample Mls. The phyla Proteobacteria, Actinobacteria, Nitrospirae, Spirochaetes, Gemmatimonadetes and Firmicutes were found higher in the sample Pzk. Sample Mls showed higher population of phyla Chloroflexi and Acidobacteria while sample Chr shows a comparatively higher

population of phyla Nitrospirae and Bacteroidetes. The population belonging to phylum Tenericutes was found only in the sample Chr.

A remarkable population of Acidobacteria was observed in all the samples. A selective increase in the population of Acidobacteria can be seen in the oligotrophic habitat than the copiotrophic ecosystem (Lee *et al*., 2008). It was also reported that the competition in the rhizosphere ecosystem has no effect on the members of phylum Acidobacteria (da Rocha *et al.*, 2013). The bacterial population belonging to class Ktedonobacteria was found comparatively less in the sample Pzk. The Ktenonobacterales and Acidobacterales were the found to be the dominant orders in all the samples. The comparison of prominent genera among the samples was carried out and the sulphur oxidizing *Desulfobacca* was found abundant in all the soil samples. The predominant genus found in the sample Mls was *Sulfuricurvum* while in the sample Chr, the genus *Ktedonobacter* was observed to be abundant. The genus *Anaerolinea* was found higher in Mls than Chr and *Desulfobacca* was found to be higher in Pzk than Chr. The ecological roles of some of the predominant genera are shown in the Table 19. Unknown prokaryotic population was found abundant at species level. The PGPR like *Bacillus, Paenibacillus, Azospirillum, Pseudomonas, Arthrobacter,* etc. were observed in the sample Mls in a notable amount. The *Arthrobacter* of phylum Actinobacteria was reported to be useful in the bioremediation of soils contaminated with pesticides (Qingyan *et al*., 2008). Few members of the genus Arthrobacter are known to have phosphorous solubilizing capacity (Chen *et al*., 2006). A study by Govindasamy *et al.* (2010) reported that the wide adaptation of *Bacillus* and *Paenibacillus* in soil and plant ecosystem was found to suppress plant pathogens. The genus *Pseudomonas* has always been one of the most exploited microorganisms in agriculture. Aside from the production of phytohormone, they are also known to produce phloroglucinol that can suppress the plant pathogens (Raaijmakers and Weller, 1998). The genus *Pseudomonas* was also found to induce systemic resistance in host plants by the production of 2,3-butanediol (Bakker *et al*., 2007). The proportion of unclassified and novel taxa was found higher in

all the samples. This high proportion of unclassified taxa might indicate the majority of undiscovered soil bacteria.

Table 19. Ecological functions of prominent genera from the samples

Phyla	Prominent Genera	Ecological Functions
Proteobacteria	*Desulfobacca* *Sulfuricurvum* *Thioalkalispira*	Geo-chemical cycling of S
	Rhodoblastus *Rhodobacter* *Rhodopila*	Photosynthesis under acidic conditions (purple non-sulphur bacterium)
	Hyphomicrobium *Pedomicrobium* *Bauldia*	Biofilm formation in waste water treatment plants
	Sphingomonas	degrade refractory contaminants, serve as bacterial antagonists to phytopathogenic fungi, and secrete the highly useful gellan exopolysaccharides
	Sulfurospirillum *Desulfobacca* *desulfomonile*	Sulphur reduction
	Sulfurospirillum *Halothiobacillus*	Sulphur oxidation
	Methylocaldum *Methylomonas* *Methylogaea*	Oxidation of methane
	Sulfurimonas	Nitrate reduction Oxidation of sulphur and hydrogen
	Acinetobacter	Phosphate solubilization

	Azospirillum	Associative nitrogen fixation
	Pseudomonas	Plant growth promotion Antagonistic activity against plant pathogens Nitrogen fixation
	Psuedolabrys	Unidentified
Bacteriodetes	*Paludibacter*	Production of propionate
	Acetobacteroides	Carbohydrate fermentation and hydrogen production
Chloroflexi	*Anaerolinea* *Ktedonobacter*	Waste water treatment
Acidobacteria	*Geothrix* *Thermoanaerobaculum*	Geo-chemical cycling of metals (Fe, Mn)
Actinobacteria	*Frankia*	N- fixation
	Streptomyces	Antibiotic production
Firmicutes	*Paenibacillus*	Nitrogen fixation Plant growth promotion
	Bacillus	Plant growth promotion Nitrogen fixation Biological control Phosphate, potassium and zinc solubilization
Nitrospirae	*Nitrospira*	Nitrite oxidation (nitrification)
	Thermodesulfovibrio	Sulphate reduction under anaerobic conditions

The archaeal phylum Euryarchaeota was found to be exceptionally high in the sample Pzk unlike Mls and Chr where Crenarchaeota was found to be the predominant archaeal phylum. The highest archaeal density and diversity was recorded by the sample Pzk. The only genus constituted the phylum Thaumarchaeota in all the samples was

Nitrososphaerae with higher population recorded by the sample Pzk. A study by Ke *et al.* (2014) reported that the members of Thaumarchaeota were found to inhibit the growth of methanogens in paddy fields. The presence *Thermococci* was found only in the sample Pzk. The prominent genera of Euryarchaeota, *Methanolinea* was found higher in the sample Pzk while *Methanocella* was found higher in the sample Mls. *Methanocella* was found to be the predominant archaeal genera of sample Chr.

Even though the samples were collected from different locations of Kole lands of Thrissur, all the samples housed almost similar bacterial diversity with slight variations in its count. The diversity of all the three samples where compared by listing the total number of bacterial taxa at each taxonomic level. The comparison of bacterial diversity among the samples revealed that the phylum level diversity was higher in the sample Chr with populations belonging to 21 phyla. The phylum level diversity of bacterial population was found less in the sample Chr which harbors the population of 18 phyla. The prokaryotic diversity from all the samples was analyzed using Shannon, Chao1 and observed species metrices. The Chao1 metric was used to estimate the species richness and Shannon metrics to estimate the observed OTU. The observed species metric provided the count of unique OTUs identified in the sample. As per the results obtained, the species diversity was found to be higher in the sample Chr and the species richness in the sample Pzk. Species diversity is determined by the number of species within a biological community and the relative abundance of individuals in that community (species richness).

From the present study, it is evident that the samples were of low pH and deficient in primary and secondary nutrients except S. Fe content was high in all the three samples. Though within the acidic range, sample Pzk was found to have slightly higher pH. The organic carbon content and the microbial biomass C were found to be notably higher in the sample Pzk. The culturable microflora did not show any notable variations among the samples but the population was slight highest in the sample Pzk. The bacterial diversity analyzed using metagenomics revealed that the Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, Nitrospirae and Bacteroidetes constituted the predominant

bacterial phyla of all the samples obtained from the rice rhizosphere soil of Thrissur Kole lands. A similar study was conducted by Sah *et al*. (2014) to understand the bacterial diversity of acid saline '*Pokkali'* soil using metagenomic approach. The *Pokkali* soil which has high acidity and high organic C content as that of Kole land soil, also revealed the predominance of Proteobacteria, Chloroflexi and Acidobacteria with halophiles, acidophiles, S oxidizers, sulphate reducers, thermophiles, denitrifiers, aerobes, strict anaerobes and mesophiles. Another study by Arjun and Harikrishnan (2011) also suggested that the rice rhizosphere has the dominance of Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes and Planctomycetes. In 2019, Ashwini *et al.* analyzed the long term effect of fertilizer regimes *viz*., organic inputs (ONM), integrated inputs (INM) and inorganic (INF) inputs on bacterial community in paddy soils using NGS sequencing of 16S rRNA and the results showed that the relative abundance of bacterial communities varied across organic and inorganic fertilizer treatments. The dominant phyla found across all the treatments were Actinobacteria followed by Acidobacteria, Proteobacteria and Firmicutes.

The sample Chr also showed a slight higher population of phyla Verrucomicrobia. All the samples showed diverse oligotrophic bacterial population. Archaea represents an important component of the plant microbiome and their impacts on hosts are still unclear (Moissl-Eichinger *et al*., 2018). The archaeal diversity was constituted by populations of three phyla: Euryarchaeota, Crenarchaeota and Thaumarchaeota with slight variations in their count. The phylum Euryarchaeota included methanogens, halobacteria and thermophiles. It is suggested that the phylum Crenarchaeota is ubiquitous to most environments (Barns *et al*., 1996).

The proportion of unclassified taxa was found high in all the samples indicating the presence of a vast undiscovered bacterial diversity. The function of unclassified prokaryotic taxa needs to be revealed to know whether they have any effect on plant health. The role of unclassified taxa can only be understood by whole metagenomic sequencing which aids to identify the functional genes and compare it with suitable

genomic database. Enhancement of native microflora along with nutrient management based on soil analysis can improve the health of soil as well as plants.

This study revealed the community diversity of bacteria and archaebacteria in Kole lands of Thrissur, which is the first report of its kind. The investigation revealed the presence of sulfur oxidizers (*Thioalkalispira, Sulfuricurvum* and *Thioalkalispira*), sulfur reducers (*Desulfobacca*), bacteria that are important in geo-chemical cycling of metals like Fe and Mn (*Geothrix* and *Thermoanaerobaculum*), nitrifying bacteria (*Nitrospira*), N-fixing actinomycete (*Frankia*) and bacteria which could be used in waste water management (*Anaerolinea* and *Ktedonobacter*). Presence of methanotrophic bacteria like *Methylobacter* and *Methylomonas*, methane-oxidizing bacteria (*Methylocaldum*) and moderately halophilic bacteria (*Salinicola*) was also observed.

Nitrogen fixing bacteria like *Bacillus, Paenibacillus, Azospirillum, Frankia* and *Pseudomonas* from the acidic soils of Kole wetlands could be cultured and screened for their efficiency in nitrogen fixation as well as plant growth promotion, and further exploited for biofertilizer production, as nitrogen fixers and PGPR. Generally the biofertilizer microorganisms proliferate well under neutral pH conditions. However, if efficient, indigenous isolates of these bacteria could be obtained from Kole lands, then these could be successfully employed as biofertilizers in this area, as these isolates could be acid tolerant ones. Similarly, many bacteria which have earlier been reported to solubilize phosphate, potassium and zinc (*Arthrobacter, Bacillus, Pseudomonas, Burkholderia, Acinetobacter* and *Paenibacillus*) have also been detected by using metagenomics tools, in the present investigation. Potential isolates from these bacteria could be screened and taken forward for developing acid tolerant biofertilizers or PGPR consortia. Such biofertilizer or PGPR consortia will be a boon to Kole wetland farmers, since no location specific biofertilizers have been identified or commercialized for this unique tract in Kerala.

Bacteria like *Pseudomonas* have earlier been reported to trigger the resistance of plants to biotic and abiotic stress conditions like plant pathogens, moisture

stress etc. The isolates of *Pseudomonas, Bacillus* or any other Genus with direct or indirect benefit to plants in overcoming abiotic or biotic stress conditions could be exploited in future for improving plant health and thereby yield. In a similar manner, potential antagonistic agents could also be identified and utilized for use as biocontrol agents.

All these have individual ecological roles in the ecosystem. A thorough investigation into these functional groups may enable us to carry out rhizosphere engineering with the objective of improving soil and plant health, thereby enhancing the yield of rice crop.

Summary

6. Summary

The study entitled "Metagenomic analysis of bacterial diversity in the rice rhizosphere of Kole lands of Thrissur" was conducted in the Department of Agricultural Microbiology, Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Radio-Tracer Laboratory, College of Agriculture, Vellanikkara during the period 2019-2021. The objective of the study was to analyze the diversity of bacterial communities in the rice rhizosphere of Kole lands of Thrissur using metagenomics. The salient findings of the study are summarized below:

Rhizosphere soil samples were collected from three different locations of Kole lands of Thrissur viz. Puzhakkal (Pzk), Mullassery (Mls) and Cherpu (Chr). The soil samples were collected by uprooting the plant along with the rhizosphere soil at maximum tillering stage (at a depth of around 15-20cm) and used for microbial analysis and metagenomic DNA isolation. Analysis of the samples for physico-chemical parameters revealed that the soils were acidic. An extreme low pH can reduce the availability of major and secondary nutrients in the soil. The sample Pzk showed higher content of organic C which can enhance the soil microbial activity.

Analysis of samples for biological properties revealed that microbial biomass C was comparatively higher in the samples Pzk and Mls. Population of culturable microflora enumerated by serial dilution plate method revealed that microbial population was also comparatively higher in samples Pzk and Mls, corresponding to the microbial biomass C. Twenty four predominant bacterial isolates were purified and subjected to PGP characterization *viz.* phosphate solubilization and production of IAA and ammonia. Ten isolates recorded phosphate solubilization, eight were ammonia producers and 13 were IAA producers. Bacterial isolates with multiple PGP activities are considered to be valuable in future.

As only less than one per cent of the total microorganisms are culturable, the diversity of bacteria was analyzed using a culture-independent metagenomic approach. The metagenomic DNA from the samples was extracted using DNeasy PowerSoil kit, as the direct method employed showed a high humic acid concentration. After checking the quality and quantity, the metagenomic DNA samples were subjected to metagenomic analysis and the V3-V4 region of 16S rDNA was amplified. The amplicons obtained were subjected to PCR-clean up and index PCR followed by library quantification and denaturation. Then the denatured library was exposed to Illumina MiSeq sequencing. The raw reads in fastq format, after demultiplexing was subjected to FastQC program to check the quality of the reads with default parameters like base composition and GC distribution. Filtration processes were carried out to eliminate mismatches, chimeras and singletons to obtain total number of OTUs.

The total number of OTUs was found to be comparatively high in the sample Pzk followed by Mls and Chr. The taxonomic assemblage of the bacterial diversity was analyzed using the tool QIIME. The representative sequences from each clustered OTUs were aligned against SILVA core set of sequences using PyNAST program. *In silico* tools, MEGAN and MG-RAST were used to get the bacterial diversity using NCBI taxonomy and RDP database respectively. Sequences that do not have any alignment against taxonomic database were categorized as Unknown.

The sample Pzk exhibited abundance of phyla Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, Nitrospirae and Bacteroidetes. Proteobacteria are reported to be active part of soil microflora, with the ability to utilize a broad range of organic compounds. The prominent genera included *Desulfobacca*, *Thermoanaerobaculum*, *Thioalkalispira*, *Anaerolinea*, *Gemmatimonas* and *Psuedolabrys*. Dominant archaeal phyla were found to be Euryarchaeota and Crenarchaeota and the prominent genera were found to be *Nitrososphaerae, Methanolinea* and *Methanocella*. An abundance of unclassified bacterial and archaeal genera was also observed in this sample.

The sample Mls showed predominance of phyla Chloroflexi, Acidobacteria, Proteobacteria, Actinobacteria, Bacteroidetes and Nitrospirae with prominent genera *Sulfuricurvum, Anaerolinea, Ktedonobacter, Syntrophobacter, Haliangium, Desulfobacca* and *Thermoanaerobaculum*. Genera like *Bacillus, Paenibacillus, Psuedomonas, Azospirillum* and *Arthrobacter* were also observed. These genera are reported to exhibit PGP activities. Crenarchaeota was found to be the most dominant archaeal phyla with unclassified genera. *Nitrososphaerae* and *Methanocella* were found to be the most abundant archaeal genera in sample Mls.

Phyla Chloroflexi, Proteobacteria, Acidobacteria, Nitrospirae, Bacteroidetes, Verrucomicrobia and Actinobacteria were found abundant in the sample Chr. The major genera were *Ktedonobacter, Anaerolinea, Desulfobacca, Geobacter, Thermoanaerobaculum, Syntrophobacter* and *Syntrophorhabdus.* The archaeal population was dominated by phyla Crenarchaeota and Euryarchaeota. *Methanocella* and *Methanobacterium* constituted the abundant genera of Euryarchaeota.

Comparative analysis among the samples revealed an increased population of archaea and bacteria was observed in the sample Pzk. The phylum level bacterial diversity was found highest in the sample Chr with 21 phyla. Proteobacteria, Chloroflexi and Acidobacteria were the most predominant phyla in all the samples. The abundance of Acidobacteria might be because of the low pH of soil samples. The samples housed huge population of bacteria that are found abundant in oligotrophic environments like ocean water, aquifiers, hot springs, etc.

Euryarchaeota and Crenarchaeota were the dominant phyla in all the samples. The population of Thaumarchaeota, which are ammonia oxidizers, was also remarkable in all the samples. These can reduce the population of methanogens by the oxidation of ammonia.

Microbial diversity analysis using matrices like Shannon, Observed species and Chao1 showed that the species richness was higher in sample Pzk. Species diversity was highest in the sample Mls.

The present study revealed the community diversity of bacteria and archaebacteria in Kole wetlands of Thrissur, which is the first report of its kind. The presence of sulfur oxidizers, sulfur reducers, bacteria that are important in geo-chemical cycling of metals like Fe and Mn, nitrifying bacteria, N-fixers and bacteria which could be used in waste water management could be observed. Methanotrophic bacteria like *Methylobacter* and *Methylomonas* and methane-oxidizing bacteria (*Methylocaldum*) were observed. All these have individual ecological roles in the ecosystem. Biofertilizer organisms like *Azospirillum, Paenibacillus, Cellulosimicrobium* and biocontrol agents like *Bacillus* and *Pseudomonas* could be detected, which could be cultured and used as potential acid tolerant biofertilizers and PGPR.

The proportion of unclassified taxa was high in all the samples, indicating the presence of a vast undiscovered bacterial diversity. The use of whole metagenomic sequencing in functional gene identification provides better understanding about the role of unclassified taxa. The functions of unclassified prokaryotic taxa have to be revealed to know whether they have any influence on plant health. Enhancement of native microflora together with nutrient management practices based on soil analysis can improve the health of soil as well as plants.

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Annexures

Annexure I

Equipment used in the present study

Sterilization of culture media – Equitron SLEFA and NatSteel horizontal autoclave

Incubation of cultures – GeNei OS-250

Centrifugation – Eppendorf 5804R and SPINWIN MC-02

pH of culture media and buffers – Eutech pH Tutor

Visualization of agarose gel

Photomicrography – Leica ICC50

Storage of microbial cultures and metagenomics DNA

Annexure II

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

1. Extraction buffer

A.	100 mM Tris HCL	-	10 ml
	1 M Tris HCL (pH – 8.0)	-	1 ml
	Distilled water	-	100 ml
B.	100 mM EDTA	-	0.372 g
C.	1.5 M NaCl		
	1 M NaCl	-	0.75 ml
	Distilled water	-	100 ml

2. Lysis buffer

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

3. Phenol : Chloroform : Isoamyl alcohol (25:24:1)

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

4. Chloroform : Isoamyl alcohol (24:1)

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

5. Potassium acetate 7.5 M

A.	Potassium acetate	-	20.412 g
B.	Distilled water	-	50 ml

6. Chilled ethanol (70%)

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

7. Sterile distilled water - 20-50 µl

Annexure III

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

1. Extraction buffer
    - A. 200 mM Tris (pH – 8.0) - 0.2 ml
    - B. 25 mM EDTA (pH – 8.0) - 0.5 ml
    - C. 25 mM NaCl - 0.375 ml
    - D. 0.5% SDS - 0.005 ml
2. Phenol : Chloroform : Isoamyl alcohol (25:24:1)

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

3. Ice-cold isopropanol

    Equal volume of isopropanol

4. 70 per cent 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 refrigerator at 4^0C

2. Ethidium bromide (Intercalating agent)

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
0.5 M EDTA (pH – 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored in room temperature.

Annexure V

Media used and composition

a) Actinomycete isolation media

Sodium caseinate	-	2.00 g
L-asparagine	-	0.10 g
Sodium propionate Dipotassium phosphate	-	0.50 g
Magnesium sulphate	-	0.10 g
Ferrous sulphate	-	0.001 g
Agar	-	20.00 g
Distilled water	-	100 ml

b) Ashby's Mannitol agar

Mannitol	-	20.00 g
Dipotassium phosphate	-	0.20 g
Magnesium sulphate	-	0.20 g
Sodium chloride	-	0.20 g
Potassium sulphate	-	0.10 g
Calcium carbonate	-	5.00 g
Agar	-	20.00g
Distilled water	-	1000 ml

c) 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

d) 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

e) Martin's Rose Bengal agar

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

f) Nutrient agar

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

g) 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
$MgSO_4.7H_2O$	-	0.10 g
KCl	-	0.20 g
Yeast extract	-	0.50 g
$MnSO_4.H_2O$	-	0.002 g
$FeSO_4.7H_2O$	-	0.002 g

Distilled water	-	1000 ml
Agar	-	20.00 g
pH	-	7.0

h) Potato Dextrose agar

Potato infusion	-	200.00 g
Dextrose	-	20.00 g
Agar	-	20.00 g
Distilled water	-	1000 ml

Abstract

METAGENOMIC ANALYSIS OF BACTERIAL DIVERSITY IN THE RICE RHIZOSPHERE OF KOLE LANDS OF THRISSUR

by

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ABSTRACT OF THE THESIS

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Abstract

Kole wetlands of Kerala are a complex ecological system and are known for the higher productivity of rice. The Kole lands remain submerged under flood water for about six months in a year and this seasonal alteration gives it both terrestrial and water related properties which determine the ecosystem structure. Though several studies have been conducted for exploring the diversity of fishes, birds, flora, butterflies, etc., in Kole lands, no systematic studies have been made on rhizosphere microbial diversity.

This study was intended to analyze the bacterial diversity in the rice rhizosphere ecosystem of Kole lands of Thrissur. Rhizosphere soil samples were collected from three locations of Kole lands of Thrissur viz. Puzhakkal (Pzk), Mullassery (Mls) and Cherpu (Chr) and analyzed for physico-chemical and biological properties. Culturable microflora was enumerated using serial dilution plate method for bacteria, fungi, actinomycetes, N-fixers, P, K and Zn-solubilizers. Twenty four predominant bacterial isolates were purified and screened for PGP activities including production of IAA and ammonia and phosphate solubilization. The bacterial diversity of the rhizosphere samples was analyzed by metagenomic library construction and sequencing of V3-V4 regions of the 16S rRNA gene, using Next Generation Sequencing (NGS) technology. The sequences thus obtained were analyzed for the Operational Taxonomic Units (OTUs) using MEGAN and MG-RAST server.

The analysis of physico-chemical parameters showed a comparatively low pH in all the samples. An extreme low pH can reduce the availability of major and secondary nutrients in the soil. The sample Pzk showed higher content of organic C. Culturable microflora and microbial biomass C analysis also showed a slight increase in the sample Pzk. The soil organic C content and microbial biomass C are reported to be positively correlated. The microbial biomass C is the measure of the weight of the organisms present.

The predominant bacterial phyla in the rice rhizosphere of Kole lands of Thrissur included Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, Bacteroidetes and Nitrospirae. The bacterial population was found higher in the sample Puzhakkal and comparatively lower in the sample Chr. Phylum Proteobacteria was found to be the most predominant bacterial phylum in Pzk while, Chloroflexi was more predominant in Mls and Chr. The classes Acidobacteria and Ktedonobacteria were found dominant in the samples Mls and Chr and the Pzk sample was dominated by Acidobacteria and Deltaproteobacteria. The phylum level bacterial diversity was found highest in the sample Chr with 21 phyla while the genus level bacterial diversity was highest in the sample Mls. The abundance of genera *Desulfobacca*, *Thermoanaerobaculum, Thioalkalispira, Anaerolinea, Ktedonobacter, Gemmatimonas, Puedolabrys, Sulfuricurvum, Syntrophobacter, Haliangium, Geobacter* and *Syntrophorhabdus* was observed in the Kole land rice rhizosphere samples. Many of these genera are involved in geo-cycling of nutrients like Fe, S and Mn and a few are used in waste water treatment. The species-level bacterial diversity was found to be highest in the sample Mls as indicated by the Chao1 and observed species indices.

The predominant archaeal phyla in the rice rhizosphere of Kole lands of Thrissur included Euryarchaeota, Crenarchaeota and Thaumarchaeota. Archaea are still an under-detected and little-studied part of the soil, so their full influence on global biogeochemical cycles remains largely unexplored. This study has thrown light on the diversity of bacterial and archaebacterial communities in the peculiar ecosystem of Kole lands of Thrissur. Many of the biofertilizer organisms like *Azospirillum, Paenibacillus, Cellulosimicrobium* and biocontrol agents like *Bacillus* and *Pseudomonas* could be detected, which could be cultured and used as potential acid tolerant biofertilizers and PGPR. Many of the ‘Unclassified’ genera could be novel bacteria and more research is needed to identify their taxonomic position and functional role in the ecosystem.