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REVIEW ARTICLE: MICROORGANISMS-DIVERSITY ANALYSIS AND APPROACHES

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ABSTRACT

As microbes are 3.5×10^9 years old dominant species prevailing on this earth in varied places even with temperatures ranging from 10-100°C. Soil life has a continuous interface between minerals and the organic life. The microorganisms regulate the physio-chemical and other biological properties of the ecosystem. The microbial community residing in the soil is, directly and indirectly, involved in various processes like the biogeochemical cycles, symbioses and nutrient recycling. Therefore, to understand the role of these minute creatures in the soil, their microbial diversity plays a critical role. Numerous techniques have been developed to unravel the information about the microbial community. The molecular techniques have made the identification of novel microorganisms convenient and robust. Hence, this review aims to highlight the importance of microbes and microbial diversity and discuss the molecular methods for gaining better insight into the microbes.

Keywords : Microbial Diversity, Mines, Molecular Techniques, Soil

INTRODUCTION

The existence of life on the planet Earth dates back to 4.1 billion years ago with the evidence of prokaryotic life to 3.5 billion years ago (Schopf, 2006). During this vast travel through time life forms have improved. However, from the origin of the first prokaryotic cell to the domination of gigantic reptiles of Jurassic era and the development of ganglion to the network of scintillating neurons in the human mind, one thing is frequent and constant. They are the tiny invisible creatures sharing the journey of time with all the life forms present on the Earth with incredible potential and sustainability- 'Microorganisms'.

Microorganisms are the smallest creatures of the living world. Having a size range of 0.2 µm to 8 µm, they are invisible to our naked eyes except for *Thiomargarita namibiensis* (Breuer, 2001; Schopf, 2006). Their presence was suspected a long time ago and mentioned in various ancient Indian and ancient Greek literature but, the actual knowledge on the microbes was produced only after the discovery of one of the essential instruments in the life sciences, the microscope. Discovery of the microscope in 1674 by an enthusiastic German lens maker '*Antoine van Leeuwenhoek'* took the curiosity about smallest organism to a whole new level and led to the development of a new area of biological studies dedicated to these organism known as 'Microbiology' (Leeuwenhoek, 1701). Since then, in 200 years, much work has been done to know more about the microbes. From the era of knowledge of sterilization given by *Spallanzani* in the 18th century, to the postulation of the Germ theory of disease by *Robert Koch* in 1876 (Fildes, 1951), microbiology has come a long way to provide the knowledge about the importance of these microorganisms and their impact on human life. Work done by inspiring researchers like *Louis Pasteur* on the structure and applications of microorganism could show that these tiny creatures have a high potential to significantly affect the life of multicellular organisms like humans. In this period, which is referred to as the 'Golden Era of Microbiology', researchers found pieces of evidence about various benefits as well as the harmful effects of the microorganisms on other life forms (Robbins, 2001).

Although information about the morphological and physiological details about the microorganisms started gathering and increasing day by day, with the help of new scientific techniques developed in the 20th century, the last century was the highly significant. Because in the last 100 years, we have gathered valuable information about the structure, morphology, physiology, genetics and ecology of microorganisms (Franco-Duarte *et al.,* 2019).

The current review highlights the importance of microbes and microbial diversity and discusses the molecular methods for gaining better insight into the microbes.

Classification of Microbes

The classification of microbes provides us with a way to know the place and distribution of microorganisms in the tree of life. In the starting of last decade of 20th century, a three-domain classification of all the living things has been given by microbiologist *Carl Woese* indicates that a large share of the ecosystem is held by the microorganisms (Woese *et al.,* 1990). Among these three domains, two domains *viz.* Archaea and Bacteria represent the microorganisms on the

planet earth. In this system of classification, the bacteria are separated from their ancestors Archaea which are the microbes living in extreme conditions like hot springs, thermal vents, among others. Archaea are morphologically and genetically different from bacteria, which enables their survival in extreme environmental conditions (Orellana *et al.,* 2018). Archaea are natives to the extreme environments, but they are also found in various other environments such as the oceans, river bands, hills and soil system.

Domain bacteria consists of prokaryotic organisms with a single cell and without any membrane-bound cell organelle. They are diverse in morphology and habitat (Cabeen and Jacobs-Wagner, 2005). The primary shapes of bacteria are spherical (coccus), rod-shaped (bacillus), twisted (spiral) and comma-shaped (vibrio). Various other shapes like diplococci (aggregate of two spherical cells), streptococci (chains of spherical bacteria), staphylococci (aggregate ball-like the structure of cocci), streptobacilli (Chain of rod-shaped bacterial cells) are found in the bacteria based on these four-fundamental shapes (Cabeen and Jacobs-Wagner, 2005).

Bacteria are found in almost every inch of the planet; from the cold Arctic Circle to the desert of Sahara, from the amazon to the profound world of Indian Ocean. The morphology and genetic ability to sustain in almost every environment make the bacteria one of the most diverse population to ever inhabit the planet (Barea *et al.,* 2005). According to an estimation, 50% of the total biomass load of the planet is made up of microbial cells (Kallmeyer *et al.,* 2012). There are a greater number of bacteria (10^{29}) estimated in the ocean than the stars in the Universe (10^{21}). However, the critical point about our knowledge of microbial diversity is that till date we have information about only 1% of microbial species amounting to around 3100 in an estimated number of 300,000 to a million! Only 3100 microbes have been cited and documented in the available literature. Around 99% of the microbial population is still awaiting exploration (Riesenfeld *et al.*, 2004).

The distribution of bacterial species throughout the planet contributes to the microbial diversity. They are found in every ecosystem of the planet with a diverse range of species and properties. This diversity of microbes contributes to the overall biodiversity of the living world and poses a significant impact on the ecosystem and other organism's life. Due to different forms of bacterial cells and physiology, they can metabolize a large number of compounds and act as decomposers in the environment (Barea *et al.,* 2005; Bhatia *et al.,* 2015). Based on the physiology, utilization of carbon and energy source, there are different types of bacterial populations found in the biosphere. Bacterial populations present in the deep oceans and found in the field soil have very different diversity and physiology. Their properties are dependent on the requirement of survival and environmental conditions (Hibbing *et al.,* 2010).

Importance of Microbes

Soil ecosystem is among the most diverse in terms of composition, heterogeneity, texture, nutrients composition, among others. Therefore, it provides a favourable habitat to the microorganisms that are full of nutrients and ambient growth conditions. Soil is present in most terrestrial niches like the grasslands, fields, river bands, dessert, hills, mountains, forest and plateau and is a home to a vast range of microbial diversity (Ettema, 2000). The bacterial population is distributed in the soil according to the composition and texture of the soil. Although bacteria have been reported in almost every type of soil composition, but there are several diverse microbial populations, which are limited to specific areas. This happens due to the natural ability of specific microbes to uptake the nutrients and regulate physiological processes while ensuring their own survival (Kallmeyer *et al.,* 2012).

Microbial metabolism has critical importance in the recycling of nutrients and various elemental cycling. Methanogens, methane oxidisers and autotrophs like cyanobacteria help in the carbon cycle and carbon dioxide fixation. On the other hand, bacteria such as *Nitrosomonas, Nitrobacter* and *Nitrospira* play a role in the nitrogen cycle and helps in the atmospheric nitrogen fixation (Ettema, 2000), nitrification, denitrification, and decomposition. The bacterial population found in the mineral-rich areas can metabolize mineral-rich compounds and high survival capacity despite scarce organic nutrients. They are nature's bioremediation agents that help in the elimination of heavy metals and other compounds from the soil and water systems (Joutey *et al.,* 2013).

Apart from these, having a tremendous microbial diversity is helpful in various other areas of human life such as healthcare, medicine, pharmaceuticals, food and beverage industries and chemical industries. Different types of microbes are the sources of enzymes and life-saving antibiotics. However, in the modern era when we are surrounded with problems like antibiotic resistance, global warming, water pollution, air pollution (Ogunseitan, 2007), shortage of effective drugs. It is therefore imperative that we care about the unculturable (99%) population of microorganisms which have not been explored yet (Riesenfeld *et al.*, 2004). Thus, the analysis of the microbial diversity is essential in this scenario. Due to its necessity in this era, many pieces of research work have been reported and ongoing on the exploration of microbial diversity all around the world (Anand *et al.,* 2019).

Therefore, the exploration and characterization of microbial diversity using molecular techniques is an essential area of research in today's scientific era. It has the potential to solve significant problems faced by humanity in recent decades (Dhanjal and Sharma, 2018).

Importance of Microbial Diversity

Habitat is one of the most critical requirements to nurture any life form, and microorganisms are no exceptions to that. However, microbes have a unique ability to survive in extreme conditions which makes them proliferate in almost every area of the planet (Dhanjal *et al.,* 2017). Unaffected by the sub-zero temperature conditions of the Arctic and the scorching environment of Sahara Desert, their habitat ranges from mountains to deep oceans and from rainforests to hyperthermal geyser vents. This contributes to the formation of one of the most diverse life forms existed till date (Xu, 2006). Microbial diversity was explained as the scattering of genetic information within the microbial species and diversity of species like this among the microbial community**.** Estimations suggest that 50% of all biomass of the planet are microorganisms (Nannipieri *et al.,* 2008).

Microbial diversity is not only crucial for the survival and sustainability of microbes themselves, but also other life

forms surrounded by them. This diversity contributes to helping in the execution of an enormous number of processes required for bio-geochemical operations and maintenance of the ecosystem (Sarangi *et al.,* 2019). Microbes play an essential role in the recycling of nutrients, decomposing waste, obliteration of pollutants and bioremediation of harmful substance present in the environment. Apart from this, they are a potent source of various secondary metabolites and compounds, including life-saving antibiotics and pharmaceutical principles (Alex *et al.,* 2019; Kokaz *et al*., 2019; Sallawad *et al.,* 2017; Sarbeen and Gheena, 2016). These contributions of microbes help us to achieve goals for better personal health care and environmental improvement (Kalia and Purohit, 2008). On the industrial front also, the diversity among these tiny creatures helps us to produce various essential enzymes and chemicals.

Studies also suggest that on the microbial ecology front, this diversity of microbes helps them to enhance the interaction among them and the environment for up-keeping the diversity itself (Konstantinidis and Tiedje, 2005).

In the recent years, the studies in the area of microbiology were focused on the analysis of the microbial diversity to check the variability among microbial life forms for a better understanding of the intra-species and inter-species interactions and the same with the environmental factors and conditions. This has led to the production of such diverse speciation and identification of new strains for the betterment of humanity (Anand *et al.,* 2019; Dhanjal *et al.,* 2017; Dhanjal and Sharma, 2018).

Importance of Studying the Coal and Mineral Rich Areas

This vast biodiversity in the region includes the potential to harbour a rich microbial diversity also. Various ecosystems present in the ecoregion display the ability to nurture predominant as well as unique microflora inside them. The mineral-rich soil of the region is a suitable place for the dynamic living system of soil, due to presence of essential organic nutrients as well as inorganic minerals like Phosphorous, Potassium, Nitrogen, Sodium, Calcium, Magnesium, among others. The proliferation of various microbial strains becomes unique and exciting from the exploration point of view. Studies have been conducted to explore microbial diversity previously in mineral-rich mining regions worldwide (Polasky *et al*., 2004). In India, many studies have been done on the mining soil to see the effects of mining on biodiversity, assessing the plant diversity of the mining region.

Impact of mining on the environment and mine soil has been studied, and the critical finding indicating the deteriorating effect of mining on the environment has been identified. (Studies have been conducted on Southeast-Asian biodiversity, and the impact of various man-made factors, including mining-related activity on the biodiversity has been reported (Sodhi *et al.,* 2004). Some studies reported about the disturbance in the properties of soil due to mining like activities can lead to the disintegration of soil and contamination of water, which creates a tremendous negative impact on the biodiversity of the region (Bell and Donnelly, 2006). If we concentrate on studies based on Indian mining area, we found considerable reports about the effect of mining on biodiversity (Gupta and Paul, 2015). Scientists focus on the physicochemical properties of the mining soil and assessment of deteriorating effects of various activities on biodiversity around Indian mines.

Exploration of the microbial diversity in the mines is gradually becoming an exciting area of research nowadays because the microbial diversity not only contributes to themselves but also give a significant impact on the biodiversity around them (Gupta and Paul, 2015). The adverse conditions that appear due to mining can affect the microbial diversity both positively and negatively. Microbes are adversely affected by their changing surroundings, but they can also adapt well to the changes. Example: antibiotic-resistant microorganisms, spore-formers, among others.

Bacterial community structure and diversity may alter during a decade, and change in the microbial population has been observed in a study of coal mines in China (Li *et al.,* 2014). Studies of Coal mine drainage in Hunan province showed the importance of minerals, especially iron, for the microbial diversity. Minerals only limited to survival but also play an essential role in the selection of bacterial species by the environment for bioremediation of certain metals present in the soil. Studies of microbial diversity from Raniganj coalfields, West Bengal, reported the diverse range of gram-negative and gram-positive bacterial populations present in the soil of coal fields according to the distribution of nutrients and minerals. Apart from these, studies were also done on the symbiotic associations of microorganism with plants in the coalfield areas for re-vegetation of the mining-affected area (Li *et al.,* 2014).

Analysis of the previously conducted studies outlines the importance of exploration of microbial diversity in the coal and mineral mining area and the assessment of bacterial species native to them. However, most studies solely depended on the culture-dependent approach for the analysis of microbial diversity from the region. The culturable approach is crucial in the determination of the physio-chemical effect of minerals and elements present in the soil on the microbes as well as the effect of mining on the microbial diversity. However, from these approaches, the study became limited to the culturable microbial populations only. We must expand our area of study enough to know the microbial diversity of non-culturable microbes present in the soil (Cabeen and Jacobs-Wagner, 2005). As a result, we will be able to make a better assessment of microbial diversity, as well as identification of new or modified strains, could be possible.

Microbes are the primary source of various essential products. From industrially relevant chemicals to life-saving antibiotics, microbes are the producers of several secondary metabolites. However, nowadays, the efficiency of microbes for production and the quality associated with effectiveness for the antibiotic drugs are under threat. Antibiotic resistance, shortage of fuel and environmental pollution are some of the major challenges in today's world. To counter these, we must look for the unknown unculturable microbial population which may possess the potential to produce new metabolites and antibiotics. So, for a better and broader analysis of microbial diversity, the combination of culture-dependent as well as culture-independent approaches can be utilized.

Molecular Approaches for the Analysis of Microbial Diversity

There are several techniques involved in the exploration of microbial diversity. However, the molecular techniques for characterization of microbial diversity are useful and accurate methods for the determination of microbial diversity (Kirk *et al.,* 2004)**.** Analysis of the genome and conserved sequences like ribosomal RNA are among those modern techniques, which are in practice nowadays for the analysis and characterization of microbial diversity. These techniques not only provide us information about the microbial population and their distribution but also equip us to identify novel bacterial strains, which may have the ability to produce novel secondary metabolites useful in various aspects of human life (Kirk *et al.,* 2004).

In the modern times, various methods are being used for the analysis of microbial diversity. However, the use of molecular techniques and methods in combination with traditional methods provide us new insights about the microbial diversity.

Although the practice of using molecular techniques started in the mid-80s, their frequent use started only two decades ago. Studies have been done for the development of new techniques for the analysis of microbial diversity consisting of genomic characterization (Karaky *et al*., 2014). Analysis of diversity through molecular methods provide us with a way to know more about previously known but less understood microbial strains (Head *et al*., 1998).

The use of molecular methods and their approaches may vary with the individual requirement, ease of use, reproducibility, application and objectives of the study (Head *et al*., 1998). However, some of the studies show biases toward the predominant and abundant microbial strains present in the soil during the sampling of soil.

The identification of bacterial species from environmental samples like soil, water, and the ocean is a time-taking and fastidious task to perform. It requires much effort to identify and purify bacterial strains from the sample with 100% accuracy. Identification of the novel isolates through traditional methods like dichotomous key does not always give assurance to the identification and description of newly identified bacterial genera. Here genomics plays a crucial role. Genomic identification of any species is a method of full reliability (due to accuracy and specificity) and an approach with wider approachability, sophistication and application (Head *et al*., 1998; Karaky *et al*., 2014). The molecular techniques utilized for diversity analysis are summarized in figure 1.

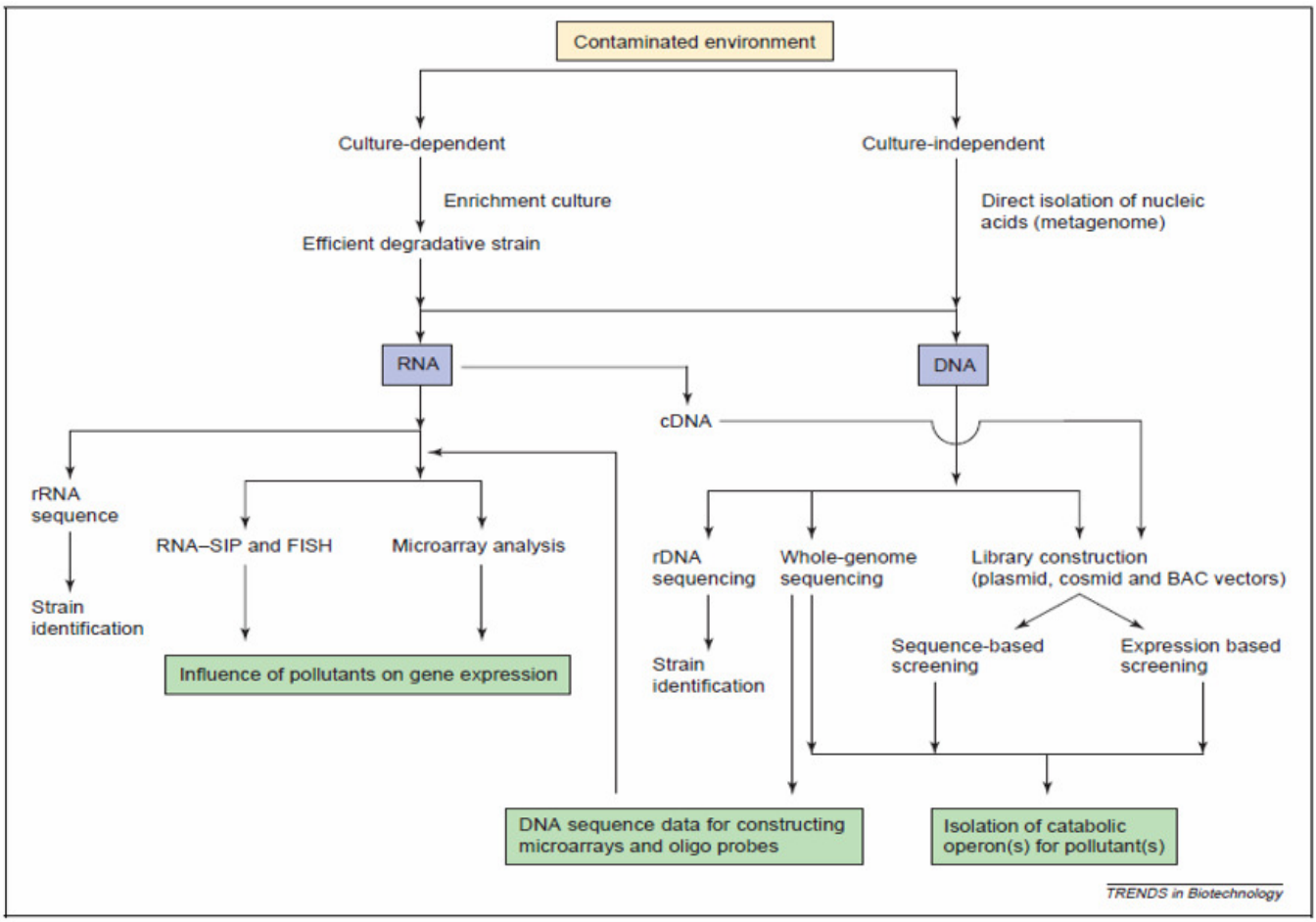

Fig. 1: Modern Molecular Techniques used for the analysis of microbial biodiversity (Paul *et al.,* 2005).

Molecular techniques have been developed and widely used in the characterization of functional diversity in a various different environment (Paul *et al.,* 2005).

Ribosomal RNA (rRNA) analysis is one of the most widely used and accurate techniques for the analysis of microbial diversity and identification of bacterial species. Ribosomes are made up of RNA and proteins. 16S rRNA found in prokaryotes is used as a molecular marker. It has

been observed that these small transcripts are among most conserved biomolecules present on the planet. They are universally found in all bacterial species. This uniqueness makes them the primary target molecule for analysis of the microbial diversity (Head *et al*., 1998). Study of the rRNA gene (ribosomal DNA; rDNA) and its sequence in different bacterial species is utilized for the assessment of microbial diversity from various places. Using molecular techniques like PCR, Genome sequencing, Transcriptome sequencing, ARDRA, RFLP, AFLP, RAPD, the characterization of bacterial diversity and species distribution has been done many time (Aquilanti *et al.,* 2004; Chandna *et al*., 2013).

DNA Isolation – Isolation of the nucleic acid is the primary step for any molecular analysis method. For any successful and reproducible molecular analysis, the nucleic acid must be isolated using the standard protocol and must be having of good quality. Isolation of the DNA from a soil sample is always a tedious task. The chances of contamination of isolated DNA is high in the process due to presence of lots of contaminants like the humic acids, RNA, proteins and other compounds due to presence of lots of complex components in the soil system (Miao *et al.,* 2014). A suitable and effective method of DNA isolation from soil has been reported in various studies; these studies suggest the ways to isolate DNA from the humus-rich soil with less or no contamination (Williams *et al.,* 1995). Isolation of DNA from different soil samples has also been reported, and the findings indicate that different protocols and strategies were used to isolated pure DNA from the soil samples without contamination. One can see the modification and adjustment done for isolation according to the soil sample and contents (Zhou *et al*., 1996). Studies not only isolated the DNA from soil-enriched culture media but also from the soil itself.

PCR- the polymerase chain reaction is one of the most essential and powerful tools of genomics in today's era. Since the development of a novel multiplication technique of DNA using two short oligonucleotide primers by Kary Mullis in 1980s, PCR is used for the amplification of DNA in molecular biology (Mullis., 1994)**.** With the help of thermo-stable *Taq* DNA polymerase nucleotide base pairs have been added to the growing chain of nucleotide and copy of the gene is generated. After each cycle of amplification, the synthesized new copy again denatured to separate, and the new cycle begins.

Various studies have been conducted on microbial diversity analysis using PCR as a valuable tool for the amplification of the desired gene. Apart from the conventional PCR technique and its various forms like RT-PCR, Reverse Transcriptase PCR, Nested PCR, new techniques like Parallel PCR are emerging at the horizon (Bhardwaj and Sharma, 2014), which may be useful in the area of molecular biology for amplification of the desired gene.

ARDRA- Amplified Ribosomal DNA Restriction Analysis is a new technique developed in 1993 by Vaneechoutte *et al.* ARDRA is extended version of RFLP related to the 16S Ribosomal RNA amplification and then restriction digestion of the product (Vaneechoutte *et al.,* 1992). In ARDRA the 16S rRNA gene of bacterial species is amplified using specific or universal primers. After amplification, the amplicons are subjected to restriction digestion with a single or multiple restriction enzymes. The patterns obtained after digestion is further analyzed for the phylogeny of the species (Vaneechoutte and Heyndrickx, 2001). ARDRA has been widely used in the studies for the determination of bacterial species present in the sample (Vaneechoutte *et al.,* 1995). Identification and analysis of isolates from the different environmental samples have indicated the potential of ARDRA in the identification of bacterial species (Ventura *et al.,* 2001). Characterization of nitrogen-fixing bacteria from various soil isolates led to the identification of bacterial species with the analysis of different strains of that family of nitrogen-fixing bacteria using ARDRA proved its accuracy and range**.** In some studies, ARDRA was used as a rapid identification technique for the bacterial species with a high success rate (Vaneechoutte *et al.,* 1992). Studies have reported the ability of ARDRA in the differentiation of bacterial load and the presence of bacterial species in samples from activated sludge treatment.

RAPD- Random Amplified Polymorphic DNA is another technique for the amplification of the DNA, but in this method, a random segment of the DNA is amplified. Arbitrary random primers are used for the amplification of large DNA fragment (Williams *et al.,* 1995). RAPD has been used many times for the assessment of microbial diversity and determination of bacterial species. The accuracy and success rate of this method is higher than the conventional methods of microbial diversity analysis (Vaneechoutte *et al.,* 1995; Koeleman *et al.,* 1998; Vaneechoutte and Heyndrickx, 2001; Sathish and Sundareswaran, 2010). Studies have been used a combination of these methods for detection of microbial species and diversity analysis of microbes from various sampling area (Koeleman *et al.,* 1998). Comparative studies have been conducted using these methods to determine the potential and efficiency of these methods in microbial diversity analysis.

CONCLUSION

Analysis of the microbial diversity can be a robust solution for the various problems related to health and environmental care. Exploration of various strains from a new and unexplored areas can lead to the discovery of new species or related species that can produce new secondary metabolites or compounds having antimicrobial potential. Diversity in the composition of the soil, as well as presence of diverse ecosystems, makes a place to nurture microbial population efficiently. Molecular methods of analysis have emerged as better and efficient ways to analyze microbial diversity than other methods. The uncultivable microorganisms form a new target group for microbial diversity analysis, and with the help of molecular techniques, we can achieve better results for this target group. Due to its abundance and conserved nature, 16S rRNA gene gives the researcher an accurate perspective with a broader range of approachability about the microbial diversity exploration. Isolation and amplification of 16S rRNA genes and sequencing of the amplicons give us the data about the microbial population present in the sample regardless of their cultivable or uncultivable nature. So, the molecular analysis method of microbial diversity assessment can provide reproducible result in the sampling area.

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