Diversity analysis of bacterial community in acid saline '*Pokkali*' soil of Kerala through metagenomic approach

Sarveshwar Sah[1], D. Girija[1]*, K. Deepa[1], P. A. Nazeem[2], P. M. Firoz[1] and E. Sunil[1]

[1]*Department of Agricultural Microbiology, College of Horticulture, Kerala Agricultural University, Thrissur, Kerala-680 656.*

[2]*Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Thrissur, Kerala-680656*

Received 29 August 2014; received in revised form 28 October 2014; accepted 19 November 2014.

Abstract

Diversity of bacterial community in acid saline *Pokkali* soil from Vyttila, Kerala was analyzed through metagenomic approach. Environmental DNA was isolated from soil by direct extraction. The gene encoding 16S rRNA component of 30S subunit of prokaryotic ribosome was used as barcode for bacterial identification. 1500bp fragment of 16S rDNA was amplified by polymerase chain reaction. Amplicons were ligated in plasmid vector pGEMT and cloned in *E. coli*. Sequence analysis of 30 randomly picked clones from the metagenomic library revealed that 23 clones (76.7%) were of unculturable bacteria. The most predominant phylum was Proteobacteria, which included Ectothiorhodospiraceae, *Azospira* sp., *Stenotrophomonas maltophilia, Thiobacillus prosperus, Levilinea saccharolytica*, Desulfobacteraceae, *Thioalkalivibrio* sp. and Rhodocylales. Other phyla included Chloroflexi, Acidobacteria and Cyanobacteria. A diverse group of bacteria including acidophiles, halophiles, denitrifiers, S oxidizers, sulphate reducers, aerobes, strict anaerobes, thermophiles, mesophiles and photosynthetic bacteria were detected. Six clones of the unculturable bacteria shared homology with no sequence in NCBI database, indicating that these could be novel. Phylogenetic tree based on partial 16S rRNA gene placed 30 clones in three major clusters- Proteobacteria, Chloroflexi and Acidobacteria. Metagenomic approach successfully revealed the composition and diversity of bacterial community in *Pokkali* soil.

Keywords Metagenomics; 16S rDNA; Unculturable, *Pokkali*

Introduction

There is a constant demand for novel genes, enzymes and compounds in biotechnology. Studies have demonstrated that natural diversity is the best source for these novel molecules. The global microbial diversity presents an enormous, largely untapped genetic and biological pool that could be exploited for the recovery of novel genes, biomolecules for metabolic pathways and various valuable products (Cowan, 2000).

Traditional methods of culturing microorganisms limit analysis to those that grow under laboratory conditions (Rondon, et al., 2000). However, it is widely accepted that up to 99 per cent of the microbes in the environment cannot be readily cultivated (Hanada, 2003; Rappe and Giovannoni, 2003; Kamagata and Tamaki, 2005; Sekiguchi, 2006). Thus, most microbes have not been described and assessed for biotechnological or industrial uses. To overcome the limitations associated with cultivation techniques, different DNA-based molecular methods have been developed for characterizing microbial species and assemblages and these have significantly influenced our understanding of microbial diversity and ecology. In general, methods based on 16S rRNA gene analysis provide extensive information about the taxa and species present in an environment. The ability to recover and analyze 16S rRNA genes directly from environmental DNA, otherwise called

*Author for correspondences: Tel: +91-487 2438674; E-mail: devakigirija@gmail.com.

metagenomic DNA, provides a means to investigate microbial populations without the need to culture them (Ward et al*,* 1990; Amann et al., 1995; Dojka et al., 2000; Ghazanfar and Azim 2009). The 16S rRNA gene analysis has been used to study diverse bacterial and archaeal communities in extreme environments with a wide range of salinity, pH and temperature.

Metagenomics is an emerging field in which the power of genomic analysis (the analysis of the entire DNA in an organism) is applied to entire communities of microbes, bypassing the need to isolate and culture individual microbial species. Metagenomics is a rapidly growing field of research that aims at studying uncultured organisms to understand the true diversity of microbes, their functions, cooperation and evolution, in environments such as soil, water, ancient remains of animals, or the digestive system of animals and humans (Ghazanfar and Azim, 2009; Girija et al., 2013).

Pokkali lands, known after the renowned salt tolerant land race of rice, are acclaimed for the unique way of reclamation and management of soil salinity and also for the integrated farming system involving rice - fish/prawn. It includes coastal paddy fields of Ernakulum, Thrissur, Alleppey and Kottayam districts of Kerala state. These areas are confluent with the Vembanad Lake through canals and subjected to tidal influence. *Pokkali* soils are tidal wet lands of Kerala, characterized by the accumulation of salts by tidal action over an underlying acidic soil. The tides that occur twice a day play an important role in the fertility and productivity of this agro ecosystem, as it contains high concentration of Na^+, K^+, Ca^{2+}, and Mg^+ that are important for the physiological processes in plant cells (Kramer, 1984).

Based on the above information, the present study entitled "Metagenomic approach to assess diversity of bacterial community in saline *Pokkali* habitats of Kerala" was taken up with the objective to analyze the bacterial community, in the acid saline *Pokkali* soils of Kerala through a culture–independent 16S rDNA sequencing approach.

Materials and methods

Extraction of environmental DNA from 'Pokkali' soil

Direct method of DNA extraction by soft lysis method (Siddhapura et al., 2010) was employed for obtaining environmental DNA from acidic saline soil representing *Pokkali* fields.

Amplification and cloning of 16S rRNA gene

The total DNA extracted was used for 16S rRNA gene amplification in an Eppendorf Master Cycler, Gradient (Eppendorf, Germany). 25µl PCR reaction mix contained 25 ng template DNA, 1× reaction buffer, 10 picomoles of primers, 0.4 mM dNTPs and 0.6 U of Taq DNA polymerase (Genei, Bangalore). The primers for 16S rDNA amplification were Forward (5 -AGA GTT TGA TCC TGG CTC AG- 3) and Reverse (5 -ACG GCT ACC TTG TTA CGA CTT- 3), as per Siddhapura et al. (2010). Amplification conditions consisted of 94°C for 90s, 55°C for 40s and 72°C for 90s. PCR was carried out for 35 cycles, in addition to an initial denaturation of 95°C for 3min and a final extension at 72°C for 10min. PCR product was loaded on one per cent (w/v) agarose gel and the desired band eluted by using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences Pvt. Ltd). The cloning and transformation of eluted specific 16S rDNA fragment was carried out with the pGEMT vector system supplied by Promega, USA. The ligated PCR product was added to 100µl of thawed competent cells (*E.coli* JM109) and the recombinant clones were identified by blue-white selection on X-Gal/ IPTG ampicillin (100µg/ml) Luria-Bertani agar. Thirty white recombinant colonies were selected at random and the presence of insert in the plasmid was confirmed by performing colony PCR with T_7 and SP_6 primers contained in pGEMT vector.

DNA sequencing and phylogenetic analysis

Thirty clones carrying 1.5 kb insert were sequenced using automated ABI 3100 Genetic Analyser at

Bangalore GeNei with universal primers T_7 and SP_6. Similar sequences in NCBI database were detected to study homology with different bacterial species using Blastn search. Identification to the species level was defined as a 16S rDNA sequence similarity of ≥ 97% with that of the prototype strain sequence in GenBank and identification at the genus level was defined as a 16S rDNA sequence similarity of ≥ 95% with that of the prototype strain sequence in GenBank. Sequence data were aligned with the ClustalW(version 1.8) package. Phylogenetic tree was constructed using the neighbour-joining method using Mega software.

Nucleotide sequence accession numbers

Thirty retrieved 16SrDNA sequences were deposited in Genbank.

Results and discussion

The main objective of the study was to analyze the bacterial community, both culturable and non-culturable, in the saline *Pokkali* soils of Kerala through 16S rDNA sequencing approach. The soil was characterized by a low pH of 3.79 and an EC of 4.03 dSm^{-1}. The soft lysis method of metagenomic DNA extraction from soil yielded good quality DNA, as assessed by agarose gel electrophoresis and spectrophotometry. 403 ng μl^{-1} DNA was obtained from one g soil. A single band of 1.5 kb was obtained on PCR amplification with 16S rDNA specific primers. Of the 30 clones sequenced, 23 showed homology with uncultured bacteria and seven with culturable bacteria (Fig. 1). Of the unculturable ones, six sequences did not show homology to any of the accessions in the NCBI database, indicating that these could be novel isolates. Diverse group of bacteria including Proteobacteria, Chloroflexi, Acidobacteria, Cyanobacteria and unidentified bacteria were detected. Of the seventeen unculturable clones, Acidobacteria was most predominant with five clones (Fig. 2). Four clones showed homology with uncultured Chloroflexi and three with uncultured Desulfobacteraceae. Two clones were grouped under uncultured Ectothiorhodospiraceae and one under uncultured *Acaryochloris*, one uncultured Rhodocyclales and one uncultured γ-Proteobacteria. None of the unculturable bacteria could be identified up to the species level. Only one clone was classified up to Genus (*Acaryochloris*) and five clones up to Family (Ectothiorhodospiraceae-two and Desulfobacteraceae-three). One clone could be identified up to Order (Rhodocyclales) and ten clones up to Class (γ-Proteobacteria-one, Acidobacteria-five and Chloroflexi-four).

Among the seven clones of culturable bacteria, four were identified up to species level (*Stenotrophomonas maltophilia*, *Thiobacillus prosperus* and *Levilinea saccharolytica*-two) and three up to Genus (*Azospira*--two and

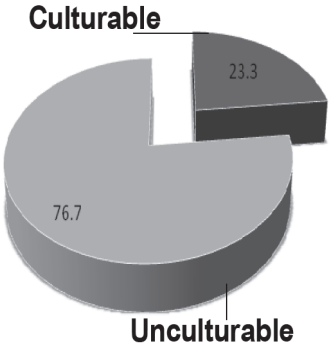

Figure 1. Per cent distribution of culturable and unculturable bacteria in *Pokkali* microbiota

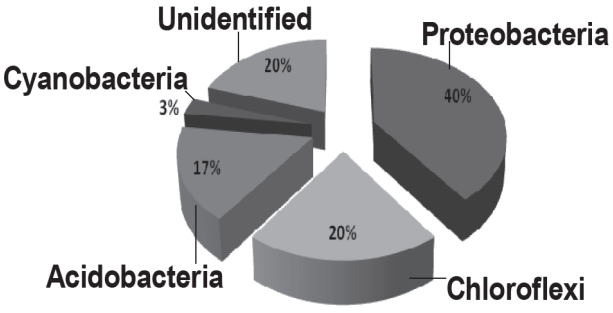

Figure 2. Distribution of bacterial phyla in *Pokkali* microbiota

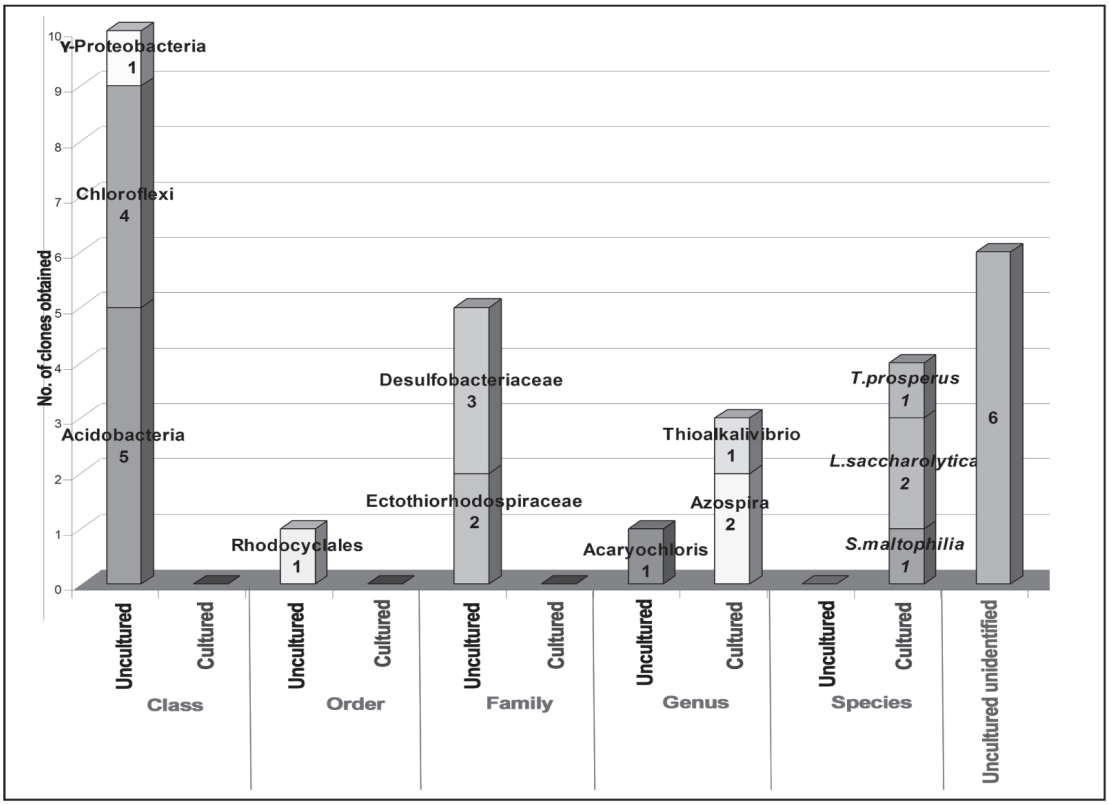

Figure 3. Distribution of taxonomic units in Pokkali microbiota

Thioalkylivibrio-one) (Fig.3). Brief description of the different groups of bacteria identified in the present study is given below:

Proteobacteria group comprised the largest number of bacteria identified from the *Pokkali* microbiota, in the present investigation (Fig. 2). Of the 30 clones sequenced, twelve were grouped under Phylum Proteobacteria (seven unculturables and five culturables). These were further grouped as γ-Proteobacteria-six; δ -Proteobacteria-three and β-Proteobacteria-three.

The non-culturable γ-proteobacteria included two clones of Ectothiorhodospiraceae, which are purple sulphur bacteria. These photoautotrophic bacteria use reduced sulphur compounds as electron donors for anoxygenic photosynthesis and deposit S globules outside the cells (Frigaard and Dahl, 2009).

The three culturable bacteria under γ-Proteobacteria included *Stenotrophomonas maltophilia*, *Thiobacillus prosperus* and *Thioalkalivibrio* sp. *Stenotrophomonas maltophilia* is a ubiquitous, aerobic, non-fermentative, gram-negative bacillus that is closely related to the *Pseudomonas* species which is found in various aquatic environments. It is an uncommon pathogen in humans which causes ailments like respiratory infections, urinary disease, and wound infections. It was first isolated in 1943 and, at that time, was named *Bacterium bookeri*. It was later classified within the genus *Pseudomonas*, then *Xanthomonas*, and then finally *Stenotrophomonas* in 1993 (Falagas et al., 2009). *S. maltophilia* is the only species of *Stenotrophomonas* known to infect humans, whereas its closest genetic relatives are plant pathogens. It is frequently isolated from soil, water, animals, plant matter, and hospital equipment

(Looney et al., 2009).

Thiobacillus prosperus, an obligate chemolithoautotroph is Fe oxidising, acid and salt tolerant bacteria. A metal-mobilizing bacterium similar to *T. ferrooxidans,* it grows aerobically on pyrite, sphalerite, chalcopyrite, and galena as well as on H_2S. It shows vigorous extraction of metal ions from sulfidic ores and is resistant to cobalt. Due to its high tolerance to salt, *T. prosperus* is suitable for industrial leaching in salt containing environments.

The third clone was identified as *Thioalkalivibrio* sp. It is reported to be extremely salt tolerant, chemolithoautotrophic, sulphur oxidizer which is closely related to *Thioalkalivibro denitrificans.* Isolated as a dominant sulfide-oxidizing species

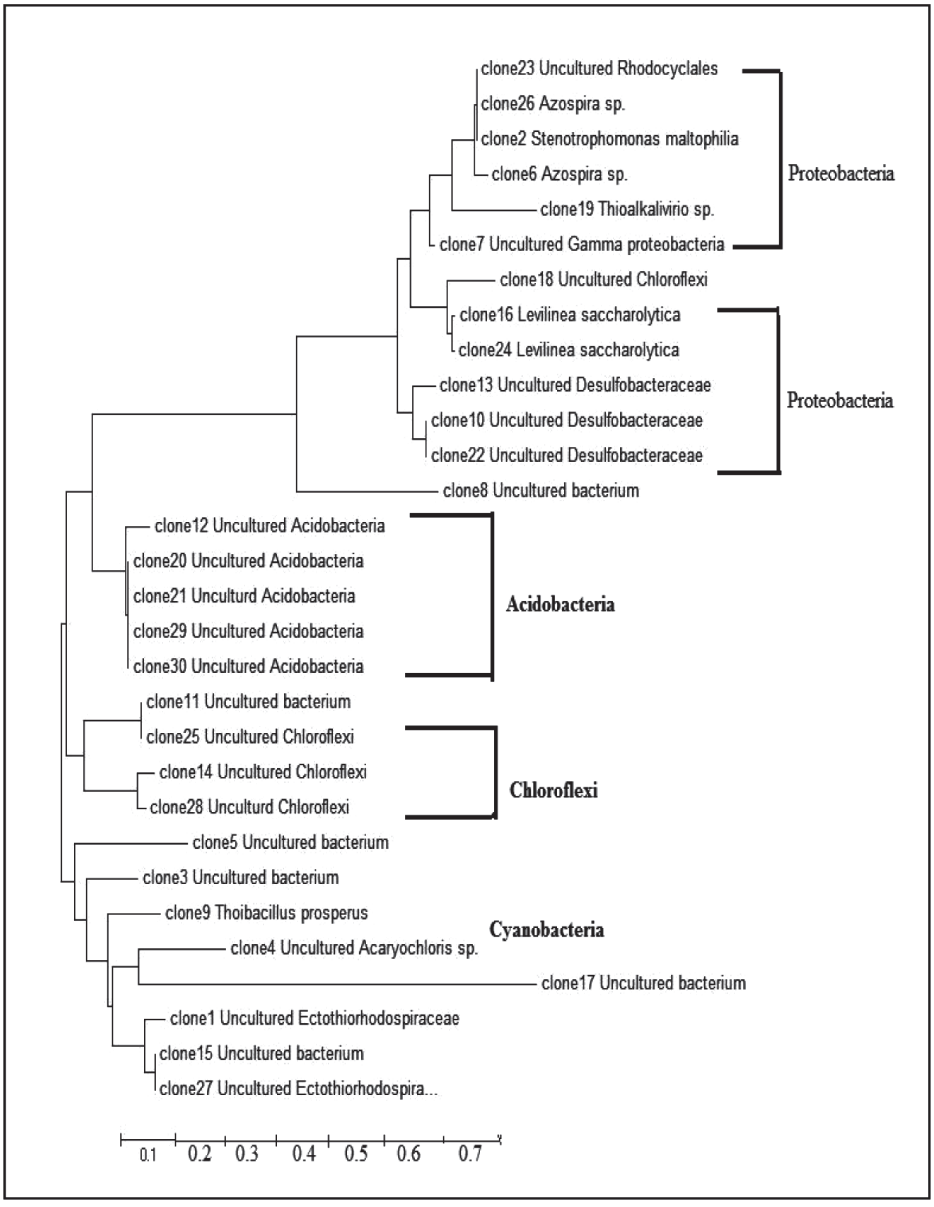

Figure 4. Distribution of taxonomic units in Pokkali microbiota

from a full-scale bioreactor, it was capable of growth up to 4M of sodium and 3.8M of potassium (Sorokin and Kuenen, 2005).

Three cloned sequences classified as β-Proteobacteria included one uncultured Rhodocyclales bacterium. It is an aerobic denitrifying bacteria found in oligotrophic conditions like aqueous environments. Two clones were identified as *Azospira* sp., a nitrogen fixing bacterium found in deep waters. The genus *Azospira* includes a single validly published species, *Azospira oryzae*, strains of which have been isolated from surface-sterilized roots of Gramineae or resting stages (*Sclerotia*) of a basidiomycete (Reinhold et al., 1986). The Genus *Azospira* is also a member of Rhodocyclales and belongs to Family *Rhodocyclaceae*. This family of Gram-negative bacteria includes many genera previously assigned to the family *Pseudomonadaceae*. The family contains mainly aerobic or denitrifying rod-shaped bacteria, which exhibit very versatile metabolic capabilities. Many occur in waste water and play an important role in biological remediation in waste water treatment.

Three cloned sequences shared homology with uncultured Desulfobacteraceae (δ-Proteobacteria) which are sulphate reducing bacteria (reduce sulphate to sulphide). They are strictly anaerobic chemoorganotrophs, chemolithoheterotrophs, or chemolithoautotrophs with respiratory metabolism. Growth occurs only in the presence of sulphate, sulphite, or thiosulfate as electron acceptors. Elemental sulphur inhibits its growth. According to Bak and Pfennig (1987), *Desulfobacter* sp. is most common in anoxic brackish or marine sediments and it is not surprising that it is present in acid saline *Pokkali* soil.

Chloroflexi was the second largest group of bacteria present in *Pokkali* soil which includes four uncultured and two culturable bacteria - aerobic thermophilic anoxygenic phototrophs, previously described by Sekiguchi (2003). The culturable bacteria (Clones 16 and 24) under the Class Chloroflexi included *Levilinea saccharolytica*- a filamentous anaerobic, non- photosynthetic bacterium.

Acidobacteria was the third major group of bacteria present in *Pokkali* soil which includes five cloned sequences, among the 30 clones under investigation. Acidobacteria has a widespread occurrence in a variety of ecosystems and it represents the second most abundant phylum after the Proteobacteria in several soils (Barns et al., 1999 and Janssen, 2006). Previous reports reveal that genomic and culture traits of Acidobacteria are indicative of use of carbon sources that span simple sugars to more complex substrates such as hemicellulose, cellulose, and chitin. The genomes encode low-specificity major facilitator superfamily transporters and high-affinity ATP-binding cassette transporters (ABC-transporter) for sugars, suggesting that they are best suited to low-nutrient conditions.

One cloned sequence has shown maximum homology with uncultured *Acaryochloris* sp., a unique cyanobacterium that is able to produce chlorophyll *d* as its primary photosynthetic pigment and thus efficiently use far-red light for photosynthesis. *Acaryochloris* species have been isolated from marine environments in association with other oxygenic phototrophs, which may have driven the niche-filling introduction of chlorophyll *d* (Miyashita et al., 1996).

Six of the cloned sequences (clones 3, 5, 8, 11, 15 and 17) shared homology with uncultured bacteria, indicating that these bacteria were unculturable and unidentified. These could be novel bacteria since they shared no homology with any of the sequences in the database. However, in the phylogenetic tree these clones were clustered as follows: Clone 8: Proteobacteria, Clone 11: Chloroflexi, Clones 5 and 3: *Thiobacillus prosperus*, Clone 17: Acaryochloris-1 and Clone 15: Echothiorhorospiraceae. Further investigations are required to establish the identity of these clones.

The phylogenetic tree based on partial 16S rRNA gene placed 30 clones from *Pokkali* soil sample in four major clusters:Proteobacteria, Acidobacteria, Chloroflexi and Cyanobateria (Fig. 4). Bacterial population changes in response to the changing environment in a coastal system, such as dissolved organic carbon (Takenak et al., 2007). This is the first report on the microflora in acid saline and acid sulphate soils of Kerala based on metagenomic studies. The present investigation clearly indicates that structure based approach in metagenomics can reveal the diversity of culturable as well as unculturable microbiota in *Pokkali* soils. By providing suitable media and culture conditions, it may become possible to culture the so called unculturable bacteria and even identify the unidentified unculturables.

In the present study, bacterial diversity was assessed successfully from saline *Pokkali* habitats of Kerala through culture independent 16S rDNA metagenomic approach. Total environmental DNA was isolated directly from saline soil and 16S rRNA genes were amplified. The cloned sequences which showed homology with several bacteria were grouped under Proteobacteria, Chloroflexi, Acidobacteria and Cynobacteria in which most of them were saline tolerant, acidophilic, nitrogen fixing, sulphur reducing, sulphur oxidizing, strict anaerobes and pathogenic. Twelve clones out of 30 were grouped under Proteobacteria and these belonged to β-Proteobacteria (10 per cent), γ-Proteobacteria (20 per cent) and δ-Proteobacteria (10 per cent). 20 per cent of total cloned sequences were found as Chloroflexi types; 17 per cent as Acidobacteria types; 3 per cent as Cyanobacteria types. 20 per cent of total cloned sequences were found as unknown uncultured bacteria sequence types. Sequencing of more clones may give a clear and complete picture of microbiota in *Pokkali* soil. Further investigations are required for the function derived approach for gene prospecting (acid and salt tolerance).

Acknowledgement: The financial support from Jawaharlal Nehru University (JNU), New Delhi is acknowledged.

References

Amann, R.I, Ludwig, W. and Schleifer, K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Bak, F. and Pfennig, N. 1987. Chemolithotropic growth of *Desulfovibrio sulfodismutans* sp. *nov*. by disproportionation of inorganic sulfur compound. Arch. Microbiol. 147:184-189.

Barns, S. M, Takala, S. L. and Kuske, C. R. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl. Environ. Microbiol. 65:1731-1737.

Cowan, D. A. 2000. Microbial genomes:the untapped resource. Trends. Biotechnol. 18:14-16.

Dojka, M .A., Harris, J. K. and Pace, N. R. 2000. Expanding the known diversity and environmental distribution of an uncultured phylogenetic division of Bacteria. Appl. Environ. Microbiol. 66:1617–1621.

Falagas, M. E., Kastoris, A. C. and Vouloumanou, E. K. 2009. Attributable mortality of *Stenotrophomonas maltophilia* infections: a systematic review of the literature. Future Microbiol. 4:1103-1111.

Frigaard, N.U. and Dahl, C. 2009. Sulfur metabolism in phototrophic sulfur bacteria. Adv. Microb. Physiol. 54: 103-200.

Ghazanfar, S. and Azim, A. 2009. Metagenomics and its application in rumen ecosystem: Potential biotechnological prospects. Pak. J. Nutr. 8:1309-1315.

Girija, D., Deepa, K., Xavier, F., Antony, I. and Shidhi, P.R. 2013. Analysis of cow dung microbiota-A metagenomic approach. Indian J. Biotechnol. 12:372-378.

Hanada, S. 2003. Filamentous anoxygenic phototrophs in hot springs. Microbes Environ. 18:51-61.

Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72:1719–1728.

Kamagata, Y. and Tamaki, H. 2005. Cultivation of uncultured fastidious microbes. Microbes Environ. 20:85-91.

Kramer, D. 1984. Cytological aspects of salt tolerance in higher plants. In: Staples, R. C., Toenniessen, G.

H. (eds) Salinity tolerance in plants-strategies for crop improvement. John Wiley and Sons, New York, pp 3-17.

Looney, W. J., Narita, M. and Muhlemann, K. 2009. *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. Lancet. Infect. Dis. **9**: 312-320.

Miyashita, H. Ikemoto, H. Kurano, N., Adachi, K.,. Chihara, M. and Miyachi, S. 1996. Chlorophyll *d* as a major pigment. Nature. 383:402-408.

Rappe, M. S. and Giovannoni, S. J. 2003. The uncultured microbial majority. Annu. Rev. Microbiol. 57:369-394.

Reinhold, B., Hurek, T., Niemann EG. and Fendrik, I. 1986. Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. Appl. Environ. Microbiol. 52:520-526.

Rondon M, R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., Minor, C., Tiong, C. L., Gilman, M., Osburne, M. S., Clardy, J., Handelsman, J. and Goodman, R. M. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. 66:2541-2547.

Sekiguchi, Y. 2006. Yet-to-be cultural microorganisms relevant to methane fermentation processes. Microbes. Environ. 21:1-15.

Sekiguchi, Y., Yamada, T., Hanada, S., Ohashi, A., Harada, H. and Kamagata, Y. 2003. *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. Int. J. Syst. Evol. Microbiol. 53:1843-1851.

Siddhapura, P. K., Vanparia, S., Purohit, M. K., Singh, S. P. 2010. Comparative studies on the extraction of metagenomic DNA from the saline habitats of Coastal Gujarat and Sambhar Lake, Rajasthan (India). Int. J. Biological Macromol. 47:375-379.

Sorokin, I. D. and Kuenen, J. G. 2005. Haloalkaliphilic sulfur-oxidizing bacteria in soda lakes. FEMS Microbiol. Rev. 29:685-702.

Takenak, T., Tashiro, T., Ozaki, A. and Takakubo, H. 2007. Planktonic bacterial population dynamics with environmental changes in coastal area of Suruga Bay. Microbes. Environ. 22:257-267.

Ward, D. M, Weller, R. and Bateson, M. M. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature. 345:63-65.