ABSTRACT

The biological diversity of Indian sub-continent is one of the richest in the world owing to its vast geographic area, varied topography and climatic conditions. Because of this India is recognized as one of the 12 mega diversity regions of the world. The most important diversity zones are Western Ghats, North-Eastern hill regions, Andaman & Nicobar Islands, Mangrove forests of Sunderban, Silent valley of Kerala, Chilka lake of Orissa, Sonar lake of Maharashtra and the Himalayan region. In the present study we have made an attempt to study the bacterial diversity from the cold desert of Spiti valley of the Indian Himalayas by a polyphasic approach. Soil samples of a glacier located in Spiti valley, a remote Indian Himalayan valley, which is known as a cold desert and remained as an un-explored region.

In the chapter 1 and 2 highlight the importance of biodiversity, in general and the need for exploration and conservation of biological species, especially Bacteria (Introduction and Review of literature). The following are the aim and objectives:

Polyphasic approaches to study bacterial diversity of cold Himalayan desert.

1. To study the culturable bacterial diversity by polyphasic approach
2. To study the total bacterial community by molecular phylogenetic approach
3. Identification of unculturable bacterial species by molecular systematic studies

Chapter 3 deals with the Materials and Methods used, including the source of chemicals, list of bacteria and the protocols adopted for performing the experimesnts. In chapter 4, 5 and 6, all the experimental results, discussions and summary were given, which includes the detailed species description of the novel species and the total community analysis using molecular systematic study.

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Abstract of the present study:

A total of 52 bacterial strains were isolated from soil samples and the same were studied further in detail by a polyphasic approach. The complete characterization of the 52 isolates indicated that they belong to 21 different genera including *Planococcus, Kocuria, Rhodococcus, Dietzia, Agrococcus, Ornithinimicrobium, Enterobacter, Paenibacillus, Flavobacterium, Exiguobacterium, Bacillus, Arthrobacter, Microbacterium, vibrio, Brevibacterium, Pseudomonas, Staphylococcus, Janibacter, Klebsiella, Gordonia,* and *Methylobacterium* (Fig. 36). Among the culturable isolates, 6 new species were already established and 2 are proposed (*Planococcus stackebrandtii, Rhodococcus kroppenstedtii, Dietzia kunjamensis, Agrococcus lahaulensis, Kocuria himachalensis, Ornithinimicrobium kibberense*, Mayilraj *et al.*, 2005; 2006, *Exiguobacterium himgiriensis, Paenibacillus mashelkarii*, under revision). For un-culturable analysis, 58 clones were randomly selected and sequenced and a total of 10 major phyla were obtained. These phyla spanned a wide range within the domain Bacteria, occupying representation from the phyla, *Actinobacteria* (26%), *Proteobacteria* (20%), *Acidobacteria* (12%), *Firmicutes* (12), *Verrucomicrobia* (7%), *Bacteroidetes* (7%), *Chloroflexi* (6%), *Gammatimonadates* (5%), *Planctomycetes* (3%) and *Nitrospiraae* (2%) Fig. 37. Majority (approximately 95 %) of the cloned sequences show little affiliation with known taxa (<97% sequence similarity).

6 Summary

6.1 Diversity of bacteria

The biological diversity of Indian sub-continent is one of the richest in the world owing to its vast geographic area, varied topography and climatic conditions. Because of this India is recognized as one of the 12 mega diversity regions of the world. The most important diversity zones are Western Ghats, North-Eastern hill regions, Andaman & Nicobar Islands, Mangrove forests of Sunderban, Silent valley of Kerala, Chilka lake of Orissa, Sonar lake of Maharashtra and the Himalayan region. In the present study an attempt was made to study the bacterial diversity from the cold desert of Spiti valley of the Indian Himalayas by a polyphasic approach. Based on the observations reported in this work, the un-culturable clones recovered by cloning techniques and culturable bacteria recovered by isolation techniques from the soil sample were different. These results also suggest that microbial cultivation has not yet been employed exhaustively for determining the taxonomic identities and distributions of soil bacteria.

6.1.1 Diversity of culturable bacteria

A total of 52 bacterial strains were isolated from soil samples and the same were studied further in detail by a polyphasic approach. Upon the complete characterization of the 52 isolates indicated that they belong to 22 different genera including *Planococcus, Kocuria, Rhodococcus, Dietzia, Agrococcus, Ornithinimicrobium, Enterobacter, Paenibacillus, Flavobacterium, Exiguobacterium, Bacillus, Arthrobacter, Microbacterium, vibrio, Lysinibacillus, Brevibacterium, Pseudomonas, Staphylococcus, Janibacter, Klebsiella, Gordonia,* and *Methylobacterium* (Fig. 36). Among the culturable isolates, 6 new species were already established (*Planococcus stackebrandtii, Rhodococcus kroppenstedtii, Dietzia kunjamensis, Agrococcus lahaulensis, Kocuria himachalensis, Ornithinimicrobium kibberense,* Mayilraj *et al.*, 2005; 2006) and 2 are proposed *Exiguobacterium himgiriensis, Paenibacillus mashelkarii,* under revision). Table 6 shows the taxonomic status of these 52 isolates.

6.2 Discovery of New Taxa

6.2.1 Characterization of *Planococcus stackebrandtii* sp. nov. Strain K22-03^T

The taxonomic position of an actinobacterium strain K22-03^T was analyzed by a polyphasic approach. The strain designated as K22-03^T matched all the phenotypic characteristics of thegenus *Planococcus* and represents a novel species. The sequence of the almost complete 16S rDNA (1464 bases) was compared with those of previously studied *Planococcus* type strains and confirmed that the strain belongs to the genus *Planococcus*.

[illegible] DNA-DNA hybridization level it showed low genomic relatedness
th *Planomicrobium mcmeekinii* (MTCC 3704^T, 23%), *Planococcus psychrophilus*
ITCC3812^T,61%), *Planococcus antarcticus* (MTCC 3854^T, 45%) and *Planomicrobium*
eanokoites (MTCC 3703^T, 51%), the four species with which it is closely related at the
S rDNA sequence level (97–97.5% similarity). Therefore, the strain K22-03^T should be
cognized as a novel species, for which the name *Planococcus stackebrandtii* sp. nov. is
pposed. The type strain is strain K22-03^T (= MTCC 6226^T =DSM 16419^T = JCM 12481^T).

2.2 Characterization of *Rhodococcus kroppenstedtii* sp. nov. Strain K07-23^T

The taxonomic position of an actinobacterium strain K07-23^T was established by a
lyphasic approach. The strain exhibits phenotypic characters which are typical of the
nus *Rhodococcus*. Comparison of the 16S rRNA gene (1467 bases) sequence confirmed
t the strain K07-23^T belongs to the genus *Rhodococcus*. The 16S rRNA sequence
ilarity studies showed that the isolate is very closely related to *Nocardia*
ynebacterioides (98.6 %), which has been recently reclassified as *Rhodococcus*
ynebacterioides (MTCC 699^T). It showed sequence similarity of 94.4 %-96.6 % with
aining species within the genus *Rhodococcus*. However, genomic relatedness between
in K07-23^T and *Rhodococcus corynebacterioides* as revealed by DNA-DNA
ridization study was low (62 %). Based on the polyphasic analysis, the strain could be
rly distinguished from other validly described species and formed a separate lineage
in the group. It is proposed that the strain K07-23^T be classified as a novel species of
dococcus as *Rhodococcus kroppenstedtii*. The type strain is K07-23^T (MTCC 6634
SM44908 ^{T}= JCM13011^T).

3 Characterization of *Kocuria himachalensis* sp. nov. Strain K07-05^T

A red-orange bacterium, strain K07-05^T was was studied by polyphasic approach.
organism had morphology and chemotaxonomic properties consistent with its
sification in the genus *Kocuria*. Phylogenetic analysis of the 16S rRNA gene sequence
ved that the strain K07-05^T was closely related to *Kocuria rosea* DSM 20447^T and
uria polaris MTCC 3702^T (98.1 % and 97.8 % respectively), whereas sequence
larity values with respect to rest of the validly published species belonging to the genus
uria were between 96.4 % to 94.2 %. However, genomic relatedness, as shown by
A-DNA hybridization of strain K07-05^T with *K. polaris* is 49.5 % and *K. rosea* is 24.0
he DNA G+C content of the strain is 75.3 mol %. The above data in concert with
otypic distinctiveness of strain K07-05^T clearly indicate that the strain represents a new

ies, for which the name *Kocuria himachalensis* sp. nov., is proposed. The type strain is -05^T (= MTCC 7020^T= DSM 44905^T= JCM 13326^T).

4 Characterization of *Dietzia kunjamensis* sp. nov. Strain K30-10^T

A coral-red pigmented actinobacterium strain K30-10^T, was isolated from a soil ıple from a cold desert of the Himalayas, India. The organism had chemical and notypic properties consistent with its classification in the genus *Dietzia*. It showed 97.9 16S rDNA sequence similarity with *Dietzia maris* MTCC 7011^T, the similarity with the e strains of three other species of the genus, *Dietzia natronolimnaea*, *Dietzia ıchralcaliphila* and *Dietzia cinnamea* ranged from 94.4 % to 96.0 %. The DNA atedness between K30-10^T and the closely related strain *D. maris* was 59.2 %. The G+C ntent of the DNA was 67.0 mol %. Based on physiological, biochemical tests and notypic differences between the strain K30-10^T and the closest phylogenetic relatives, it proposed that the strain be classified as a new species of *Dietzia* as *Dietzia kunjamensis* ı. nov. The type strain is K30-10^T (=MTCC 7007^T =DSM 44907^T=JCM 13325^T).

2.5 Characterization of *Agrococcus lahaulensis* sp. nov. Strain K22-21^T

The taxonomic position of a lemon-yellow pigmented actinobacterium, strain K22-1^T, isolated from a soil sample from Lahaul-Spiti Valley, located in the Himalayas, India, ıas determined using a polyphasic approach. The organism had phenotypic properties nclude chemical properties consistent with its classification in the genus *Agrococcus*. 16S DNA similarities of strain K22-21^T with the three validly published species namely *Agrococcus jenensis* DSM 9580^T, *Agrococcus baldri* DSM 14215^T and *Agrococcus citreus* DSM 12453^T were 98.5 %, 96.8 % and 96.6 % respectively. However, the level of DNA relatedness between strain K22-21^T and the closest species *A. jenensis* was 55.1 %. The novel strain was distinguished from the type strains of the three validly published species of the genus *Agrococcus* using DNA-DNA relatedness and phenotypic data. Based on these differences strain K22-21^T (MTCC 7154^T= DSM 17612^T), should be classified as the type strain of a novel species of *Agrococcus*, for which the name *Agrococcus lahaulensis* sp. nov. is proposed.

6.2.6 Characterization of *Ornithinimicrobium kibberense* sp. nov. StrainK22-20^T

A buff-yellow pigmented bacterium strain K22-20^T, which was isolated from a cold desert of The Himalayas, India, was subjected to a polyphasic taxonomic study. The organism had phentotypic properties include chemical properties consistent with its classification in the genus *Ornithinimicrobium*. The major fatty acids of the strain are iso-$C_{17:1}\omega9c$ (cis15-methyl 7-hexadecenoic acid), iso-$C_{15:0}$ (13-methyl-tetradecanoic acid), iso-

(14-methyl-pentadecanoic acid) and iso-$C_{17:0}$ (15-methyl-hexadecanoic acid). The
tent of the genomic DNA is 71 mol %. According to the 16S rDNA sequence anal
closely related to *Ornithinimicrobium humiphilum* (97.7 %). However, geno
dness between strain K22-20^T and *Ornithinimicrobium humiphilum* as revealed
-DNA hybridization study was low as 64.5 %. Based on the polyphasic data, it
sed to classify strain K22-20^T in a new species of the genus *Ornithinimicrobium*, fo
the name *Ornithinimicrobium kibberense* is proposed. The type strain is K22-20
CC 6545^T=DSM 17687^T =JCM 12763^T).

Characterization of *Paenibacillus mashelkarii* sp. nov. Strain K22-17^T

A Gram-positive, endospore producing bacterium strain K22-17^T isolated from soil
studied by polyphasic taxonomy. The isolate had morphology and chemotaxonomic
rties consistent with its classification in the genus *Paenibacillus*. 16S rRNA gene
nce analysis confirmed that the strain K22-17^T had the signature sequences PAEN
and PAEN 862F,confirmed that the strain K22-17^T had the signature sequences PAEN
and PAEN 862F, which were found in the genus *Paenibacillus*. The G+C content of
NA is 50.5 mol %. The 16S rDNA (1456 bases) sequence analysis shows that the
formed a distinct phylogenetic lineage with *Paenibacillus terrae*, *Paenibacillus*
ae, *Paenibacillus peoriae* and *Paenibacillus polymyxa*. The strain K22-17^T showed
st phylogenetic affiliation to *P. terrae*, with 16 S rRNA gene sequence similarity value
7.9 %. The other four closely related strains exhibited 16S rRNA gene sequence
larity values between 95.9 - 96.8 %. However, the result of DNA-DNA hybridization
revealed low genomic relatedness of 35.6 % with the closest type strain *P. terrae* JCM
6^T. Based on data from phenotypic tests and genotypic differences of the strain K22-
with its closest phylogenetic relatives, it is evident that this should be regarded as a new
ies. It is proposed that the isolate should be classified in the genus *Paenibacillus* as a
l species, *Paenibacillus mashelkarii* sp. nov. The type strain is K22-17^T(= MTCC
^{T} = JCM 14261^T).

Characterization of *Exiguobacterium himgiriensis* sp. nov. StrainK22-26^T

The taxonomic position of an orange colored bacterium, strain K22-26^T isolated
a soil sample was studied by using a polyphasic approach. Phenotypic and chemical
erties of the strain K22-26^T were consistent with its classification in the genus
guobacterium. Phylogenetic analysis of the16S rRNA sequence showed that the strain
-26^T was closely related to *Exiguobacterium aurantiacum* (98.9 %) followed by
guobacterium mexicanum (98.7 %), *Exiguobacterium aestuarii* (98.0 %) and

Exiguobacterium marinum (97.9 %), whereas the sequence similarity values with respect to other *Exiguobacterium* species with validly published names were between 92.2–93.2 %. However, the level of DNA-DNA relatedness showed that strain K22-26^T belongs to a novel species. The DNA G+C content of strain K22-26^T is 50.1 mol %. The novel strain was distinguished from its closely related type species of the genus *Exiguobacterium* using DNA-DNA relatedness and phenotypic data. Based on these differences, the strain K22-26^T should be classified as a novel species of the genus *Exiguobacterium*, for which the name *Exiguobacterium indicum* sp. nov. Strain K22-26^T = (MTCC 7628^T = JCM 14260^T), is proposed.

The culturable diversity part concluded with the following:

From a total 52 isolates, so far 8 novel species were identified and characterized. Few more strains were considered to be novel isolates and further characterization work will be done to find out their exact taxonomic affiliation. The results indicated that the 52 isolates belong to 22 different genera, which were, identified as species of the genera *Planococcus, Kocuria, Rhodococcus, Dietzia, Agrococcus, Ornithinimicrobium, Enterobacter, Paenibacillus, Flavobacterium, Exiguobacterium, Bacillus, Arthrobacter, Microbacterium, Vibrio, Lysinibacillus, Brevibacterium, Pseudomonas, Staphylococcus, Janibacter, Klebsiella, Gordonia,* and *Methylobacterium.*

a. Strain designated as K22-03^T, a Gram-positive actinobacterium was identified as *Planococcus stackebrandtii*, published as a novel species.
b. Strain designated as K07-23^T, a Gram-positive actinobacterium was identified as *Rhodococcus kroppenstedtii*, published as anovel species.
c. Strain designated as K07-05^T, a Gram-positive actinobacterium was identified as *Kocuria himachalensis*, published as a novel species.
d. Strain designated as K30-10^T, a Gram-positive actinobacterium was identified as *Dietzia kunjamensis*, published as a novel species.
e. Strain designated as K22-21^T, a Gram-positive actinobacterium was identified as *Agrococcus lahaulensis*, published as a novel species.
f. Strain designated as K22-20^T, a Gram-positive actinobacterium was identified as *Ornithinimicrobium kibberense*, published as a novel species.
g. Strain designated as K22-17^T, a Gram-positive firmicutes was identified as *Paenibacillus mashelkarii*, proposed as a novel species.

h. Strain designated as K22-26^T, a Gram-positive firmicutes was identified as *Exiguobacterium himgiriensis*, proposed as a novel species.

i. Strain designated as K22-02^T, a Gram-negative Bacteroiodetes was identified as *Flavobacterium sp.*.

j. Strain designated as K22-15^T, a Gram-positive firmicutes was identified as *Exiguobacterium sp.*

k. Strain designated as K22-24^T, a Gram-positive firmicutes was identified as *Enterococcus Sp.*.

l. Other remaining isolates belonging to the known species of the genus, which were already validly published.

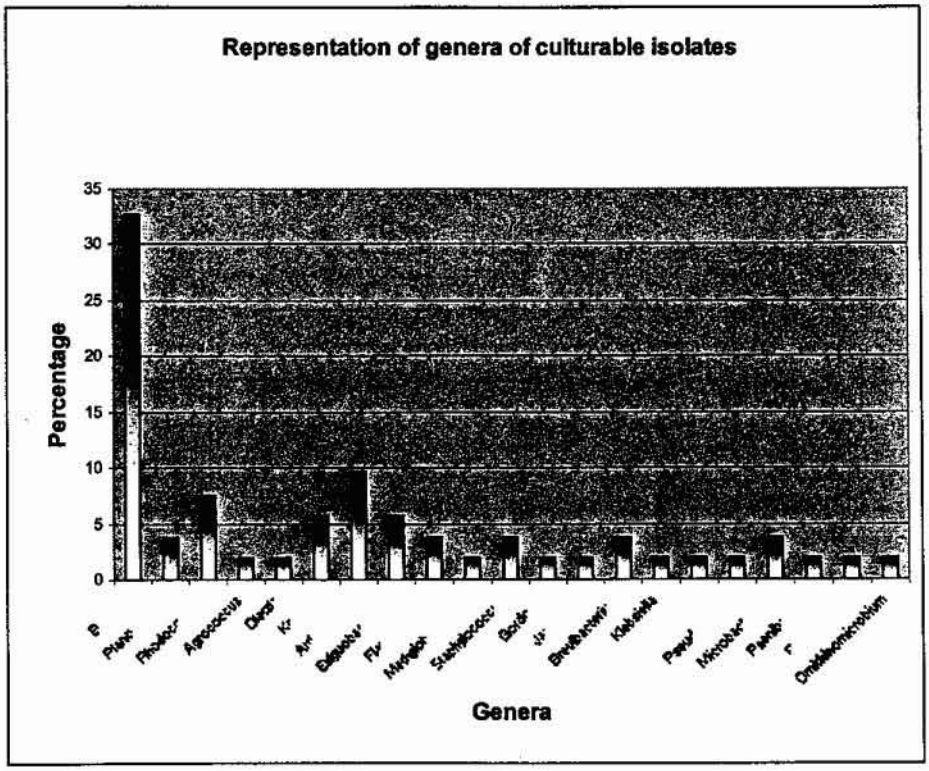

Figure. 36. Representation of genera of culturable isolates

6.3 Identification of unculturable bacterial species by molecular systematic studies

Community DNA was extracted directly from soil. 16S rRNA genes were amplified by PCR using primers specific to the domain Bacteria. The PCR products were cloned and sequenced. For the analysis, 58 clones were randomly selected from 150 clones and sequenced and a total of 10 major phyla were obtained. These phyla represented a wide range within the domain Bacteria, occupying representation from the phyla, *Actinobacteria* (26%), *Proteobacteria* (20%), *Acidobacteria* (12%), *Firmicutes* (12), *Verrucomicrobia* (7%), *Bacteroidetes* (7%), *Chloroflexi* (6%), *Gammatimonadates* (5%), *Planctomycetes* (3%) and *Nitrospirae* (2%). Majority (approximately 95 %) of the cloned sequences show little affiliation with known taxa (<97% sequence similarity).

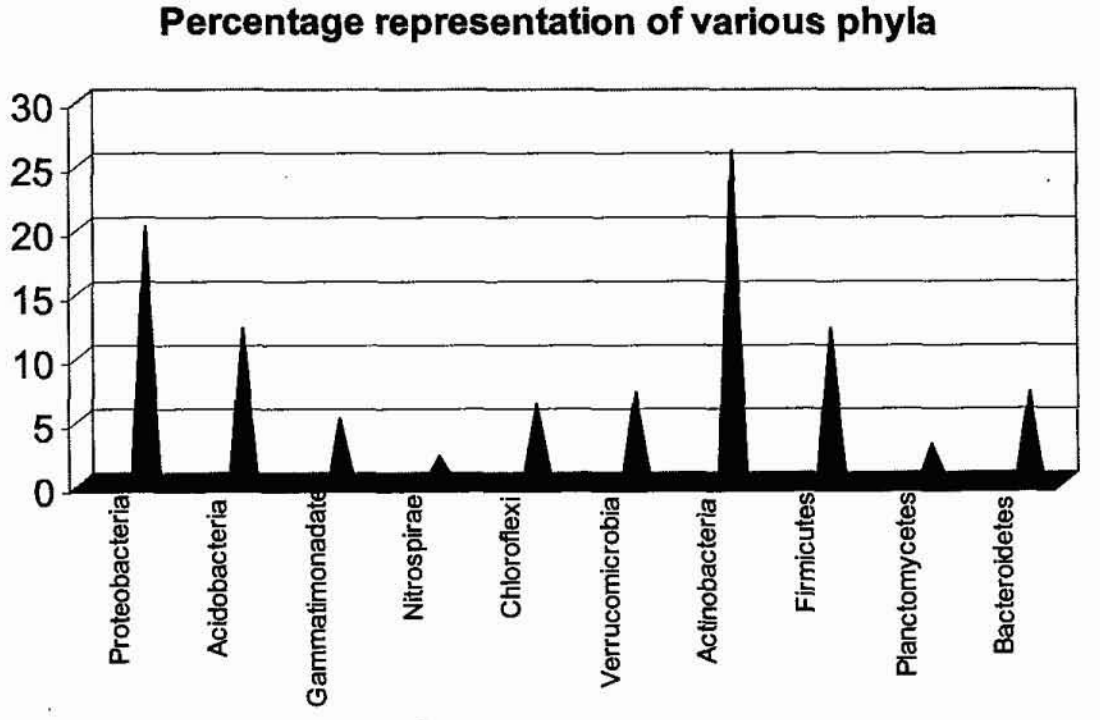

Figure. 37. Percentage representation of various phyla present in the sample

6.4 Rarefaction analysis

The graph (Figure. 35) in the result chapter shows that the data do not seem to have reached the saturation point, indicating that the number of clones studied was not sufficient to conclude the total diversity of bacterial community from the soil sample and analysis with more number of clones may reveal the exact diversity of bacterial community. Thus the polyphasic approach for characterization of the culturable diversity and assessing the unculturable component of the microbial diversity by molecular approach gave insight into the distribution of microbes belonging to different taxa. Further, studies will help in understanding the role/interaction of these populations in maintaining the characteristic ecology of the cold desert of Lahaul-Spiti valley.