Amit Bhattacharya (2009). ***Study of prokaryotic diversity fromn selected niches of western ghats of India.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Microorganisms constitute the oldest, vast and almost unexplored reservoir of natural resources and are likely to provide innovative applications in challenging areas like food, energy and climate change. They are vital for sustaining life on the biosphere directly or indirectly which sustain life on this planet. In spite of their immense importance, prokaryotic diversity did not draw as much attention because of their invisibility. As the existence of the microbial life was recognized only relatively recently in history (about 300 years ago), the knowledge gained is still rudimentary. Microorganisms still represent the largest reservoir of undescribed biodiversity. Moreover, conventional study of prokaryotes requires them to be cultured in the laboratory, but many lines of evidences suggest that most of them (more than 99 %) are not amenable to cultivation. Fortunately, techniques are now available to detect them and to a certain extent tap their gene pool by culture independent approach. Although, such culture independent studies of prokaryotes have highlighted their huge diversity and functions in the last few years, on the whole, their diversity and functions still remains poorly known and understood.

India has a rich diversity of macroscopic life forms (fauna and flora) due to its unique geographical location and possesses two megadiversity "hot spots". One of which is in the North Eastern part and the other is along the Western Ghats. Unfortunately, very little or no systematic study of prokaryotic diversity from any of these hot spots or other regions of this country has ever been taken up.

In this context, the present study was aimed at a systematic study of the prokaryotic diversity of Western Ghats samples (water, sediment and mangrove swamp sediment); using both culture dependent as well as by culture independent approaches. Since, prokaryotes represents huge reservoir of technologically important biomolecules therefore, some isolates (60) from water (WCW) and sediment (WCS) samples were also subjected for the screening of few enzymes (lipase, esterase, protease, tyrosinase protease and amylase).

Chapter 1 commences with an introductory chapter highlighting the present status of our knowledge about prokaryotic diversity. The chapter also highlights features of Western Ghats in terms of geography, climate, flora and fauna.

Chapter 2 describes the materials and methods employed in this investigation.

Chapter 3 presents description of samples, their processing, selection of the samples for DGGE analysis and the data obtained from analysis of the warm spring water sample, sediment and mangrove swamp sediment sample by culture dependent as well as by culture independent approaches in 11 sections. It also includes some discussions of the results in the context of present understanding of bacterial diversity.

Section 3.1 and 3.2 describes characters of the samples collected and initial processing of samples, serial dilution plating of the samples, DGGE analysis and selection of samples for

further detailed study on the basis of total CFU count, number of morphotypes appeared on plates and DGGE analysis. In brief 14 samples were subjected to serial dilution plating on three types of media [TS (tryptic soy agar; TSA), TD (tryptic soy agar diluted 100 times; TSBA100) and PC (plate count agar; PCA)], enumeration followed by isolation, purification and preservation of culturable isolates. Maximum number of colonies (2.45×10^8) appeared on TSBA100 (pH9) from sediment (WCS) sample and lowest number (2.60×10^2) on TSBA from the water sample (WCW) collected from the same site. It was found that in general nutritionally poor medium like TSBA100 supported maximum number of colonies. Altogether 1024 culturable strains were isolated. It was observed that some isolates survived one or two subculturing but were not able to grow upon subsequent subculture on their respective media. In order to have some idea about viability of environmental isolates percent survival of isolates were determined by counting the number of growing colonies after streaking on the respective plates and proper incubation at the appropriate temperature. It was observed that in general nutritionally poor media (TD) provided better survival upon subculture than nutritionally rich media. Operational Taxonomic Units (OTUs) reflected in the number of bands in the DGGE profile ranged from 6-24 in soil and sediment samples. It was observed that bacterial diversity in sediment was more compared to soil and water; in latter the number of bands varies from 10-13. It was also observed that in many samples, especially sediment and few water samples, there were one or two intense thick band(s). The major found in DGGE profile of WCW may be related to that of *Thiovirga* sp. which was found to be dominant in culture independent approach (discussed in section 3.4). DGGE profiles of archaeal community were also generated for WCS and SAS2 samples. It was observed that both SAS2 as well as WCS samples are rich in archaeal diversity as shown by number of bands appeared in DGGE profiles. DGGE profile of archaeal diversity of WCW sample could not be generated when similar set of primers and conditions were employed. Considering the uniqueness of two samples, in terms of large number of culturable bacteria, morphotypes and more number of operational taxonomic units in DGGE analysis, WCS (sediment of warm water spring) and SAS2 (soil of mangrove) samples, were selected for detailed study. WCW sample (water of the same warm spring) was also taken into consideration for detailed study so as to get a comparative and comprehensive idea of bacterial diversity of water and its sediment of the warm spring.

Subsection 3.3 describes detailed analysis of water sample (WCW). Thirty nine isolates were selected for characterization. Upon preliminary characterization these isolates were placed into 16 groups. These 39 isolates were further subjected to detailed characterization that included morphological and physiological tests, oxidation pattern of 95 substrates using Biolog system (could be done for 35 isolates), Fatty Acid Methyl Ester (FAME) analysis and partial 16S rRNA gene sequencing. In summary, diversity of culturable bacteria of this warm water (WCW sample) suggests presence of both Gram-positive (61%) as well as Gram-negative taxa (39%). Four strains

DWCW2, 9, TSWCW18 and 25) were found to be novel species of the genera (*Bacillus, enibacillus, Exiguobacterium* and *Acidovorax*).

Representatives of other genera represented in WCW sample were *Streptomyces, crobacterium, Brachybacterium, Brevibacterium, Pseudomonas, Acinetobacter, Rheinheimera, phylococcus, Cohnella* and *Enhydrobacter*. Culture dependent analysis of diversity WCW sample caled that it is dominated by members of class *Bacilli* of phylum *Firmicutes* and class *mmaproteobacteria* of phylum *Proteobacteria* and these two phyla constitute 41% and 39% of the l culturable bacteria. Members belonging to phylum *Actinobacteria* cover 20% of this population.

Section 3.4 describes culture independent analysis of water sample (WCW). A total of 47 es were analysed by BLAST search and their sequences were deposited in GenBank. From the ysis of the sequence data it appears that diversity of the bacteria present in WCW sample is ed to two classes *Gammaproteobacteria* and *Betaproteobacteria* of the phylum *Proteobacteria*. esentatives of the phyla *Firmicutes* and *Actinobacteria* which altogether constituted 61% of the ed isolates conspicuous by their absence in culture independent approach. Culture dependent sis also showed more than one third (39%) of cultured bacteria of the spring water belong to *obacteria* However, Generic compositions obtained using two different approaches were not me. The 16S rDNA clone library was overwhelmingly (34 out of 47 or 72%) dominated by lated to *Thiovirga* sp. with sequence similarities varying from 95-99%. Members of the genus *ga* which dominate this habitat were not recovered in the culture independent analysis. This is of the choice of media for plating which might not be suitable for specialised groups of like the one found in WCW sample.

ction 3.5 describes bacterial diversity of culturable bacteria of sediment sample (WCS) as by culture dependent approach. The sample was plated out on 5 different media (TSBA, 00, PCA, and TSBA of pH 9.0 and TSBA100 of pH9.0). A total of 148 isolates were rized phenotypically taking 10 characters into consideration and 48 groups were formed for more biochemical and physiological characterizations were done. All the isolates were d to Biolog analysis, fatty acid methyl ester analysis (FAME) and 16S rRNA gene ng. In brief, the bacterial diversity of WCS sample is represented by both Gram-positive well as Gram-negative taxa (35%). The culturable diversity of the sample was distributed in ial phyla namely *Firmicutes, Proteobacteria, Actinobacteria,* and *Bacteroidetes*. Within positive lineages, the isolates were distributed into two bacterial phyla: *Firmicutes* and *ria*. *Firmicutes* constitutes 55% of the total cultured bacterial population and majority belong to genus *Bacillus* (57% of the *Firmicutes*), other genera within this phylum were by *Paenibacillus, Brevibacillus, Lysinibacillus, Staphylococcus* and *Exiguobacterium*. the phylum *Actinobacteria* constitutes 10% of the population and 86% of members belong to genus *Arthrobacter* followed by *Microbacterium* (14%). Among

members of Gram negative lineages, isolates are represented from phyla *Proteobacteria* (26%) *Bacteroidetes* (9%). Majority of the isolates under the phylum *Proteobacteria* belong to class *proteobacteria* (89%) followed by members of class *Alphaproteobacteria* (8%) and *mmaproteobacteria* (3%). Within the class *Betaproteobacteria,* majority of the isolates belong to us *Acidovorax* (76%). Among the members of the phylum *Bacteroidetes,* majority of the bers belong to the class *Flavobacteria* (79%) followed by *Sphingobacteria* (21%). In this ple one strain (TSWCSN5^T) merits the rank of a novel genus at least 9 isolates were found to be ntial candidates to be described as novel species. Most of them (6) are novel species within the us *Bacillus.* Novel strains TSWCSN 6, 7, 8, 14, and 16 belong to genus *Bacillus* and WCSN16 to genus *Paenibacillus.* Two strains, namely THWCSN29 and THWCSN35, represents species of the genera *Rhodobacter* (class *Alphaproteobacteria*) and genus *Undibacterium* *Betaproteobacteria*) respectively. One strain THWCSN34 deserves to be a novel species the genus *Flavobacterium* under the phylum *Bacteroidetes.*

Section 3.6 describes the bacterial and archeal diversity of sediment sample (WCS) as revealed lture independent approach. Two clonal libraries were constructed for the WCS sediment le (one archaeal and one bacterial). A total of 48 clonal sequences were used for determination logenetic status of the bacterial clones. All 48 clone sequences were deposited in the GenBank e. Sequence analyses for the 48 clones were carried out using the BLASTN program of the ebsite. In short, culture independent analysis of WCS sample revealed that among five phyla *bacteria, Deinococcus -Thermus, Bacteroidetes, Nitrospira, Chloroflexi*), majority of clones o phylum *Proteobacteria* and thus making it a dominant phyla in the sample. It comprises the total population when revealed by culture independent approach followed by *ccus-Thermus* (4%), *Chloroflexi* (4%) and *Bacteroidetes* (2%) and *Nitrospira* (2%) of the pulation of all the clones examined. Among the clones belonging to *Proteobacteria* majority the clones belong to class *Gammaproteobacteria* within this class 25 clones showed their lationship with *Thiovirga sufuroxidans* which is a potent sulfur oxidizer. Other Classes of *bacteria* detected in the sample are *Betaproteobacteria* (28%), *Epsilonproteobacteria* (5%) *haproteobacteria* (2%). Among members of *Epsilonproteobacteria,* Clones are related to *rvum kujiens* which is also a sulfur oxidizing species. Among members of *Chloroflexi,* long to two classes *Dehalococcoides* and *Anaerolineae* which are strict anaerobe and take in bioremediation. Approximately 4 % of the clones studied showed ≤ 85.0 % sequence o the sequence of any cultured representative in the NCBI database. These clones could ned to any existing bacterial phyla probably represent lineages of yet uncultured novel cterial phyla. The section also revealed analysis of 25 archaeal clone sequences of WCS library. Sequence and phylogenetic analysis results revealed that these 21 clones fell rchaeal phyla *Crenarchaeota* (8%) and *Euryarchaeota* (76%) while 4 clones (16%) laced to any existing phyla. Within *Euryarchaeota,* 17 clones were grouped in only

class of methanogenic archaea i.e. *Methanomicrobia* and 2 clones were found to be unknown of *Euryarchaeota.* Among clones that belong to *Methanomicrobia methanogenic* bacterial up, majority (94%) of the clones belong to order *Methanosarcinales* followed by *thanomicrobiales* (6%). Clones belonging to phylum *Crenarchaeota* were found to be distantly ed with their closely related cultured representative archaeal sequences; therefore, these clones identified only up to phylum level only. Few clones (16%) did not show more than or even l to 85% sequence similarity with a cultured representative and thus may belong to novel idate archaeal phyla. Sequence analysis of 19 dissimilatory sulfite reductase gene clones were analysed that showed the presence of clones related to A subunit as well as B subunit of DSR and strengthen the hypothesis of an active sulfur cycle regulation in this spring ecosystem by tivities of both sulfur oxidizing and sulfur reducing bacteria. The section 3.6 also describes a rison of bacterial diversity of water and sediment sample as revealed by culture dependent and independent analysis.

ection 3.7 describes culture dependent analysis of mangrove swamp sediment sample (SAS2). e was plated on TSBA, PCA and TSBA 100 supplemented with or without 2% NaCl. A total olates were selected for characterization by phenotypic methods, FAME analysis 16S rRNA equence analysis. Culture dependent analysis revealed that majorities (71%) among the were Gram Positive and the rest (29%) were Gram negative. The culturable diversity of the was found to be restricted to three bacterial phyla namely *Firmicutes, Proteobacteria,* and *acteria.* Within the Gram-positive lineage the isolates were distributed into two phyla: *es* and *Actinobacteria. Firmicutes* constitutes 68% population and majority of the isolates to the genus *Bacillus* (77%). Other genera within *Firmicutes* were represented by *illus* (16%), *Paenibacillus* (5%), *Brevibacillus* (2%) as determined by their 16S rRNA gene analysis, phylogenetic analysis, phenotypic characters and presence of major fatty acids.

nalysis of 16S rRNA gene sequence revealed that among the members of Gram negative hat constitute approximately one third (29%) of the total population, all of them belonging ylum *Proteobacteria.* Interestingly, all isolates under the phylum *Proteobacteria* belong to *maproteobacteria* (Table 3.101) and majority of them belong to genera *Pseudomonas* llowed by *Enterobacter* (22%). Members related to genera *Acinetobacter* (17%) and (17%) are equally represented and only one strain, PCSAS2-4 belongs to the genus stituting 5% of the total population of Gram negative bacteria.

AS2 sample two isolates may represent novel taxa. More detailed biochemical, l and chemotaxonomical evidences are needed to describe these two strains as novel

n 3.8 describes culture independent analysis of mangrove swamp sediment sample 16S rRNA gene sequence analysis of 39 clones revealed majority of the clones belong *teobacteria* and thus making it the dominant phyla in the sample. It constitutes almost

half (41%) of the total population when revealed by culture independent approach followed by *Firmicutes* (13%), *Choloroflexi* (10%) and *Actinobacteria* (8%). *Bacteroidetes* and *Planctomycetes* constitute 5% and 2% respectively of the total population of all the clones examined while 21% clones could not be placed to any existing bacterial phyla and may represent novel candidate phyla. Among the clones belonging to *Proteobacteria* majority of the clones belong to class *Alphaproteobacteria* (44%). Other Classes of *Proteobacteria* detected in the sample are *Gammaproteobacteria* (31%), *Betaproteobacteria* (13%), *Deltaproteobacteria* (6%), and *Epsilonproteobacteria* (6%). About 23% showed similarity to uncultured clone sequences retrieved from mangrove sediment and estuary sample. Few clones showed very high sequence similarities with cultured bacteria of *Bacillus* sp., *Shwanella* sp., and *Pseudomonas* sp. members related to *Bacillus* sp. and *Pseudomonas* sp. were also recovered in culture dependent analysis. In this section the analyses of 22 clones of archaeal library of SAS2 sample were also described. All 22 sequences analysed in this study indeed belong to the domain *Archaea*, and most of them were related to uncultured archaeal members. Sequence similarity of these clones to the closest relative ranged from 80% to 98%. Twenty three percent clones could easily be placed in the phylum *Euryarchaeota*. Four clones are very likely to be members *Methanomicrobia* class of *Euryarchaeota*. These clones showed 94% to 97% sequence similarity with species of *Methanosarcina* sp. while the other had 97% similarity with *Methanobacterium bryantii*. Mangrove swamp sediment produce significant amounts of methane therefore, the presence of this group of organisms were expected in mangrove sample. One clone although fall within the clad of *Methanomicrobia*, could not be placed in any existing phyla because of low (83%) sequence similarity. Twelve clones showed their association with *Thermoplasmata* related clones and showed no cultured representative. There was no representation of *Korarchaeota* or *Nanoarchaea* in this study this may be due to the small number of clones analysed in the archaeal library or to the fact that members of those groups were isolated from high temperature environments. The closest relatives of many SAS2 archaeal clones were reported from salt marsh sediment and from mangrove sediment. This suggests the archaeal community in Salim Ali Bird Sanctuary mangrove soil is relatively high similar to studied by other workers. In this section a comparison of culture dependent analysis with culture independent analysis is made that revealed a bit more overlap of bacterial phyla in SAS2 sample than samples in previous section in both approaches employed to study bacterial diversity but it would be conclude that both these approaches are complementary to each other and could reveal in better way when employed together.

The site specific comparison of bacterial diversity of all the three samples (water, sediment and rhizosediment) analyzed by culture dependent and culture independent analysis and archaeal diversity in SCS and SAS2 sample by culture independent analysis is presented in section 3.9. The comparison with culture dependent and independent approaches did not show much overlap (since the laboratory conditions used for cultivating isolates rarely simulate the natural habitat) but very

complemented each other and clearly indicated more diversity in sediment samples (WCS and SAS2) than in the water sample (WCW). Culture dependent analysis revealed almost similar pattern of diversity in all the samples studied where *Firmicutes* were found to be dominant population followed by *Proteobacteria* and *Actinobacteria.* Members of *Proteobacteria* were found to be dominant in all the samples as uncovered by culture independent approach. In the present study culture independent analysis showed members of some bacterial phyla and their classes were found to be present specifically to a particular niche like members of phyla *Deinococcus-Thermus* and *Spirochaetes* were only found in WCS sample while members related to *Planctomycetes* were present only in SAS2 sample.

Section 3.10 describes roles and reliabilities of Biolog, FAME and 16S rRNA gene sequence analysis for characterization and identification of culturable bacteria it revealed in many cases (20/39 in WCW sample and 60/148 in WCS sample) all three methods lead to the same genus although not necessarily to the same species.

Section 3.11 showed results of screening for enzyme activity from cultured isolates. In brief, strains isolated from the warm spring were screened for the enzyme activities by plate based assay. The enzymes selected were lipase, esterase, tyrosinase, chitinase, protease and amylase. Out of isolates screened for lipase 14 were found to be lipase positive that belong to WCS sample and only was found to be lipase positive tested from WCW sample. Among lipase positive strains, PCWCS1 (a novel member of *Bacillus* sp.) and PCWCS22 (a member of *Brevibacillus brevis*-*formosus* sub cluster) showed maximum lipase activity as determined from the size of clearing zone around the colonies. Chitinase activity was shown by 14 isolates isolated from WCS sample and chitinase positive strain was found in WCW sample. Among all chitin degraders, isolate PCWCS2 was found to be most active. Many strains were found to be positive for the activity of esterase (35 isolates), Amylase (32 isolates), Protease (35 isolates) and Tyrosinase (10 isolates) and belong to both WCS sample as well as WCW sample. On the basis of screening for enzyme activity it can be concluded that the Western Ghats region represent a huge microbial resources for industrially important enzymes.

Chapter 4, of the thesis presents major finding of the work and overall conclusions drawn from the study of bacterial diversity of a warm spring water sample, sediment sample and mangrove sediment of Western Ghats.