Riddles of soil Actinobacteria in tea gardens and selected other biotopes

Thesis submitted to the University of North Bengal for the Award of
Doctor of Philosophy
in
Tea Science

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2022

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Document Information

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Submitted	2022-02-09T12:10:00.0000000
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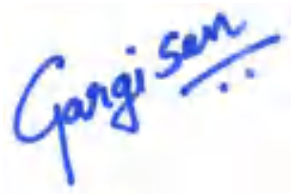
Declaration

I declare that the thesis entitled “Riddles of soil actinobacteria in tea gardens and selected other biotopes” has been prepared by me under the supervision of Dr. Malay Bhattacharya, Assistant Professor of the Department of Tea Science, University of North Bengal. No part of the thesis has formed the basis for the award of any degree or fellowship previously.



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Abstract

Actinobacteria which were previously known as actinomycetes or ray fungi form an important constituent of the microbial biome. It comes next to proteobacteria in terms of number and distribution. Actinobacteria are mostly aerobic, gram-positive to gram variable with high G+C content and occupy a diverse microbial niche. They share some characteristics with fungi, such as colony morphology, mycelial growth and musty smell, on the other hand, peptidoglycan cell wall structure is common with bacteria. A phylogenetic study based on 16SrRNA classifies actinobacteria into six classes i.e., Acidimicrobia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, Thermoleophilia and Actinobacteria.

Actinobacteria form an integral part of the microbial community which is involved in antibiotic and secondary metabolite production, bioremediation, nitrogen fixation, etc. They are in constant interaction with other organisms as symbiotic or pathogenic microbes altering the immediate environment. Bioactive compounds produced by microbes exhibit pharmacological or toxicological effects in other organisms. Actinobacteria have been considered as one of the significant groups of microorganisms as they represent a broad range of valuable and prominent sources of pharmaceutically active metabolites. These metabolites are detected by GC-MS technique and are utilized as antimicrobial and anti- cancerous agents. In nature, actinobacteria play a pivotal role in the cycling of organic compounds and have also been associated with soil organic matter production, owing to their black pigments called melanin, which are related in

respect to soil humic acid.

Among actinobacteria, *Streptomyces* is so far the largest genus with approximately 900 species. Hence, this genus is a valuable resource for novel secondary metabolites (SMs). The most widely distributed genus of actinobacteria, Streptomyces has been the focus of research by biologists because of the commercial applicability of the substances produced. S*treptomyces*, a soil-dwelling, high GC content, gram-positive,actinobacteria continue to be a promising microorganism for biosynthesizing several clinically important secondary metabolites including antibiotics, antiviral, anti-fungal, anti-tumoral, anti-parasitic and immune-suppressors.

Human activities are creating new compounds and the microbial community is constantly evolving to cope up with this stress. The mechanism of action of the microbial community *in vitro* and *in vivo* conditions may deduce the remedy for degradation of the environment as well as the drug resistance in organisms. Agrochemicals such as pesticides and fertilizers are essential inputs to improve the productivity of the farmer in terms of farm produce, but their use is also associated with chemically induced injuries to health. In order to overcome this problem Integrated pest management utilizing microbial pesticides is gathering momentum. This is because they are more specific, have low relative cost and are more eco-friendly.

The ongoing proliferation of whole -genome sequences is a stepping stone for systems biology which aims to study the integrated network constituted by the complete repertoire of genes (genome), the population of transcripts (transcriptome), the population of proteins (proteome), the population of metabolites (metabolome), and fluxes of an organism or cell, in relation to intrinsic and environmental

stimuli.Codon usage patterns and preferences vary significantly within and between organisms.

Recent advances in genome sequencing-based approaches have revealed that most of the secondary metabolite gene clusters (SMGCs) of actinobacteria are condition-dependent and are inactive under normal laboratory conditions. Thus, the SMs producing capacity of actinobacteria has been under-estimated. The proportion of uncultivable microbes to cultivable microbes is still very high. To circumvent such cultivation limitations, strategies have been developed based on the extraction of microbial DNA directly from an environmental sample and its subsequent analysis or exploitation (for biotechnological purposes) independent of its original host. This approach, which is based on the recovery of a sample's microbial metagenome (the sum of all microbial genomes), has, in theory, great potential for biotechnological and ecological studies of the system under investigation, for example soil. Such a metagenomics-based view of the community will aid in enhancing our understanding of microbial functioning and interactions in a soil ecosystem.

Bioremediation and bio-augmentation are the techniques for the improvement of the capacity of contaminated soil or water to remove pollution by the introduction of specific capable strains of microorganisms. In this context, it is vital to identify and characterize the microbial consortia mainly consisting of actinobacteria from native as well as pristine biotopes with promising qualities for bioaugmentation. This will help to improve the production and quality of tea and increase its demand in the international market.

Hence, the objectives of this present study were to identify and characterize the actinobacteria from different biotopes. Molecular

documentation of different actinobacterial strains using16S rRNA primers and Whole-genome sequencing of a few potential actinobacteria such as *Streptomyces* were carried out. Comparative analysis of different genomes through bioinformatic tools was also part of this research. Apart from this, Bioassays of selected strains and the GC MS analysis of microbial cultures for their metabolites were also done. Another aspect of this current study was the Metagenomic analysis of soil microbiome from different biotopes such as tea gardens, mangroves, rubber plantations, paddy fields, forests and vegetable fields. Moreover, a survey of the health condition of tea garden workers in the Terai and Dooars region was carried out with the help of resident tea garden physicians and other concerned authorities through interviews and literature surveys.

Characterization of the isolated microbes by slide culture technique and growth in different media was studied along with Gram's staining and spore chain morphology studies using standard protocols. Biochemical studies were done for, nitrate reduction, melanin production, starch hydrolysis, etc. Molecular Identification of Actinobacterial Isolates by 16S rDNA amplification using actinospecific primers was executed. Sequence similarity studies and comparative genomics were carried out using the available algorithms.

We could isolate several actinobacteria from various biotopes but the further characterization was limited to *Streptomyces* isolates. There were 25 *Streptomyces* isolates from soil samples of mangroves, 10 from tea garden, 8 from the vegetable field, and 2 each from Rubber estate and forest soil which was isolated on selective media- Streptomyces media and International

Streptomyces Project (ISP 4). The isolate VRA 1 showed maximum inhibition to all of the test pathogens and the antagonistic activity of the newly isolated strains proved their potential to be utilized as antimicrobial agents in the future. The ability of the isolates to produce industrial enzymes like lipase, gelatinase, amylase and cellulase makes them industrially important. The isolated strains were found to have PGPR activities such as Phosphate solubilization, IAA, Ammonia production. The ability of the strains to produce extracellular enzymes cellulase further adds to their PGPR potential as cellulase is known to contribute to their antagonism against phytopathogens. The isolates with PGPR were found to improve shoot length, root length, fresh weight of the mung bean plant. It is promising because the actinobacterial strains with PGPR reported in this study can be exploited for biofertilizer production. GC-MS analysis of the extract produced by the selected strains, it was found that there were many bioactive compounds with antimicrobial, antifungal and antioxidant activities. Thus, the isolate with the potential to produce antimicrobial compounds has been further explored for its bioactivity against pathogens that cause various diseases. Further, the antimicrobial compounds were then assessed by in silico molecular docking approach for Tuberculosis and enteric disorders that were prevalent among the tea garden workers. We found that compounds Celidoniol,Pentacosane, Deoxyspergualin, Nonalactone showed the highest binding affinity with tuberculosis proteins (1USL) and enteric toxin(2NRJ).The potency of these compounds can be further validated by *in vivo* studies and could be utilized by the pharmaceutical industry.

The five isolates were sent for the

whole genome sequencing. The sequencing was done by MicrobesNG at the University of Birmingham, the UK using Illumina sequencing technology. The codon usage of the four sequenced isolates (VRA 1, VRA 16, TEA 02 and TEA 10) and the selected *Streptomyces* strains along with an out-group *Kitasatospora* indicated towards the biased use of GC rich codon. This shows that GC richness is an important factor that determines codon usage. The effective number of codon (Nc) of the genomes suggests that these high GC-rich genomes exhibited considerable heterogeneity in codon usage. A strong positive correlation between CAI and Fop was observed in all the cases revealing the utilization of the majority of optimal codons by PHX rather than PLX ($p<0.01$). CAI values also showed a significant positive correlation with GC3 and Nc values attributed to the strong compositional constraints among the genes. RSCU was also found to correlate strongly with Fop, GC3 and CAI This correlation pointed towards the preferred usage of GC3 rich optimal codons and a certain level of translational selection on these genes. Hence, we may hypothesize that GC compositional constraints along with the translational selection have an influence on the codon usage as well as expression pattern. The pan- core plot has been given in a total of 18759 genes were accumulated in pan-genome. However, the core gene count was reduced to 152. The core genes were found to be the housekeeping genes involved in some major metabolic and signal transduction pathways.

The antiSMASH studies of strains showed that they harbor several secondary metabolite biosynthetic gene clusters (BGCs). These findings may help to explore and

utilize the secondary metabolites in agriculture, the pharmaceutical industry, etc.

The metagenomic study of the soil samples from different biotopes provided us with valuable insights into the soil microbiome. The study of soil samples from different altitudes of Darjeeling hills along with the rhizospheric soil from an actinorhizal plant *Alnus nepalensis* showed the abundance of diazotrophs in the rhizosphere.

We also targeted two very popular tea gardens of Darjeeling hills- Makaibari (Mak) and Casselton (Cas) to study the soil community through a metagenomic approach. Our studies showed that the abundance profile of pathogenic microbes was much higher in Cas than in Mak. This also underlined our survey results which has shown the prevalence of tuberculosis and gastric disorders among the tea garden workers.

Based on the OTU number, Chao1 bacterial species abundance index, and Shannon microbial diversity index, α-diversity analysis was conducted on the microbial diversity of various samples. The metagenomic analysis of paddy field, tea garden, and other selected biotopes showed the abundance of *Candidatus* solibacter of *Acidobacterium*. The actinobacterial population was much reduced in the studied biotopes. Among the bacterial population, the genus *Isophaera* showed high density, followed by *Gemmatimonas*, *Bfidobacterium*, *Frankia*, *Streptomyces* etc.

It is apparent from the current work that actinobacteria has the potential to deliver innumerable metabolites which could solve the problem of emerging antibiotic resistance, help in novel drug production. The quality of tea can be improved by the use of organic manure and biofertilizers which will reduce the MRL. This will in turn boost the tea industry which aids in the development of the tea

garden community. The metagenomic studies indicate the reduced species diversity in the agricultural fields and plantation areas. It is an alarming situation, where the microbial population is affected by manmade activities and this may pose threat to our own existence. However, it has also shown that use of organic manure favours the microbial population which is promising.■

Preface

When you have a dream, you have to grab it and never let it go.

Carol Burnett

This journey which I have embarked in the year 2018 that would not been successful without the help and support of the array of well wishers in my life. These four years have been a learning experience for me. I was indeed blessed with a very supportive Supervisor Dr. Malay Bhattacharya, Assistant Professor, Department of Tea Science, University of North Bengal. Words are not enough to express my heartfelt gratitude towards him. I would like to express my deep sense of appreciation to Dr. Chandra Ghosh, Head, Department of Tea Science who was always there to help me in all possible ways. I would also like to thank Dr. Sonali Roy for encouraging me in this venture. The major part of my work was executed in the Bioinformatics facility and Molecular Genetics laboratory at Department of Botany. Here I was privileged to have a group of young, enthusiastic and talented lab mates. Among all, I would like to mention few names without their help this journey would not have been possible. I was fortunate to have Dr. Indrani Sarkar in our team whose able guidance enabled me to complete the *In Silico* work effortlessly. She was always there with immense patience to solve my doubts. Mrs. Saroja Chettri, who has become a younger sister to me in this short period of time and her passion for research in Actinobacteria, bonded us together. Her valuable succour will be cherished throughout my life. Dr. Pallab Kar, Dr. Ayan Roy and Dr. Saubashya Sur with their meticulous and systematic approach was always an inspiration and I am also thankful for their unconditional support. I would also like to

acknowledge the timely assistance from my juniors, Sandipan Ghosh, Swarnendra Banerjee, Sutapa Dutta, Reha,Labar, Saumita Bhattacharjee and Moushika Lala. Although I had a very short interaction, I was always enriched with the lab mates of Tea Science Department, Sahadeb Sarkar, Sourav Chakraborty, Sumedha Saha, Sukanya Acharya, Soumya Majumder and Arindam Ghosh. Special thanks are due to Mr. Basudeva Sangha for his sincere and selfless assistance during this tenure. This endeavour in the interim of my life was always watched with an apprehensive eye by the majority of the people. But my family and friends remained as lifeline in all odd situations. I would like to specially mention my late father in law, Sri Paresh Ch. Sen, who could not be the part of this joy but always conferred his blessings from heaven. I would like to express my deep sense of gratitude to my, mother in law for her constant encouragement and blessings. A very special person who took all the pain and efforts to enable me to accomplish this task is my soul mate Prof. Arnab Sen. Our children Abhishikta and Amartya have risen to the occasion and endured our absence at home with smiling faces.

This venture wouldn't have been possible without the blessings and endless prayers of my parents, sisters and dear and near ones whose names are not mentioned separately. Their strong belief in me has helped me to sail forward in the troubled waters. I am thankful to the Almighty who bestowed me with his love and strength that capacitated me to reach up to this point.■

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Chapter 1

Introduction

The true laboratory is the mind, where behind illusions we uncover the laws of truth.

Sir J.C.Bose

Soil is the source of innumerable life. It provides habitat for beneficial microbes and there exists a synergistic relationship between the microbial communities. Soil is essential for the conservation of biodiversity and it is vital for the existence of life on this planet. Soil can be considered as an ecosystem itself, as they support a variety of life and all accompanying complex interactions among organisms (Curtis & Sloan 2005; Ponge, 2015). The major organisms in soil are prokaryotes and can form the largest part of the soil biomass (Sikroski 2015; Daniel 2005; Hassink et al. 1993). It has been found that one gram of soil comprises approximately 10^{10} bacterial cells and estimated species diversity of between $4X10^{3}$ to $5X10^{4}$ species

(Raynaud 2014; Roesch et al. 2007; Torsvik et al. 1990). Fertile soils are the outcome of team work of microorganisms, which directly affect the biological fertility of that soil. In addition to fertility, soil microorganisms also play essential roles in the recycling of nutrients that are essentially central to life on the planet. Soil microbial communities play a vital role in maintaining soil quality, function and the ecosystem sustainability (Sapkota 2020; Jacoby et al. 2017; Waldrop et al. 2000; Nannipieri et al. 2003). Many previous studies revealed that soil microbial communities were affected by various factors, including plant species, soil types and agricultural practices (Zhang et al. 2021; Xue et al. 2018; Garbeva et al. 2004; Jangid et al. 2008; Berg and Smalla 2009). However, the age-old practices of conventional agriculture may not be helpful in promoting the healthy populations of microbiome, limiting production yields and intimidating sustainability. Scientific studies are exploring new and exciting prospects for the restoration and promotion of healthy microbial populations in the soil. A definite understanding of the microbiota of an ecosystem helps in sustainable agriculture, ecological restoration, reclamation of land, pathogen resistance, nutrient acquisition, and stress tolerance of the host plant (Liu et al. 2019).

Actinobacteria is one of the dominant phyla present in the soil microbial community and it is poorly understood. Actinobacteria are widely distributed in various biotopes such as soil, water, permafrost, mammals, arthropods, plants etc. (Sen et al. 2014). Furthermore, various lifestyles are encountered among Actinobacteria, and the phylum includes pathogens (e.g. *Mycobacterium* sp. *Nocardia* sp. *Tropheryma* sp. *Corynebacterium* sp. and *Propionibacterium* sp.), soil inhabitants (*Streptomyces* sp.), plant commensals (*Leifsonia* sp.), nitrogen-fixing symbionts (*Frankia sp.*), and gastrointestinal tract (GIT) inhabitants (*Bifidobacterium*) (Ventura et al. 2007).

Actinobacteria form the integral part of microbial community which is involved in antibiotic production, secondary metabolite production,

bioremediation, nitrogen fixation, etc. (Girao et al. 2019). They are in constant interaction with other organisms as symbiotic or pathogenic microbes altering the immediate environment. Bioactive compounds produced by microbes exhibit pharmacological or toxicological effects in other organisms. Actinobacteria have been considered as one of the significant groups of microorganisms as they represent a broad range of valuable and prominent sources of pharmaceutically active metabolites. Actinobacteria mostly inhabit in soil, freshwater, and marine habitats playing an important role in decomposition of organic materials, such as cellulose and chitin, thereby playing a vital part in biogeochemical cycles, replenishing the supply of nutrients in the soil, and helps in humus formation (Bhatti et al. 2017). Its diversity and distribution studies will help us to reveal about microbial ecology and its applications in agriculture and pharma sector.

Human activities are creating new compounds and the microbial community is constantly evolving to cope up with this stress. The mechanism of action of microbial community *in vitro* and *in vivo* conditions may deduce the remedy for degradation of environment as well as the drug resistance in organisms. The metabolic path ways involved in xenobiotic degradation is not yet studied in a comprehensive manner (Mishra et al. 2021, Maurice et al. 2013). The detailed study of xenobiotic activity of pathogenic and non-pathogenic Actinobacteria in different niches may help us to understand the intricate mechanism of microbial community with the environment. It may open up new avenues of understanding that could have many applications in fields diverse as agriculture, biotechnology, ecosystem monitoring. In nature, Actinobacteria play a pivotal role in the cycling of organic compounds and have also been associated with soil organic matter production, owing to their black pigments called melanins, which are related in some respects to soil humic acid (Anandan et al. 2016; Schafer et al. 2010).

The most widely distributed genus of Actinobacteria, *Streptomyces* has been the focus of research by biologists

because of the commercial applicability of the substances produced. Among the biological control agents derived from different microbes, Actinobacteria especially *Streptomyces* sp. are one of the most important microbial resources which can provide potential new bioactive compounds for use as insect-control agent (Kaur et al. 2013; Gadelhak et al. 2005). So, the main focus of this study is mainly limited to *Streptomyces* genera of Actinobacteria from selected biotopes of Indian subcontinent.

An area with uniform environmental conditions providing a living place for a specific assortment of plants and animals. It has its origin from the Greek word combining "bios" (meaning life) and "topos" (meaning place). The German scientist Friedrich Dahl introduced the term biotope in 1908 This term was a complement of the term biocoenosis (attributed to Karl Möbius in 1877) meaning the assemblage of animals and plants living together in a specific habitat.

Furthermore, the term biotope is not limited to physical conditions that surround a community of organisms but it also comprises the relative biota. Therefore, biotope is a topographic unit characterized by similar environmental (physical) conditions and a specific assembly of plant and animal species. In this study, I have chosen different biotopes for the isolation and characterization of Actinobacteria mainly, *Streptomyces.* Diverse biotopes are selected in order to isolate new strains of the genus. The biotopes that are selected for this study are the soils from tea garden, mangroves, rubber plantation, paddy fields, vegetable garden, and natural forests.

The world's second most popular drink is tea that has been consumed every day by billions of people. Besides legendary Darjeeling tea, Assam and Dooars tea are the most popular global brands. It is unique for its strength, taste and aroma. Tea is the backbone of Darjeeling and Dooars economy which supports the majority of the population in North Bengal. It is estimated that around 28, 000 small tea growers in Terai and Dooars of North Bengal who create employment for 1.7 lakh local people of this sub-

Himalayan region (Chowdhury et al. 2016). However, the small-scale tea sector faces a number of problems such as lack of capital, improper knowledge about the agro-techniques of tea cultivation, inadequate input availability and problem of marketing (Saikia, 2019, Karmakar & Banerjee, 2005). Apart from these, tea industry as a whole is facing multiple problems in term of its quality and production. Though the large-scale producers can overcome the losses but this is having adverse impacts on small tea estate owners (Rajbangshi & Nambiar, 2019, Kumar et al. 2008). Bioremediation and the bio-augmentation are the techniques for improvement of the capacity of a contaminated soil or water to remove pollution by the introduction of specific capable strains of microorganisms. In this context, it is vital to identify and characterize the microbial consortia mainly consist of Actinobacteria from native as well as pristine biotopes with promising qualities for bio augmentation. This will help to improve the production and quality of tea and increase its demand in international market. Another aspect is the utilization of PGPR activity of Actinobacteria strains which will reduce the input of chemical fertilizers (Aldesuquy et. al. 1998; Borah & Thakur, 2020). The bio -augmentation property of Actinobacteria (Sharma et al. 2020) is highly promising to the declining tea industry of North Bengal. Identification and characterization of key metabolites from microbiome will be highly promising to improve the health status of tea garden workers which will have wider social, economic and ecological implications.

Another important biotope, which is under studied is mangrove forests. Mangrove forests are large ecosystems prevalent in tropics and subtropics. They make up over a quarter of the total coastline in the World. Mangroves form the transient ecosystem which exhibits the edge effect and shows wide species richness. They provide habitat for different flora and fauna and also a treasure trove for microbial biome. Since it is an ecotone habit, it is rich in biodiversity. There are some criteria to be fulfilled by an ecosystem to be considered it as mangrove (Tomlinson 2016). They are: complete fidelity to

the mangrove environment, plays a major role in the community, ability to form pure stands, morphological adaptation to the habitat. Such mangrove forests are estimated to cover an area of about 150,000 sq.km.in world wide (Forest survey of India, 2017). The total area of mangroves in India is estimated to be 6,740 sq. km (Kumar et al. 2008) which is drastically decreasing due to anthropogenic activities. It is a link between land and sea which serves as breeding and nursing grounds for aquatic organisms. This ecosystem is an active geographical zone which is rich in organic sediments. These sediments are the vast resources for microbial production and metabolism. Due to the presence of rich source of nutrients mangroves are called the homeland of microbes. The mangrove ecosystem is saline and highly rich in organic matter and remain mostly untapped source for screening and isolation of potential bioactive secondary metabolites (Law et al. 2019; Newman & Cragg 2007).

Rubber plantations are considered as a key ecosystem in tropical areas such as Southeast Asia, Latin America, etc. (Dechner et al. 2018). The rubber tree is an indigenous species of Amazon rainforests and utilities as the sole source of natural rubber for the industry. The site for natural rubber biosynthesis and storage is the secondary laticifers present in the inner bark of rubber tree are (Hao & Wu 2000). Rubber is widely cultivated as plantation crop in Asia, Africa. In India, rubber is mainly cultivated in Kerala.

The increase in rubber plantations led the researchers to focus on the ecological and environmental consequences of these so called, ‘artificial ecosystems’. These studies have analysed the effects of rubber plantation on soil fertility, soil organic carbon and soil microbial biomass (Diniz et al. 2020; Yang et al. 2004; Zhang et al. 2007). A soil microbial community in the rubber plantation varies according to the age of the plantation, cover vegetation of the plantation, use of agrochemicals etc. Most studies have focused on microbial biomass and activity in surface or subsurface soil of rubber plantations (Guo et al. 2015; Zhang et al. 2007). There are very few studies

available for the effects of rubber cultivation on composition of microbial population in the rhizosphere and bulk soil of rubber trees (Zhou et al. 2017). So efforts have made to isolate and characterise the indigenous microflora from the rubber plantation soil.

Rice (*Oryza sativa* L.) is the most important cereal crop in the world, feeding more than 50% of the human population and is the major human food in Asia. The rice ecosystem or paddy field may be a prime habitat for microorganisms adapted to fluctuating nutritional levels and oxygen and light availability. The application of fertilizers in paddy field to increase rice yield affect the microbial community. Soil microorganisms are crucial for nutrient cycling, soil fertility, and crop productivity (Jacoby, 2017; Garbeva et al. 2004). Maintaining the health of soil microbiota is imperative for soil fertility and optimal crop yield. Microbial communities within rice fields in different habitats vary in diversity and response to environmental changes. Microbial communities in the rice rhizosphere can be significantly affected by seasonal changes (Dong et al. 2021; Schmidt & Eickhorst 2013). Soil properties and the microbial community in rice field is also affected by fertilization treatments (Ahn et al. 2016; Pascual 2000). Chemical fertilizers are likely to stimulate the growth of gram-positive bacteria in rice soils, while organic amendments increase the relative abundance of bacteria and fungi and decrease the abundance of Actinobacteria. Zhang et al. 2012). Nitrogen fertilizers increase soil biomass in organic carbon-rich rice soils (Yu et al. 2020). It has been shown that in these agroecosystems, rice exudates and nutrients from straw incorporation were shown to influence the bacterial communities' composition. It is vital to gather the information about microbiota prevailing in this unique biotope.

The forest is an important biotope as they are abundant with microflora and also act as carbon sinks. Previous studies by various have shown that soil microbial communities are affected by the changes in land use patterns and climate (Jesus et al. 2009; Tripathi et al. 2012; Lee-Cruz et al. 2013;

Rodrigues et al. 2013; Kerfahi et al. 2016). While most of the research have focused on fungi, and minimal information is available on forest soil bacteria which play a vital role in the environment. In forest soils, bacteria inhabit numerous habitats with definite properties, which vary from bulk soil, rhizosphere, litter, and deadwood habitats, where their communities are influenced by nutrient availability and biotic interactions. Bacteria interact with plant roots in symbiotic or non-symbiotic manner and exhibit commensality property with mycorrhiza fungi as symbiotic or mycorrhiza helpers. Bacteria also facilitate multiple critical steps in the nitrogen cycle, including Nitrogen fixation. However, the understanding of bacterial communities in forest soils has advanced in current years, but it is still insufficient. The precise level of the involvement of bacteria to forest ecosystem processes will be known only in the future, when the activities of all soil community members are studied synchronously. (Lladó et al. 2017). So, the forest soil is also chosen for the isolation of Actinobacteria in this study.

The isolated microbes will be characterised at both biochemical and molecular level using the available protocols. The genome sequencing of few isolated Actinobacteria and its detailed bioinformatic analysis is proposed to be undertake in this research.

Soil being the most assorted and multifaceted microbial habitat on earth surpasses the quantity of inhabiting microbial communities than other environments. Some phylogenetic surveys on soil environs made evident that the number of prokaryotic species present in a specific soil sample is far more than the known cultured prokaryotes (Daniel 2005). Metagenomic analyses endow extensive information about the structure, architecture, and predicted gene function and behaviour of varied environmental assemblages. The first step in soil metagenomics is DNA isolation from the soil along with production and screening of clone libraries that aids us to explore the cultivation-independent genetic reservoir of soil microbial neighbourhood (Sarkar et al. 2021). The unmatched diversity of soils

promises sustained exploration of diverse industrial, agricultural and environmental agents in the future. Each environment has its unique challenges to metagenomic-investigation requiring explicitly designed approaches considering both biotic and physicochemical factors (Kakirde et al. 2010; Nesme et al. 2016). It's a promising approach in unfolding the useful potential of the soil microbial community, which might produce greater insight into the health of a soil than taxonomy-based metrics, and also delivers Information about microbial communities and their interaction (Berg et al. 2020). It's a powerful tool for accessing almost 100% of genetic resources from environment and also aids in gene level studies of the communities. Functional metagenomic strategies helps us to explore discovery of new genes, enzymes and natural products etc. (Kennedy et al. 2011).

The successful application of soil metagenomics demands high-quality DNA extraction, purification along with cloning methods for the predicted downstream bioinformatics analyses (Kakirde et al. 2010). The average insert size of a clone library or the length of sequence read for a high-throughput sequencing approach is also crucial. An appropriate metagenomics screening strategy should be adapted to address the specific questions of interest (Kakirde et al. 2010). Hence, 16S amplicon or metabarcoding analysis based on the V3-V4 region will be undertaken for the soils collected from different biotopes to have a comprehensive account of the microbiome. Qualitative analysis of the different soils of the different biotopes by physiochemical methods is also planned under this study. This may give an insight into the interaction of microbes and their environment.

Bioinformatic tools has become indispensable in the genomics era for the analysis of voluminous data. The plethora of biological data are available to the scientific community due to the advancements in sequencing technologies (Albarano et al. 2020). Since the release of the first genomic sequence of *Haemophilus influenza* in 1995 by Fleischmann, thousands of genomes are sequenced and are available in biological databases. The

completion of human genome project in 2003 was an added booster. In this scenario, Bioinformatics, a new discipline has gained popularity. It is the application of computational techniques to analyse and organise the biological data. The genome sequences which are available in databases are utilized for the study of interrelationships between various species in terms of structure and function. Codon usage study provides information about usage of codons and gene expression in genomes (Liu 2020). Codon usage patterns and preferences shows variation within and between the species (Zhou & Li 2009) The various indices employed in codon usage study are GC content, GC3 content, relative synonymous codon usage (RSCU), (Peden 1999), effective number of codons (Nc) (Wright 1990) and frequency of optimal codons (Fop) (Ikemura 1981). The basic principle is that the genetic code is degenerate and the genetic codes of different organisms are often biased towards the use of one of several codons. The codon usage bias or differences among the usage of the synonymous codons have been the important factor for the evolution of proteome diversity. The difference in the usage for synonymous codons exists widely within the genomes due to mutation, natural selection, and random drift (Shen et al. 2020). Thus, a complete understanding of the biases in codon usage can help us to explore the evolution of genes in different genomes. Codon adaptation index (CAI) is an important tool for analysing the patterns of codon usage within a gene relative to the reference set of genes, mainly, ribosomal protein genes (Sharp & Li 1987; Wu et al. 2005). CAI is used to predict the highly expressed genes in various organisms. Apart from these, comparative genomics-based analysis helps to elucidate the function and evolutionary processes that act on genomes (Lukjancenko et al. 2012). The characterisation and profiling of the core genome and pan genome of the concerned organisms is an effective method to unravel the puzzles of speciation and genomic variations. The core genome is the conserved pool of genes present in a species and the dispensable genome is the set of genes present in some strains of a species but

not present in all strains of a species (Medini et al. 2005). The pan genome is the complete sets of genes within a species, including the core and the dispensable genomes (Morneau 2021). Hence in this study, steps are taken to compared and analyse the newly isolated and sequenced genomes from various biotopes with the selected genomes from public domain.

Apart from these, GC MS analysis of few isolates are also proposed under this study. This will help to identify the potential metabolites present in the isolates.

Agricultural production is often mired by numerous biotic stresses. Various chemical pesticides are used to minimize crop loss, which are hazardous (Mittal & Gupta 2008). Agrochemicals such as pesticides and fertilizers are essential inputs to improve the productivity of the farmer in terms of farm produce, but their use is also associated with chemically induced injuries to health. One of the most important plantation crops in India is tea. The tea industry in India is one of the old agriculture-based industry since 18th century (Borborah & Gogoi 2007). As of 2019, India is ranked the second largest tea producer in the world with production of 1,339.70 million kgs. The estimated tea production in India stood at 73.44 million kgs in April 2021. The total tea export was US$ 755.86 million in April 2020 to March 2021 and for March 2021 it was US$ 53.35 million (https://www.ibef.org).Though, tea is grown in various parts of India, Darjeeling tea which is unique in its flavour is grown in Terai and Dooars region in West Bengal and is considered as an economical backbone in tea cultivation areas of Assam and Northern part of West Bengal. In Terai and Dooars area of North Bengal, tea production is the only major and key industries on which teagardens, workers, tea traders are dependent for their major economy. Tea industry provides employment opportunities to large number of people and is cultivated by small tea growers which is largely unorganized and is ignorant of occupational hazards including the agrochemical exposure. [Tea industry Annual report, 2010]. Large number of families live on the tea estates. The recent information gathering showed that tea farmers have been applying

different pesticides to reduce pest incidence in their tea garden. It is also reported that nearly more than 20 different types of pesticides being used in tea garden. Though farmers have been using WHO approved pesticides, they are practicing cocktail preparation of two or more than two pesticides without knowing their compatibility, which is of serious concern to health and environment. These are toxic chemicals and they are to be handled with extreme care. They are not easily degradable; they remain in the soil, leach to ground water and contaminate the environment. Further more, it also enters the body of organisms, which leads to bio accumulation in the food chains and adversely impairs the human health. The problem of heavy metal toxicity affects the health of the people. It has been reported that metal toxicity in the blood samples is above the permissible level among the people living in tea gardens. (NBMCH report). Moreover, occurrence of physical and genetic abnormalities and declining reproductive health has also been reported. The reports of the health status of tea garden workers makes it evident that the soil micro flora has an effect on human health. The respiratory ailments such as tuberculosis and skin disorders are prevalent among the tea garden workers (Gayathri & Arjunan, 2019). The health survey conducted among the tea garden workers of various tea gardens of Northeastern India has established the fact that workers are suffering from various ailments such as gastrointestinal disorders, respiratory disorders, skin diseases due to the unhealthy environmental condition (Rajput et al. 2021; Ahmmed & Hossain 2016; www.tezu.ernet.in/ project reports).

The bacteria and fungi produce produces secondary metabolites which are an important source of antimicrobials and other bioactive compounds. The genome mining has emerged as an effective approach in identifying and characterizing new compounds in recent years. The 'antibiotics and secondary metabolite analysis shell or antiSMASH' is extensively used tool for identifying and analysing biosynthetic gene clusters (BGCs) in bacterial and fungal genome sequences (Blin et al. 2019). It also helps to compare and coordinate

with the available secondary metabolites present in the databases. This has prompted us to explore the bioactive compounds of Actinobacterial populations from different biotopes of India. The secondary metabolites present in the selected genomes will be identified using antiSMASH software as well as by GC MS analysis.

Keeping these facts in mind,

Objectives of the present study are:

- Survey of health status of tea garden workers in Terai and Dooars region.
- Screening and Isolation of Actinobacteria in pure culture from various biotopes such as tea gardens, paddy fields, mangroves etc.
- Identification and Morphological and biochemical characterisation of isolated microbes using standard protocols.
- Molecular documentation of different Actinobacterial strains using16S rRNA primers.
- Bioassays of selected strains using different methods.
- Whole genome sequencing of few potential Actinobacteria such as Streptomyces.
- GC MS analysis of microbial cultures for their metabolites.
- Comparative analysis of different genomes through bioinformatic tools.
- Metagenomic analysis of soil microbiome from different biotopes.■

Chapter 2
Review of Literature

What you learn from a life in science is the vastness of our ignorance.

David Eagleman

2.1 Soil an Ecosystem

Soil microorganisms are greatly important in the soil food web and natural equilibrium (Neemisha 2020). Microorganisms in soil are significantly relative to healthy soil and healthy plant because they are a considerable component of soil physical and chemical processes. The soil microbial communities can improve soil structure for plant growth by enhancing soil aggregate stability (Lu et al. 2018). Bacterial polysaccharides and, fungal hypha and metabolic products play important role in binding soil particles together (Costa et al. 2018, Haynes et al. 2020; de Caire et al. 1997; Caesar-TonThat and Cochran 2000). Soil aggregation is necessary for soil quality to improve infiltration rate, water holding capacity

and plant root development. Thus, decreasing microbial biomass and their activities are a result of reducing soil aggregation (Gao et al. 2019; Jain & Saxena 2019). Numerous species of microorganisms including protozoa, bacteria, fungi and nematode are contained in the soil. However, bacteria and fungi seem to be the most important in soil nutrient cycling because they are the first organisms degrading organic materials as their energy source (Rashid et al. 2016; Kladivko 2001). Soil fertility is enhanced by increasing microbial biomass. Soil microbes strongly influence soil biogeochemical cycles because they obviously express their ability in organic matter decomposition, nutrient mineralization and nutrient cycling (Witzgall et al. 2021). Furthermore, some soil microorganisms have the potential to degrade or detoxify chemical pollutants and pesticides in soil (Raffa & Chiampo 2021). However, the structure of the microbial community depends on many factors such as climate, moisture, topography, plant growth, and quantity and quality of substrates (Deltedesco et al. 2020) Additionally, soil chemical (e.g., pH and salinity) and physical condition (e.g., texture and soil-water potential) are also influence soil microbial efficiency (Almendro-Candel 2018). Hence, the soil microbial community can respond to environmental change and it has been used for monitoring the impact of agricultural practices and ecological stresses on soil health (Alori et al. 2020). The reservoir of soil microorganisms in the pore structure within and between soil particles. The community of soil microorganisms can use as a useful indicator of soil quality and ecological stresses because of their adaptation.

2.2 The Actinobacteria

Actinobacteria, previously known as ‘Actinomycete’ or ray fungi is originated from two Greek words ‘atkis’ (ray) and ‘mykes’ (fungi). It forms an important constituent of the microbial biome and comes next to proteobacteria in terms of number and distribution. Actinobacteria are mostly aerobic, gram-positive to gram variable with high G+C content and occupy diverse microbial niche (Amin et al. 2020). The phylum also includes a few

Gram-negative species such as *Thermoleophilum* sp. (Zarilla & Perry 1986), *Gardenerella vaginalis* (Gardner & Dukes 1955), *Saccharomonospora viridis* P101T (Shin et al. 2017), *Ferrimicrobium acidiphilum,* and *Ferrithrix thermotolerans* (Johnson et al. 2009). They share some characteristics with fungi, such as colony morphology, mycelial growth and musty smell, on the other hand, peptidoglycan cell wall structure is common with bacteria. On this basis, it was named as 'Actinobacteria' by C. O. Harz and it was considered as the missing link between bacteria and fungi. Though the first Actinobacteria was discovered by Ferdinand and Cohen from human lachrymal ducts in the year 1875 it came to the limelight only after the discovery of the antibiotic, actinomycin by Dr. Selman Waksman A phylogenetic study based on 16S rRNA classifies Actinobacteria into six classes i.e., Acidimicrobia, Coriobacteria, Nitriliruptoria, Rubrobacteria, Thermoleophilia and Actinobacteria (Sen et al. 2014). So they were considered transitional forms between fungi and bacteria. Indeed, like filamentous fungi, many *Actinobacteria* produce a mycelium, which is nonseptate and slenderer and many of these mycelial Actinobacteria reproduce by sporulation (Chater & Chandra 2006). However, the comparison to fungi is only superficial: like all bacteria, Actinobacteria cells are thin with a chromosome that is organized in a prokaryotic nucleoid and a peptidoglycan cell wall; furthermore, the cells are susceptible to antibacterial agents (Smith 2005)). Physiologically and ecologically, most Actinobacteria *a*re aerobic, but there are exceptions (*Actinomycosis meyeri* and *A. israelii*). Further, they can be heterotrophic or chemoautotrophic, but most are chemoheterotrophic and able to use a wide variety of nutritional sources, including various complex polysaccharides (Shivlata & Satyanarayana 2015).The genome size of Actinobacteria ranges from 0.93 Mb (*Tropheryma whipplei*; Bentley et al. 2003) to 12.7 Mb (*Streptomyces rapamycinicus*; Baranasic et al. 2013), that exists either as a circular or linear form. Actinobacteria mostly inhabit soil, freshwater, and marine habitats playing a pivotal role in the

disintegration of organic materials, such as cellulose and chitin, thereby playing a vital part in biogeochemical cycles, refilling the supply of nutrients in the soil, and helps in humus formation.(Elbendary et al. 2018) They have been considered as one of the significant groups of microorganisms as they represent a broad range of valuable and prominent sources of pharmaceutically active metabolites. These metabolites are analyzed by GC-MS technique and are utilized as antimicobial and anticancerous agents. (Ajilogba et al. 2019)

2.3 Diversity and importance in various ecosystems

Actinobacteria are widely distributed in various biotopes such as soil, water, permafrost, mammals, arthropods, plants, etc. (Sen et al. 2014, Rego et al. 2019). The density of the Actinobacterial population is determined by their habitat and the existing climate conditions. The effect of climate on the distribution of Actinobacteria was analyzed by Hiltner and Strömer 1903, who indicated that the soil microbial flora is more in the autumn season than in spring because of the available crop residues in this season. But, during the winter, frost reduces their abundance in the population. The class Actinobacteria contains 16 orders and the order *Actinomycetales* is limited to family *Actinomycetaceae*, and the other suborders that were part of this order are now considered as specific orders. So, 43 families within the phylum Actinobacteria are allocated to a single class, Actinobacteria, whereas the other five classes together are limited to10 families (Zhang et al. 2019, Barka et al. 2015). Various lifestyles are shown by *Actinobacteria*, and the phylum has pathogens (e.g. *Mycobacterium* sp. *Nocardia* sp. *Tropheryma* sp. *Corynebacterium* sp. and *Propionibacterium* sp.), soil-dwelling (*Streptomyces* sp.), plant commensals (*Leifsonia* sp.), symbiotic nitrogen-fixing (*Frankia*), and gut inhabiting (*Bifidobacterium*). Actinobacteria, are the source of various biocatalytic tools such as acid-producing *Corynebacteria*, secondary metabolite producing *Streptomyces*, carotenoid building *Micrococcus* strains, acid fermenting *Propionibacteria*, probiotic *Bifidobacterium*, xenobiotic *Gordonia*

species, and *Rhodococci,* etc. (Tischler et al. 2019). Most of the Actinobacteria spend the major part of their life cycles as semi-dormant spores and are saprophytic, soil-dwelling organisms and the phylum has adjusted to a wide range of ecological environments (Goodfellow & Williams 1983). This phylum is also known for its diversity in chromosome topology. Genus *Frankia*, *Salinispora* possess circular chromosomes but the major genera *Streptomyces, Rhodococcus* are having linear chromosomes and may or may not have plasmids. The linear plasmid pSLA2s was first identified in *Streptomyces rochei* (Hayakawa et al. 1979) which further led to the discovery of linear plasmids in other Actinobacteria. The size of the plasmids ranges from 12 to 600 kb in size (Kinashi et al. 1987; Sakaguchi 1990) and are mega-plasmids, and the size of linear chromosomes in Actinobacteria vary from 8 to 10 Mbp (Hopwood 2006). Though the genes involved in secondary metabolite production remain in clusters on the chromosome (Hopwood 2006), studies have also identified biosynthetic clusters on the large linear plasmids (Novakova et al. 2013). Nowadays, its members are considered among the most effective settlers of all environments in the extremobiosphere, in contrast to the belief that Actinobacteria are autochthonous soil and freshwater organisms (Bull et al. 2011).

Thermophiles

The Actinobacteria which grow at high temperatures ranging from 40 to 80° are termed as thermophilic organisms (Tortora et al. 2007). They are observed in moldy hay (Corbaz et al. 1963), self-heating plant residues, cereal grains, sugar cane bagasse (Suihko et al. 2006), decaying vegetable materials, and compost heaps (Henssen & Schnepf 1967). These may be obligate thermophilic or moderately thermophilic Actinobacteria. The obligate thermophilic can grow in the temperature range between 37 and 65 ° C, but optimum growth takes place at 55–60 °C. The moderately thermophilic Actinobacteria grow at 28–60 °C and require 45–55 °C for optimum proliferation (Jiang & Xu 1993). Whereas thermotolerant Actinobacteria can thrive at temperatures up to 50 °C

(Lengeler et al. 1999). Some examples of thermophilic Actinobacteria are *Amycolatopsis ruanii* and *Amycolatopsis thermalba.* The ability to grow in high temperatures is due to the electrostatic, hydrophobic and disulfide bonds in the proteins of these organisms (Ladenstein and Ren 2006). The presence of special proteins known as chaperones (Singh et al. 2010) and other proteins that bind to DNA and inhibit their denaturation at high temperatures.

Psychrophiles

Psychrophiles are the cold-loving organisms and most copious organisms on earth in terms of its diversity and distribution (Margesin et al. 2008). Actinobacteria phylum has been considered as one of the most prominent microbial divisions in different Antarctic regions (Cary et al. 2010; Pearce et al. 2012). This phylum is recognized as a producer of a wide range of secondary metabolites with different activities including herbicides, antifungals, antitumor or immunosuppressant compounds, and anthelmintic agents (Manivasagan et al. 2014). It has been shown that old permafrost has more amount of Actinobacteria (Willerslev et al. 2004). Antarctic Actinobacteria isolates belonging to the genus *Arthrobacter, Streptomyces,* and *Rhodococcus* exhibited antifungal activities (Santos et al. 2020). The genus *Arthrobacter* was reported from alpine permafrost in China (Bai et al. 2006). The psychrophilic and psychrotolerant Actinobacteria of *Nocardiopsis and Streptomyces* were isolated from the water samples of the Polar Frontal region of the Southern Ocean (Sivasankar et al. 2018). The psychrophilic Actinobacterial isolates are able to grow at low temperatures and alkaline conditions, produce a variety of enzymes such as proteases, amylases and cellulases (Zhang et al. 2007). Psychrophiles are subjected to temperature fluctuations and frequent freeze-thaw events. This has led to the evolution of several adaptation mechanisms concerning reproduction, metabolic activities, and survival and protection strategies in these organisms. Culture dependent and culture-independent molecular methods and the advancing fields of genome and proteome analyses will reveal more

about psychrophilic lifestyle (Margesina & Miteva 2011)

Xerophiles

Desert soil is also designated as an extreme terrestrial environment and organisms growing in this extreme environment are designated as xerophiles. The distribution of Actinobacteria in sandy soil (Cario, Egypt; Falmouth, MA), black alkaline soil (Karnataka, India), sandy loam soil (Keffi Metropolis, Nigeria; Presque Isle, PA), alkaline desert soil (Wadi El Natrun, Egypt; Wadi Araba, Egypt), and subtropical desert soil (Thar, Rajasthan), are established from different studies and *Streptomyces sp*. were found dominant followed by *Nocardia, Nocardiopsis,* and *Actinobacteria* (Cundell & Piechoski 2016). The isolates recovered from a desert soil sample collected in Beni-Abbes (southwest Algeria) were named Nonomuraea sp. (Badji et al. 2007) *Streptomyces youssoufiensis* sp. nov., was identified from Moroccan phosphate mine by Hamdali et al. 2011. In 2019, Nafis et al. reported the isolation of different Actinobacterial genera (*Streptomyces, Nocardioides, Saccharomonospora, Actinomadura*, and *Prauserella)* from Moroccan desert soil of Merzouga, Draa sfar mining sites which exhibits plant growth-promoting activities. The Actinobacterial isolates from the Algerian Saharan desert (Badji et al. 2006), Atacama Desert (Rateb et al. 2011), Egyptian desert (Koberl et al. 2011), Qinghai-Tibet Plateau (Ding et al. 2013), and Thar desert (Thumar et al. 2010) were studied for bioactive metabolites. The chloramphenicol was the first antibiotic isolated from Saccharothrix sp. PAL54A strain from Saharan soil in Ghardaïa (Aouiche et al. 2012). Currently, the focus has been diverted to extremophilic Actinobacteria with the anticipation that these organisms would add a novel arena to antimicrobial product research (Zitouni et al. 2004; Dhanasekaran et al. 2014).

Man is always curious to know about the existence of life on another planet such as Mars. This has led to the establishment of the Mars Desert Research Station (MDRS) where astrobiology research has been undertaken. MDRS in southeast Utah is situated in a cold arid desert where the

conditions are comparable to those on Mars. The microbial flora of this terrestrial Mars analog revealed several extremophilic Actinobacteria similar to uncultured Actinobacteria of the cold desert of McMurdo Dry Valleys, Antarctica and methane hydrate-bearing deep subsurface marine sediments in Nankai Trough (Japan). Most of them mainly belong to order Acidimicrobiales (Xu et al. 2018) thus indicative of putative xerophilic micro-organisms. Others were related to *Sporichthya* sp. from an ice core and is psychrophilic in nature (Direito et al. 2011)

Hydrophiles

Actinobacteria are widely distributed in aquatic habitats both fresh and marine environment. The major Actinobacteria dwelling in freshwater include *Actinoplanes, Micromonospora, Rhodococcus, Streptomyces,* and endospore-forming *Thermoactinomyces* (Cross et al. *1*981). The common freshwater Actinobacteria and found to be indigenous to such habitats is *Micromonospora* where they turnover complex sugars such as cellulose, chitin, and lignin.

Halophiles-Marine and Mangrove ecosystem

Actinobacteria is part of the marine microbial community of sediment samples that originated from terrestrial habitats and were disseminated to the sea in the form of resistant spores (Goodfellow & Haynes 1984). The first marine Actinomycete species to be characterized is *Rhodococcus marinonascene* (Helmke & Weyland 1984) followed by *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia maris,* and *Verrucosispora* (Jenson et al. 2004). Grossart et al. (2004) have reported that Actinobacteria is the major phylum colonizing marine organic aggregates which helps in the disintegration and mineralization of organic matter (Magarvey et al. 2004). Recent studies established the presence of indigenous marine Actinobacteria in the oceans and in different marine habitats (Stach et al. *2*004). Marine Actinobacteria from sponges was isolated using nutrient supplements and

enzymes have been reported (Kim et al. 2005). The discovery of some strains that display specific marine adaptations and others that are metabolically active in marine sediments was described (Jensen et al. 2008). The culture-dependent studies have shown the existence of indigenous Actinobacteria in the oceans. Various marine Actinobacteria, such as *Dietzia maris, Rhodococcus erythropolis,* and *Kocuria erythromyxa,* from a subseafloor sediment core collected at a depth of 1225 meters off Hokkaido was isolated by Innagaki et al. (2003). Actinobacteria from soil samples belonging to the salt pan regions of Cuddalore, Parangipettai, India was isolated and screened for primary antibacterial activity by Dhanasekaran et al. (2005). *Streptomyces* sp. and *Saccharomonospora* sp. showed promising antimicrobial activity against different bacteria. The marine environment is a less exploited source of Actinobacterial diversity and their metabolites. Compared to the terrestrial microflora, marine Actinobacteria growing in the extremely saline environment produce different types of metabolites (Cross 1982). They thrive under high pressure and anaerobic conditions at temperatures just below 0 - 8 °C on the deep seafloor to high acidic conditions at temperatures of over 8 - 100 °C at the mid-ocean folds (Stach et al. 2005). This is reflected in the wide genetic and metabolic diversity shown by marine Actinobacteria (Bull et al. 2005).

There has been a growing interest in marine water habitat as a source of Actinobacteria that produce beneficial metabolic products that are known to be the producers of half of the discovered bioactive secondary metabolites (Berdy 2005), which may be antitumor agents (Cragg & Newman 2009) notably antibiotics, immunosuppressive agents (Mann 2001) and enzymes (Hill and Prins 2016). So it is evident that Actinobacteria is an important source of biologically active compounds and it is highly unexplored. (Lee et al. 2014). Though several studies have been conducted in this area it is far inconclusive. Exploration of a wide range of Actinobacteria communities in the marine environment will definitely answer the quest for new metabolites in the future.

Mangrove forests are large ecosystems prevalent in tropics and subtropics; they make up over a quarter of the total coastline in the World (Saddhe et al. 2016). Mangroves form the transient ecosystem which exhibits the edge effect and shows wide species richness. They provide habitat for different flora and fauna and also a treasure trove for the microbial biome. Since it is an ecotone habit, it is rich in biodiversity. It is a link between land and sea which serves as breeding and nursing grounds for aquatic organisms. This ecosystem is an active geographical zone that is rich in organic sediments remains the homeland of microbes. The mangrove ecosystem is saline and highly rich in organic matter and an untapped source for screening and isolation of potential bioactive metabolites (Newman & Cragg 2007). These bioactive compounds have a unique structure and chemical features which is not found in natural terrestrial products (Kathiresan & Bingham 2001). The microbes in mangrove habitats not only produce primary and secondary metabolites but are also involved in an important ecological role in soil organic matter decomposition and mineralization (Ghosh et al. 2011). Actinobacterial species such as *Streptomyces, Micromonospora,* and *Nocardioform* were found to be abundant, in the anaerobic mangrove rhizosphere, which is 1000 to 10000 times smaller than the aerobic population because of tidal influence (Tan & Cao 2009). *Nocardia* isolated from mangrove habitat produced metabolites that strongly suppressed human cell lines, such as gastric adenocarcinoma (Schneider 2009). Actinobacteria play an active role among the mangrove bacterial communities, because of their divergence and capacity to synthesize chemical compounds of high economic value (Watve et al. 1999) Actinobacteria participate in many important biochemical processes in the soil. From the marine ecosystem *Actinomyces, Actinopolispora, Micromonospora, Micropolispora, Nocardia, Rhodococcus*, *Streptomyces*, *Streptosporangium* and *Streptoerticillium* are reported so far (Lechevalier & Lechevalier 1970). Several studies indicate the importance of Actinobacteria in antibiotic metabolite production. The metabolites

are used as anticancer compounds, antifouling compounds, bioremediation, PGPR, immunomodulators, etc (Singh & Dubey 2018). Studies on the biodiversity of Actinobacteria from the mangrove ecosystem are important for biotechnological exploitation. Actinobacteria are well known for the production of commercially important bioactive compounds and antitumor agents in addition to enzymes of industrial interest (Khattab 2017). It has been shown that approximately 203 of the naturally occurring antibiotics are from Actinobacteria (Takizawa et al. 1993). Actinobacterial community, which resides in mangrove sediments, are poorly studied (Sivakumar 2001). Currently, enrichment techniques, new selection methods have led to the isolation of novel Actinobacteria from sediment samples (Jensen & Lauro 2008). There is a huge prospect for the isolation of novel secondary metabolites from Actinobacteria of mangrove habitat. The metabolites from these Actinobacteria possess distinct chemical structures that may lead to the synthesis of new drugs that could be used against resistant pathogens (Stach et al. 2004)

Acidophiles

The proliferation of *Actinobacteria* in the soil is preferred by low humidity, especially when the spores are immersed in water. The growth is very limited and may be halted in dry soils (Kim et al. 2003). They prefer soils with a neutral pH. and grow well in soil with pH between 6 and 9. But there are reports for the isolation of some strains of *Streptomyces* from acidic soils (pH 3. 5) (Kim et al. 2003) and acidic forest and mine drainage soil, that grow in the pH range from about 3.5 to 6.5. *Streptacidiphilus anmyonensis* sp. nov., *Streptacidiphilus rugosus* sp. nov. and *Streptacidiphilus melanogenes sp.* nov. (Cho et al. 2008) grow well in acidic soil. The acidophilic Actinobacterium is an important group to study the novel bioactive metabolites. This is evident from the genome-based studies conducted on *Streptomyces yeochonensis* CN732 and the presence of cysteine transpeptidases among the Biosynthetic Gene Clusters (BGC) which indicates their role in the biosynthesis of secondary metabolites

(Malik et al. 2020). The acidophilic Actinobacteria are considered as the sources of polyketides such as polyether ionophores that exhibit antagonistic activity against drug-resistant bacteria and parasites (Wang et al. 2011).

Lithophile (stone dwelling)

Several reports for stone-dwelling Actinobacteria are available recently which shows growing interest and suggests more studies in this arena. Actinocommunity is known for its role in ecological succession as one of the pioneer communities. The major family which has been isolated from the stone niche is *Geodermatophilaceae*, an Actinobacterial family (Sghaier 2016) which is endemic to soil (Sen et al. 2014) that consists of three genera: *Geodermatophilus, Blastococcus* and *Modestobacter* that was isolated from desert soils (Luedemann 1968), seawater (Ahrens & Moll 1970) and Antarctic regolith (Mevs et al. 2000), respectively. The omnipresence of *Geodermatophilaceae* in different biotopes including prominent rocks (Eppard et al. 1996) and desert sandy soils (Montero-Calasanz et al. 2012) and its evolutionary ability is intriguing. The soil and stone niches have provided us knowledge regarding the wide distribution of *Geodermatophilaceae* (Gtari et al. 2012; Normand et al. 2012), and has created interest in their evolutionary and adaptation mechanisms to harsh environments.

Cave dwelling Actinobacteria

Caves are seldom explored and is prevalent with different mineral structures, permafrost and previously unknown organisms that have evolved in a microenvironment with more or less constant temperature, humidity, air composition and other conditions over long periods (Culver & Sket 2000). These biomes are of great interest due to the presence of microorganisms, which have been subjected to evolution in stable conditions for a long duration (Grady 2005). Also, caves are contained zones with limited resources and have little energy exchange with the environment. Maciejewska et al. (2015) isolated the strain *Streptomyces lunaelactis* sp. nov. from moon milk speleothem collected in the cave

'Grotte des Collemboles' (Comblain-au-Pont, Belgium)

The novel species was isolated from caves and cave-related habitats and were from the genus *Streptomyces*, *Amycolatopsis* and *Nocardia*. *Antricoccus, Beutenbergia, Knoellia, Lysinibacter Spelaeicoccus* and *Sphaerimonospora* are the novel genera isolated from cave soils. The genus *Hoyosella* was isolated from the biofilm on the ceiling and wall of Altamira cave, Spain (Jurado et al. 2009). The cave environment which is extremophilic in nature, poses pressure for the inhabitant microorganisms at the genomic level, resulting in the evolution of new species and their production of more metabolites (Tiwari & Gupta 2013). Therefore, caves are considered an exquisite ecosystem for the identification of new Actinobacteria.

Another interesting aspect is the diversity of Actinobacteria in beehives in Thailand was studied by Promnuan et al. (2009). They had isolated and identified thirty-two isolates using morphological, physiological, chemical and molecular characterization. The major isolates have belonged to the genera *Streptomyces, Nonomuraea, Nocardiopsis* and *Actinomadura*.

2.4 Bioactive metabolites

Bioactive metabolites are compounds that originated from living organisms that affect the different activities of living cells. The term "bioactive" is comprised of two words: bio- and -active. Bio- from the Greek (βío-) "bios" [bio-, -bio], refers to life and –active from the Latin "activus", means: dynamic, full of energy, with energy or involves an activity (Bernard & Dromard 2011). This activity represents all phenomena of life, a functioning or a process. These effects may be progressive or deleterious depending on the substance, the dose or the bioavailability.

Out of the 22500 microbial metabolites discovered so far, about 17% (3800) are from unicellular bacteria *Bacillus* spp. and *Pseudomonas* spp.); 45% (10 100) are products of Actinobacterial origin; and about 38% (8, 600) are from fungi (Demain & Sanchez 2009). Amidst the filamentous Actinobacteria, approximately 75% (7600) of metabolites are produced by species of

the genus *Streptomyces* (Berdy J. 2005; Lam 2007). 140 Actinobacteria genera have been described to date. Genus *Streptomyces* alone produces a large number of bioactive molecules. The genera *Saccharopolyspora*, *Amycolatopsis, Micromonospora* and *Actinoplanes* produce bioactive metabolites but at a lower scale compared to *Streptomyces* (Solanki et al. 2008). It has great biosynthetic potential that remains unopposed without a possible competitor among other microbial groups. Another genus *Arthrobacter* spp. exhibit great metabolic versatility and are able to degrade pollutants and xenobiotics such as heavy metals (As, Cd, Cr, Hg). But the prospect of finding highly potential Actinobacteria from terrestrial habitats is reduced due to the wide exploitation for antibiotic production. So, attention has been diverted to unexploited and extremophilic habitats.

Human activities are creating new compounds and the microbial community is constantly evolving to cope up with this stress. The mechanism of action of the microbial community in vitro and in vivo conditions may deduce the remedy for degradation of the environment as well as the drug resistance in organisms. The metabolic pathways involved in xenobiotic degradation is not yet studied in a comprehensive manner (Maurice et al. 2013). The detailed study of the xenobiotic activity of pathogenic and nonpathogenic Actinobacteria in different niches may help us to understand the intricate mechanism of microbial community with the environment. It may open up new avenues of understanding that could have many applications in fields diverse as agriculture, biotechnology, ecosystem monitoring. Integrated pest management utilizing microbial pesticides is gathering momentum. This is because they are more specific, have low relative cost and are more eco-friendly (Kesho,2020). Many reports indicated the important role played by Actinobacteria in the management of *Spodopetra littoralis* (Bream et al. 2001), *S. litura* (Arasu et al. 2013), *Musca domestica* (Ghazal et al. 2001), *Culex quinquefasciatus* (Khawagh et al. 2011), *Drosophila melanogaster* (Gadelhak et al. 2005), *Helicover paarmigera* (Bapatla et al. 2021),

Anopheles mosquito larvae (Dhanasekaran et al. 2010). Among the biological control agents derived from different microbes, Actinobacteria especially *Streptomyces* sp. are some of the most important microbial resources which can provide potential new bioactive compounds for use as insect-control agents (Kaur et al. 2016).

2.5 Streptomyces genera

The most widely distributed genus of Actinobacteria, Streptomyces has been the focus of research by biologists because of the commercial applicability of the substances produced.*Streptomyces are* mainly soil -dwelling saprophytes having a large genome size of 6-12 Mbp (Tidjani et al. 2019) *S. cavourensis* is a producer of the antibiotic chromomycin and *S. michiganensis* is involved in the synthesis of anthelmintic acid and antiprotozoal substances (Silva et al. 2013).

Streptomyces genus of Actinobacteria has been known for its prolific production of metabolites which may help to combat antibiotic-resistant pathogens (Berdy 2005; Jones & Elliot 2017). Around 13, 700 bioactive secondary metabolites are produced from Actinobacteria in which *Streptomyces* spp. alone comprised approximately 10, 400 (75%) and (39%) of entire microbial products (Sousa et al. 2016). Notably many such secondary metabolites are potent antibiotics, a trait that has turned *Streptomyces* spp. into the primary antibiotic-producing organism exploited by the pharmaceutical industry (Atta et al. 2015).An orally active metabolite that exhibits immunosuppressive effect Everolimus is a derivative of rapamycin, originally produced by the actinomycete *Streptomyces hygroscopoicus* (Chapman & Perry 2004). A cyclic lipopeptide, Daptomycin which possesses good antibacterial activity and has been approved for the treatment of complicated skin infections was produced from *Streptomyces roseosporus*. “Delta-Indomycinone: a new Member of Pluramycin class of antibiotics from marine Actinobacteria and “Himalomycin A and B were isolated from marine *Streptomyces* sp. (Maskey et al. 2003). Studies have revealed that in spite of the occurrence

of genes that are present in antibiotic synthesis in the genome, Actinobacteria fail to exhibit antagonistic properties *in vitro.* Identification of natural conditions for the expression of dormant genes is crucial for the screening and isolation of novel antibiotics (Trenozhnikova & Azizan 2018).

Streptomyces pluripotens MUSC 137 isolated from mangrove soil in Malaysia shows antioxidative and cytotoxic activities were established by Ser et al. 2015. Similarly, antimicrobial and cytotoxic Activity of marine *Streptomyces parvulus* VITJS11 crude extract was reported by Naine et al. 2015. Bream et al. showed potent biological activity of secondary metabolites of Actinobacteria such as *Streptomyces* and *Streptoverticillum* against *S. littoralis* which caused larval and pupal mortality. Several metabolites from genus *Streptomyces,* such as avermectin, prasinons, doramectin, milbemycin, nanchangmycin, dianemycin and spinosad have been established as potential protective agents against a variety of insect pests and are friendly to the environment (Omura 2008). These microbial pesticides offer an alternative to chemical insecticides with increased target specificity and ecological safety so that they are used either uniquely or in combination with other pest management programs.

Actinobacteria have been reported to produce an extensive range of prospective commercial enzymes that can be used in biotechnological applications and pharmaceutical fields. Advances in sequencing technology and bioinformatics field have made it possible to study microbial enzyme production by using proteomics and transcriptomics (Pieper 2005). Amylases from *Streptomyces* sp. has an important role in biotechnological applications in different industries. Pectinases are the enzymes used in the food industry which had been isolated from different species of *Streptomyces* such as *S. lydicus* (Jacob et al. 2008). They find application in extraction and clarification of wines, juices, oils, flavoring compounds and preparation of linen fabrics and hemp manufacture (Horikoshi 1999). *Streptomyces* are the main producers of xylanases in the Actinobacterial phylum. Xylan is the most important component of

hemicelluloses and it is used in the improvement of the pulp and bio bleaching industry (Ghorbel & Prakash 2012). Enzymes from the Actinobacterial population find wide application in different areas of industry and are emerging as a potent source of novel enzymes. Pigments are industrially important as they are used for coloring textiles, cosmetics, etc. Efforts have been taken to replace synthetic colors with natural sources since the former has several harmful effects. Numerous pigments are synthesized by Actinobacteria using natural and artificial media. Subhash and Kulkarni (2015) could obtain red color melanin by *Streptomyces bikiniensis* using a tyrosine medium and used it as an antimicrobial agent. *Streptomyces torulosus* produces three different dyes with various colors and were used for dyeing wool and polyamide fabrics (Kheiralla et al. 2016). Recently, bio-cosmetics are pushing through the cosmetics industry in the world and the demand for biocosmetics is rising and quite significant. Screening and isolation of Actinobacteria from underexplored habitat may definitely boost the cosmetics and pharmaceutical industries in the future.

The phylum Actinobacteria with black pigments melanin is involved in the cycling of organic compounds and also in the production of soil humic acid (Schaeffer et al. 2015).It has also been reported that mixed cultures are more effective in bioremediation in comparable to pure cultures (Joutey et al. 2013).The production of various bioactive compounds has been identified however, specific metabolic pathways of synthesis and their regulation remains unexplored.

2.6 Characterization

Soil microbes are greatly important in agroecosystem, the microbial community structure assessment with accurate and reliable methodology is necessary. The methods for studying soil microbial diversity can be categorized into microscopy, biochemical-based and molecular-based methods (Kirk et al. 2004). The transmission electron microscope studies have provided much useful information about the structure of organisms belonging to various genera of the Actinobacteria Details of the

internal, vegetative and reproductive structures of several genera have been obtained from the examination of ultra-thin sections. The development of spores in sporangia-forming genera have been given by Rancourt & Lechevalier (1963) who studied *Microellobospora.,* Lechevalier & Holbert (1965) who examined *Actinoplanes* and Lechevalier & Holbert (1966) who studied the genera *Streptosporangium, Spirillospora* and *Actinoplanes.*

Gas chromatography-mass spectrometry (GC-MS) is a hybrid analytical technique that couples the separation capabilities of GC with the detection properties of MS to provide higher efficiency of sample analyses. While GC can separate volatile components in a sample, MS helps fragment the components and identify them on the basis of their mass. GC-MS provides enhanced sample identification, higher sensitivity, an increased range of analyzable samples, and faster results, which enable a whole new range of applications for GC-MS in several areas (Sahil et.al. 2011). Microbial extracts can be subjected to GC-MS for identifying and characterizing the bioactive compounds.

2.7 Tea Industry

Tea industry is one of the oldest industries in India. In India the four main tea-producing states are Assam, West Bengal, Kerala and Tamil Nadu. Tea is also grown in parts of Tripura and Himachal Pradesh. There are more than one million workers in the tea industry in India comprising mainly of scheduled castes, tribes and ethnic minorities of which more than 50% are women. (CEC report http://www.cec-india.org)

The Darjeeling hills and Dooars region of West Bengal have 283 tea gardens, employing 350, 000 permanent and casual workers, who earn Rs 176 per day besides the weekly ration. Sprawling across the districts of Darjeeling, Jalpaiguri and Alipurduars, the belt has another 40, 000 small growers who employ one lakh laborers. 1,86, 559 families residing in the tea estates of hill, Terai and Dooars areas of North Bengal. Chengmari T.E. has the highest no. of families i.e. 4, 950 whereas Girish Chandra T.E. has the lowest no. of families i.e. 05. Out of

283 Tea Estates, only 166 Tea Estates have hospitals. Out of these 166 Tea Estates only 56 Tea Estates have full time residential doctors. Other 110 Tea Estates hospitals depend on visiting doctors (Ghosh 2016). The five major health afflictions were identified among the estate workers. They are continuous cough/dry cough, low blood pressure/high blood pressure neck pain/shoulder pain, Respiratory and skin problems, Continuous Cough/ result of infection by cold and flu viruses.

Tea is one of the major plantation crops in the world. Tea plants are subjected to attack by many pests species and reduce productivity which affects the economy negatively. According to Chen & Chen (1989) 1034 species of arthropods, 82 species of nematodes 1 algal and 350 fungal diseases are associated with tea plants globally. At present, India is the largest producer of pesticides in Asia and ranks twelfth in the world for the use of pesticides with an annual production of 90, 000 tons (www.teri.res.in/ pesticide.htm). Pesticides are poisonous substances and they are to be handled with extreme care. These are not easily degradable; they remain in soils, leach to groundwater and pollute the environment to a wide extent. These enter bodies of organisms, bio concentrates in the food chains and have an adverse impact on human health. The tea farmers have been applying different pesticides to reduce pest incidence in their tea gardens. Farmers are practicing cocktail preparation of two or more than two pesticides without knowing their compatibility, which is of serious concern to health and environment (Jallow et al. 2017). Again, based on the types of pesticides used, various signs and symptoms of diseases/ disorders have been observed among the tea growers and the relative risk also observed to be high (Shreshta & Thapa 2015) Absence of adequate protective measures were noticed that have increased the declining state of the health of farmers. To reduce the pesticide environment pollution and MRLs on tea product, safer pesticides as a last resort have to be considered (Gurusubramanian et al. 2008). Recently, microbial and botanical bio-pesticides have gained popularity against different insect pests of various

crops. Among the biological control agents derived from different microbes, Actinobacteria especially *Streptomyces* sp. are one of the most important microbial resources which can provide potential new bioactive compounds for use as an insect-control agent. Many reports indicated the important role played by Actinobacteria and their secondary metabolites in the management of *Spodopetra littoralis, Culex quinquefasciatus, Helicoverpa armigera* (Yandigeri 2021).

Among biological approaches, the use of microbes with degradative ability is considered the most efficient and cost-effective option to clean pesticide-contaminated sites (Massiha et.al 2011). Several groups of Actinobacteria are capable of removing heavy metals from polluted environments.

2.8 Bioinformatic studies.

Bioinformatics tools become relevant in this aspect. The language of DNA which is the four-letter alphabet that is expressed as a triplet codon for the amino acid is the key for fundamental gene expression. The degeneracy of codons for 18 amino acids except methionine and tryptophan showed that different sequences of DNA produce identical protein sequences (Knight et al. 2001). The degeneracy mainly occurred in the third position of the codon. Data from whole-genome sequences help to study the preference of codons among organisms. It has been noticed that variation of choice of codons to represent amino acids is not only observed among species from the different taxonomic group, but also showed significant variation among individuals of the same species, across different genes in the same genome and even across regions in the same gene (Sharp et al. 2005). But, the codon bias is most prominent in species from different taxonomic groups even in proteins with identical functions. This phenomenon of species-specific codon choice is known as “codon dialect” which signifies the codon-usage bias observed across different organisms (Ikemura 1985).

CODON USAGE

The codon usage pattern is a unique feature of a particular organism. It helps us to understand the gene expression,

horizontal gene transfer and also enables to determine phylogenetic relationships between organisms. The study of codon usage (Zhu et al. 2008) patterns of several genes and genomes is a popular technique to characterize and analyze genomic trends from a bioinformatics-based perspective. Codon usage patterns and preferences vary significantly within and between organisms (Sen et al. 2007; Sharp et al. 1987).

CODON W software developed by Pedan in 1999 became very popular and widely used for studying codon usage and multivariate analysis because of its error-free analysis. The parameters such as GC content (amount of guanine-cytosine in the nucleotide sequences), GC3 content (frequency of either G or C nucleotides in the third position of synonymous codon), the effective number of codons used in a gene (Nc), frequency of optimal codons (Fop), CBI (codon bias index), GRAVY (hydrophobicity of amino acids) are included in this analysis. The most obvious factor that determines codon usage is mutational bias that shapes genome GC composition. Mutational bias is responsible not only for intergenetic difference in codon usage but also for codon usage bias within the same genome (Ermolaeva 2001). Most of the organisms with a balanced AT/GC genome have codon heterogeneity (Sen et al. 2007). Highly expressed genes contain a higher percentage of codons that are transnationally optimal (Ikemura 1985). Codon heterogeneity in the genome can be studied by GC content, GC 3 content, effective number of codons (Nc). Nc measures the overall codon bias of synonymous codons (Wright 1990). It ranges from 20 (in the case of one codon for one amino acid) to 61 (where all codons are used). The GC content estimates the amount of the guanine-cytosine in the nucleotide sequences. The GC3 infers the frequency of either G orC nucleotides present in the third position of the synonymous codon. This is not applicable to methionine, tryptophan and the termination codon. These values have a direct correlation with Nc. Its measures the synonymous codon usage of genes and its value ranges from 20-62 (Peden,1999).

The frequency of optimal codons *(Fop)* is the fraction of synonymous codons

which are optimally used. It is given by (*Fop) =Noc/ Nsc* where N represents the frequency of each codon, *Noc* and *Nsc* represents optimal codons and synonymous codons respectively. The *Fop* values range from 0 to 1. If the value of *Fop* is 1, it shows the usage of all optimal codons.

GRAVY scores determine the hydropathic indices of amino acids (Kyte & Doolittle 1982). A positive score indicates the hydrophobic nature and a negative score shows the hydrophilic nature of amino acids.

The Relative Synonymous Codon Usage (RSCU) values for the genes are calculated to understand the characteristics of synonymous codon usage without the confounding influence of amino acid composition of different gene sample (Sharp & Li 1986). The codons with RSCU values >1.0 have positive codon usage bias (abundant codons), while those with RSCU values <1.0 have negative codon usage bias (less-abundant codons); and when the RSCU values are 1.0, it means that these codons are chosen equally or randomly, indicates lack of bias (Tsai et al. 2007). The RSCU is the observed frequency of a codon divided by the frequency expected if all synonymous codons for that amino acid are used equally. The synonymous codons with RSCU more than 1.6 were thought to be over-represented, while the synonymous codons with RSCU less than 0.6 were regarded as under-represented (Wong et al. 2010). The RSCU values are particularly useful in comparing codon usage between genes that differ in size and amino acid composition.

Codon adaptation index is a widely used index for studying gene expression in general and the efficiency of translation in particular. CAI has been used extensively in biological research. It has been used to study functional conservation of gene expression across different microbial species (Lithwick & Margalit 2005), to predict protein production (Futcher et al. 1999; Gygi et al. 1999), and to optimize DNA vaccines (Ruiz et al. 2006). CAI has recently been used for detecting lateral gene transfer (Bodilis

& Barray 2006).

The CAI program in EMBOSS (Rice et al. 2000), typically referred to as the EMBOSS.cai program is most popularly used. Another software for computing CAI is the web application called CAI Calculator 2 (Wu et al. 2005). The improved CAI is implemented as a new function in DAMBE (Xia & Xie 2001, freely available at http:// dambe.bio.uottawa.ca/dambe.asp), which uses a windowed user interface. DAMBE can read 20 standard sequence file-formats including files in the simple FASTA format and the more involved GenBank format or trace files from automatic sequencers. The CAI function can be accessed by clicking 'Seq. Analysis Codon usage'. The ensuing dialog box is self-explanatory, except that, for species without a reference set of highly expressed genes, a codon table based on tRNA anticodon can be used by clicking the alternative option button.

CAI values vary from 0 to 1 and higher CAI values indicate that gene of interest has a codon usage pattern more similar to the highly expressed genes (Sharp & Li 1987).

Codon usage bias (CUB) is usually defined as a species-specific deviation from uniform codon usage in the coding regions of genomic sequences. This bias is possible due to the redundancy of the genetic code, which allows differential use of synonymous codons (Behura & Severson,2013). The particular pattern of bias observed in a given species is thought to be the product of drift and selection pressures acting on a number of parameters, but mainly on tRNA gene copy number and genomic % GC content. CUB is therefore a strong species-specific statistic with numerous applications, such as gene prediction or the identification of laterally transferred genes.

Protein energetic cost:

It can be defined as the energy consumed for the synthesis of an amino acid encoded by a specific functional codon. Mostly, the energy cost of potentially highly expressed genes is lower than the energy budget of the rest of the proteome. But it cannot be applied to all organisms. In the case of Actinobacteria, it has been shown that

energy cost varies with its niche (Sarkar et. al 2018)

CAI is a major index to measure the m RNA expression level. Generally, genes with 10% of the highest and lowest CAI value are chosen as potentially highly expressed and potentially lowly expressed genes respectively. The remaining genes are considered as potentially medially expressed genes. DAMBE software calculates the EC (Dambe ver. 6.4.81). The EC values can be analyzed statistically using ANOVA test, F-test and t-test. Heat maps are generated using R statistical software (Kim 2019).

2.9 Metabarcoding analysis

We are grossly ignorant of bacterial life on earth. Environmental microbiologists estimate that less than 2% of bacteria can be cultured in the laboratory (Wade 2002). In the mouth, we do rather better, with about 50% of the oral microflora being culturable. For other body sites, the figure is unknown but is likely to be similar to that found in the mouth or higher. For example, the colonic microflora is suspected to be predominantly uncultivable. It is therefore likely on numerical grounds alone that uncultivable and therefore uncharacterized organisms are responsible for several oral and other human infections. The best example is syphilis, caused by the spirochete *Treponema pallidum*, which remains unculturable (Radolf et al. 2016).

Most of the microbes don't grow under *in vitro* conditions. The proportion of uncultivable microbes to cultivable microbes is still very high(Nichols et al. 2010) To circumvent such cultivation limitations, strategies have been developed based on the extraction of microbial DNA directly from an environmental sample and its subsequent analysis or exploitation (for biotechnological purposes) independent of its original host. This approach, which is based on the recovery of a sample's microbial metagenome (the sum of all microbial genomes), has, in theory, great potential for biotechnological and ecological studies of the system under investigation, for example, soil(Lombard et al. 2011). It is recognized as the best option to access the microbial genetic diversity present. Such a metagenomics-based

view of the community will aid in enhancing our understanding of microbial functioning and interactions in a soil ecosystem. Shotgun metagenomic sequencing has enabled to detect and characterize the diversity and function of the microbial communities. This technology will help to discover new microbial products as well as new species. The metagenomic study involve several steps starting with the extraction of DNA from the source, library construction, sequencing, data analysis etc. Data screening can be done by either sequence based or by function driven analysis. The specifically designed primers or probes based on the sequences of previously identified bioactive compounds are used in sequence driven analysis. These primers are used for PCR amplification of the metaDNA and subsequently sequenced and cloned to the expression vectors. Contrastingly, in the function driven analysis aids us to identify novel compounds. Here in this method, DNA will be subjected to restriction digestion and a library of clones are prepared which will be further analysed for the production or synthesis of new molecules (Datta et al. 2020). It has potential for numerous industrial applications when used to isolate genes involved in the synthesis of new molecules such as antibiotics of the polyketide class by cloning of such genes into hosts that express them. The extraction of the metagenome and its sequencing will enable the physiological requirements of the dominating non-cultivated bacteria to be deciphered, which will help to determine the appropriate growth conditions for these recalcitrant bacteria (Lombard et al. 2011). This technology will also revolutionize the clinical diagnosis area. Though in the nascent stage, metagenome has the potential in the profiling of the resistomes from different environments (Sukhum et al. 2019).

Actinobacteria is the second most diverse group of microbes after Proteobacteria. Its diversity comprises its presence in biotopes of extreme habitats such as deserts, minefields, stones, deep sea beds, etc. Actinobacteria which is a primitive and prominent phylum among prokaryotes are distributed in a wide range of ecological niches. Thus, they establish a

substantial proportion of the telluric microflora which is of extensive interest to the scientific community. Some of the most important microbes include *Streptomyces* which is the biological warehouse of various antibiotics. *Streptomyces* genus of Actinobacteria has been known for its prolific production of metabolites which may help to combat antibiotic-resistant pathogens (Berdy 2005). However, specific metabolic pathways of synthesis and their regulation remain unexplored. During the last decade many genomes and their plasmids of Actinobacteria have been sequenced (13402 complete genomes, to date as per IMG database) and made available for data mining which may deliver access to several potential biocatalysts that await interpretation.

Yet, Actinobacterial research for the most part is rather recent and knowledge on many members is still elusive. This review is an attempt to give a comprehensive account of various applications of Actinobacteria especially from extremophilic habitats based on the knowledge available today. The utilization of new technologies for culture, identification and classification of microbial flora, makes the Actinobacterial research dynamic and promising. There is much more to be explored in this area. The research in this dimension will definitely provide immense information that will be highly beneficial to mankind in the future.■

Chapter 3
Materials & Methods

Science and everyday life cannot and should not be separated.

Rosalind Franklin

3.1 Survey on the health status of tea garden workers

A preliminary survey was conducted based on literature studies and other available national and international reports. Apart from that, various tea gardens were visited and questions were asked to the management and laborers. For this purpose, a survey datasheet has been prepared (Figure .3.1). The various stake holders such as resident physicians, Asha workers, health officials were interviewed.

3.2 Collection Sites

Different places of North Bengal, Kerala and Sunder ban were visited for the collection of soil. The soil samples were collected from mangroves of Sunder ban (West Bengal), Vyppin, Kochi (Kerala), Tea gardens of North Bengal, Vegetable

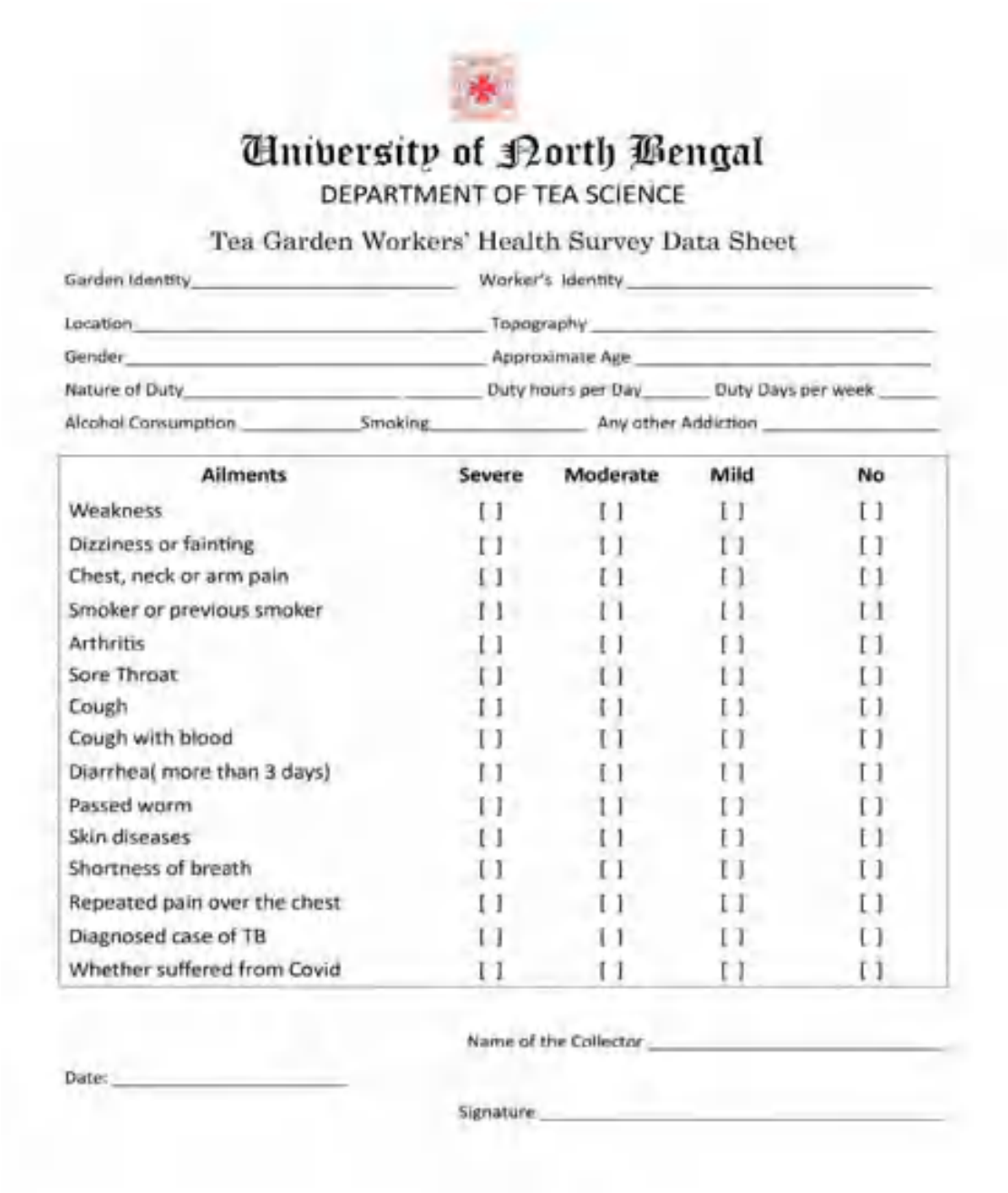
Table 3.1: Details of collection site for the present study

Biotopes	Location	District & State	Latitude	Longitude
Tea garden	Matigara	Darjeeling, West Bengal	26.7223°N	88.3810°E
Tea garden	Jalpaiguri	Jalpaiguri, West Bengal	26.5215°N	88.7196°E
Mangroves	Sunderbans	South 24 Paragnas, West Bengal	21.9497°N	89.1833°E
Mangroves	Vypin, Kochi	Ernakulam, Kerala	10.1024°N	76.2044°E
Vegetable field	Rangapani	Darjeeling, West Bengal	26.6651°N	88.3758°E
Paddy Field	Balurghat	North Dinajpur, West Bengal	25.2373°N	88.7831°E
Paddy Field	Jalpaiguri	Jalpaiguri, West Bengal	26.5215°N	88.7196°E
Rubber plantation	NBU campus	Darjeeling, West Bengal	26.7095°N	88.3542°E
Forests	Darjeeling hills	Darjeeling, West Bengal	27.0410°N	88.2663°E

fields (Rangapani), Paddy field of Balurghat, North Dinajpur (West Bengal), forest soils of Darjeeling hills, Rubber estate of University of North Bengal. For details about collection sites refer to Table 3.1 and map refer Figure. 3.2).

In isolating Actinobacteria, soil samples were collected from the study area. The soil samples were collected in sterile plastic containers, sealed tightly, and brought to the laboratory and stored at -20°C. In order to kill most of the Gram-negative bacteria, the soil was dried on the bench inside the laboratory for one week and further dried in a oven at 50°C to further destroy other vegetative microorganisms. The samples were weighed before and after drying. One gram of soil was and serially diluted using 0.85 % NaCl. Plating was then carried out using different media such as ISP4 Media, Streptomyces media, Actinobacterial isolation media (for composition refer Appendix C) onto which an antifungal agent (fluconazole 50 µg/ml) was added. The plates were incubated at 30°C for seven days.

Colonies showing resemblance with common Actinobacterial morphology were chosen from the agar plates using a sterile loop and inoculated on



University of North Bengal

DEPARTMENT OF TEA SCIENCE

Tea Garden Workers' Health Survey Data Sheet

Garden Identity_____ Worker's Identity_____

Location_____ Topography _____

Gender_____ Approximate Age _____

Nature of Duty_____ Duty hours per Day_____ Duty Days per week _____

Alcohol Consumption _____Smoking_____ Any other Addiction _____

Ailments	Severe	Moderate	Mild	No
Weakness	[ ]	[ ]	[ ]	[ ]
Dizziness or fainting	[ ]	[ ]	[ ]	[ ]
Chest, neck or arm pain	[ ]	[ ]	[ ]	[ ]
Smoker or previous smoker	[ ]	[ ]	[ ]	[ ]
Arthritis	[ ]	[ ]	[ ]	[ ]
Sore Throat	[ ]	[ ]	[ ]	[ ]
Cough	[ ]	[ ]	[ ]	[ ]
Cough with blood	[ ]	[ ]	[ ]	[ ]
Diarrhea(more than 3 days)	[ ]	[ ]	[ ]	[ ]
Passed worm	[ ]	[ ]	[ ]	[ ]
Skin diseases	[ ]	[ ]	[ ]	[ ]
Shortness of breath	[ ]	[ ]	[ ]	[ ]
Repeated pain over the chest	[ ]	[ ]	[ ]	[ ]
Diagnosed case of TB	[ ]	[ ]	[ ]	[ ]
Whether suffered from Covid	[ ]	[ ]	[ ]	[ ]

Name of the Collector _____

Date: _____

Signature _____

Figure 3.1: Sample Survey Datasheet

inorganic salts-starch agar (ISP 4 medium). plate The plates were then incubated at 30°C for 7 days. Morphologically different Actinobacterial cultures were selected and preserved using ISP4 agar slants as well as in 35% glycerol broth.

3.3 Morphological characterization

Different media were used to characterize the Actinobacterial isolates. The media used were

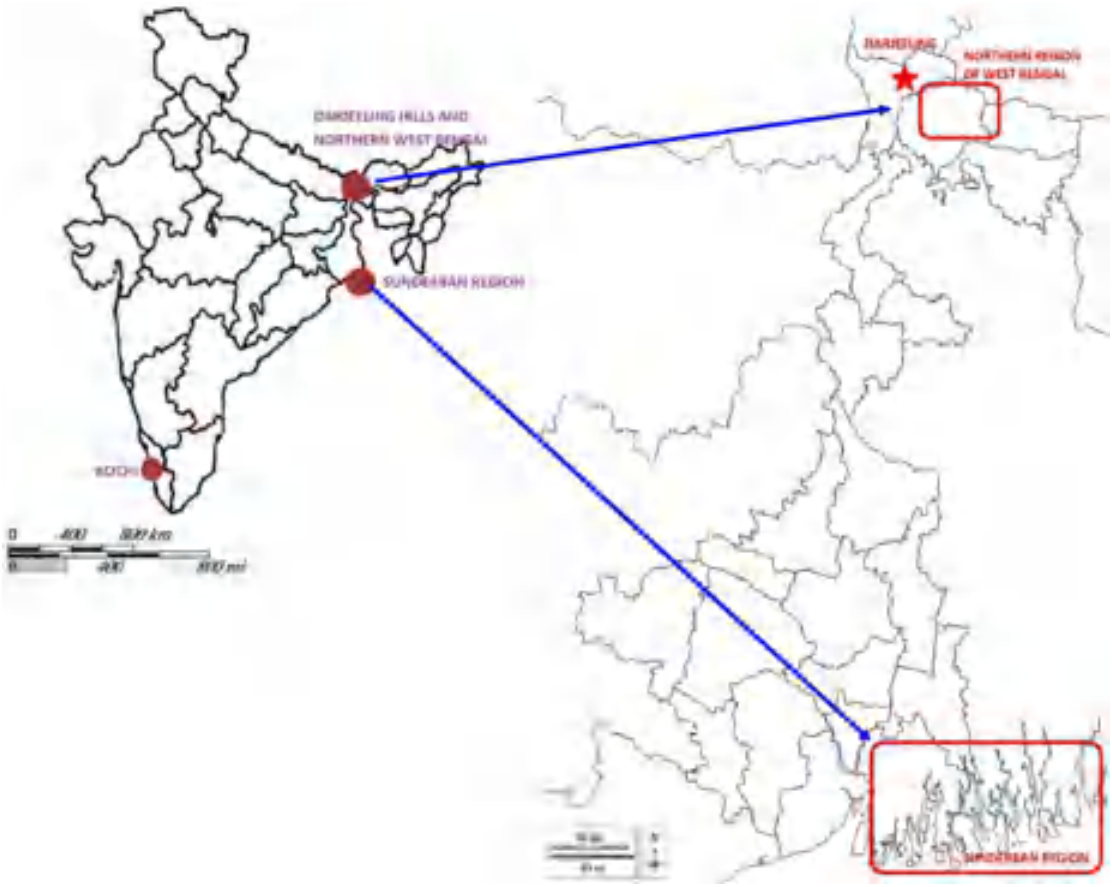


Figure 3.2: Map of collection sites

Streptomyces media (Kuster & Williams, 1964), ISP-4 (Shepherd 2010), Bennet Agar (Gordon & Smith, 1954), Nutrient Agar, (Schmitt 2005), Actinomycetes Isolation Media (Lee 2014). Compositions of the media have been provided in the Appendix C.

During retrieval and maintenance, most of the Actinobacterial cultures showed good growth on ISP4 agar and Streptomyces medium. The majority of the isolates produced powdery and leathery colonies with white or Grey color aerial mycelium. Microscopic observation revealed that the actinobacteria cultures showed the presence of both aerial and substrate mycelia in which the majority of them are *Streptomyces.* The cultures which showed only the presence of substrate mycelium were considered as non-*Streptomyces* and were not taken into consideration for further analysis.

3.4 Light Microscopy

The slide culture technique was used

to study the morphological characteristics of the isolates. The isolates were inoculated into ISP4 medium by streaking on the slide. Incubation of the slide was done at 30°C for 7 days. Staining with methylene blue was done followed by observation of the slides under the microscope (Olympus, India CX2liLEDES1).

Gram's staining

Crystal violet, gram's iodine, 95% ethyl alcohol and safranin were used in Gram staining. Aseptically, the isolated actinobacteria were separately placed on glass slides using a wire loop. Gram staining protocol was followed as per Aneja (1996). The slides were observed under a light microscope.

3.5 Electron microscopy

A standardized protocol (Saha et al. 2019) was used for SEM. The bacteria from the log phase culture (after 4 -5 days of incubation in Bennet Broth at 30°C) was taken. The bacterial cells were fixed with 3% Glutaraldehyde and 1% Osmium tetroxide, respectively, with interim phases of washing with buffered Glutaraldehyde and water. The fixed cells were attached to thin glass slides and dehydrated using an ascending alcoholic gradient. The dehydrated bacterial cells were splutter-coated with gold for 1 minute and loaded onto a metal stab with a carbon tab for SEM (JS MIT 100, JEOL Ltd., Tokyo, Japan).

3.6 Biochemical characterization:

Biochemical characteristics of the strains were determined following the methods of 'The International *Streptomyces* Project (ISP).

Melanin formation

The melanin production in microbes help in combating thermal, biochemical stresses and also also show metal chelating ability.

Melanin formation was determined as per the standardized protocol (El-Naggar and El-Ewasy 2017). Strains were inoculated into autoclaved ISP6 broth and ISP7 agar plates (Shirling and Gottlieb 1966). Plates and broth were incubated for 7 days at 30°C. The formation of black diffusible pigments around the colonies and blackening of the media indicated that

the isolates were melanin formers and recorded as a positive result.

Xanthine decomposition

Xanthine decomposition was done as per the protocol described by Berd (1973). Xanthine (0.4 g) was suspended in 10 ml of distilled water and autoclaved. The sterilized xanthine was added to 100 ml of sterile nutrient agar media, mixed properly, and poured into Petri plates. Strains were inoculated on the test plates and incubated for 7 days at 30° C. The appearance of a zone of clearance around the colony was taken as a *positive* result.

Esculin Hydrolysis

Esculin Hydrolysis was performed using the conventional protocol (Gordon et al. 1974). The strains were inoculated into Esculin agar (Esculin, 1 g; Ferric citrate, 0.5 g; Peptone, 10 g; NaCI, 5 g; Agar 10 g; distilled water to make up the volume to 1,000 ml) slants were prepared and were inoculated and incubated at 30°C for 7 days along with a control. The growth of the isolates with the blackening of the medium was recorded as a positive result.

Amylase production

The isolates were tested for Amylase production following the protocol described by Gopinath et al. (2017). Isolates were inoculated onto Starch Agar medium (Peptone 5g, Sodium chloride 5g, Yeast extract 1.5, peptone 1.500, Starch 2 g, Agar 15g, distilled water 1000 ml pH 7.4) and incubated for 4-5 days at 30°C. After incubation, the plates with growth were flooded with 3 ml of 1% iodine, the development of a clear zone around the colonies indicated the amylase production.

Tween 80 degradation

The tween hydrolysis releases the fatty acids which bind with the calcium present in the medium thereby forming insoluble crystals and the formation of those white precipitation around the colony indicated the Tween 80 degradation. (Ramnath et al. 2017). The isolates were grown on the Media containing 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 20 g agar and 10 ml (v/v) Tween 20/80 and made up the volume to 1 litre.

Nitrate reduction test

This test determines the production of an enzyme called nitrate reductase, which results in the reduction of nitrate (NO_3). The nitrate reduction test was done by the modified protocol of Maciejewska et al. (2017). Isolates were inoculated into nitrate broth tubes (Peptone 5 gm/Lit, Beef extract 3.0 gm/Lit, Potassium nitrate 1 gm/Lit, pH 7) and incubated for 5 days at 30°C along with a control. i.e. un inoculated nitrate broth. After incubation, nitrate reduction activity was detected by the addition of approximately 2 ml of sulphanilic acid (8 gms of sulphanilic in 1000ml of 5N acetic acid) and alpha-naphthyl amine (alpha-naphthylamine 5 g in 1000 ml 5N acetic acid) to the tubes. A change in coloration to red or pink indicated nitrate reduction. To further confirm reduction, zinc dust was added to the tubes to analyse, whether the strain has reduced nitrate beyond nitrite. Zinc powder catalyses the reduction of nitrate to nitrite. The change to the red color of the solution on addition of Zinc shows that nitrate was not reduced by the organism which indicate that the test organism is not capable of reducing nitrate. If there is no color change occurs after the addition of zinc, this proves that the organism reduced nitrate to one of the other nitrogen compounds and thus is a nitrate reducer (Li et al. 2016).

Gelatin hydrolysis

The production of the gelatinase enzyme by the isolates was tested by inoculating on the Gelatin agar media (Gelatin 15g/liter, Peptone 4g/liter, Yeast extract 1g/liter, Agar 15g/liter). The plates were incubated for 5 days at 30°C. After the growth, the plates were flooded with $HgCl_2$ solution (15% $HgCl_2$ in 20% HCl solution in distilled water). The formation of a clearance zone around the colonies was taken as positive for gelatinase enzyme production (Tsykerowich 1976; Saryono et al. 2019).

Starch hydrolysis

To test the hydrolysis of starch, isolates were grown on starch agar media and incubated for 5 days at 30° C. after the growth of the colonies, media was flooded with iodine solution to detect the clear zone around the colonies which is taken as positive for the test.

Table 3.2: list of antibiotics used (disk type Icosa-Universal-2 IC006, Hi-Media)

Antibiotics (mcg)	Symbol.	Conc.
Amikacin	Ak	30
Gentamicin	G	10
Netilmycin	Nt	10
Tobramycin	Tb	10
Chloramphenicol	C	30
Ceftazidime	Ca	30
Ceftriaxone	Ci	30
Cefadroxil	Cq	30
Cefoperazone	Cs	75
Vancomycin	Va	30
Nitrofurantoin	Nf	300
Erythromycin	E	15
Ampicillin	A	10
Amoxycillin	Am	10
Cloxacillin	Cx	1
Penicillin	P	10
Nalidixic acid	Na	10
Norfloxacin	Nx	10
Ciprofloxacin	Cf	5
Co-Trimoxazole	Co	25

Catalase production

The isolates were grown on nutrient slants and incubated for 5 days at 30° C. After the growth of the colonies,1 ml of 3% H_2O_2 was added directly to the fully grown cultures. The production of effervescence was taken as a positive result and was observed against a black background (Cappuccino and Sherman, 2008).

3.7 Intrinsic antibiotic resistance (IAR) test

Antibiotics

The experiment was carried out using multiple antibiotic discs Icosa-Universal-2 (IC006 - HIMEDIA). Each antibiotic strip contained 20 different discs of antibiotics. The symbols and concentrations of the respective antibiotics are given in Table 3.2

The resistance of isolates to different concentrations of antibiotics was determined using the agar disc diffusion system of Bauer *et al*. (1966). Freshly prepared Bennett Agar was poured on a 20 cm Petri plate (approx. 120 ml) each and was allowed to cool down for solidification. Approximately 2 ml of the fresh culture of isolates were poured at the center of the agar medium. The culture was spread evenly on the agar medium with a sterilized cotton swab to make a bacterial lawn. Each impregnated antibiotic disc was placed onto the agar surface adjusting the distance from the center. The plate was then left drying for 4-5 min. The plates were incubated at 30ºC for 3-4 days in an upright position.

The diameter of each zone of inhibition was observed and

recorded. A heat map was prepared for the same.

3.8 Antimicrobial studies:

The Cross streak method was used for confirmation of antibacterial activity for the active isolates (Velho -Pereira & Kamat,2011). The active isolates which were grown on Muller-Hinton agar plates were subjected to the cross streak method. The isolates were streaked in a single line on the agar plates and incubated at 30 ºC for 5-7 days. This was done to allow the production of an antibiotic substance by the isolates and for its diffusion to the agar. The distance between the test pathogen streak and the isolate was kept at 1cm. the length and width of the isolates were kept at 5cm and 0.5cm respectively. The streak of test pathogen was at a length of 3cm and width of 0.5 cm. The test pathogens namely *Escherichia coli (MTCC-452), Bacillus amyloliquefacie* (MTCC-10439), *Staphylococcus epidermidis* (MTCC-435), *Klebsiella pneumonia* (MTCC-109) were streaked perpendicular to the streak of the isolate. The plates were incubated for 10 days to observe whether there is lysis or inhibition of the growth of the test pathogen.

3.9 Molecular Characterization of isolated strains

Genomic DNA of strains was extracted and purified by the CTAB method (William et al. 2012). The composition of the solutions and the details of the chemicals used are provided in the appendix. The bacterial strains were grown in Nutrient Broth for 5 days at 30 ºC. Thirty ml of 24-48 hr. culture was put into a sterile Oakridge tube and kept in the ice bucket for at least 1hour. The tubes were centrifuged (REMI, Model No.C-24) at 5000Xg for 20 minutes at 4ºC.

The supernatant was discarded and the pellet obtained was used for further isolation. The pellet was suspended in 5.67 ml 1X TE buffer (pH 8.0). Fifty- five microliter (µl) of lysozyme (Conc. 10 mg/ml) was added to lyse the bacterial cell wall. The mixture was incubated for 5 min at room temperature.

To the mixture 300 µl of 10%, SDS was added and mixed well. Finally, 50µl of proteinase K (20mg/ml) was added and mixed well with gentle

swirling. The mixture was then incubated in a water bath (Rivotek, Cat# 50121002) for 1 hr at 37ºC. The tubes were mixed occasionally by gentle swirling in between the incubation period.

Following incubation, 1ml of 5M NaCl was added to the solution and mixed well. To that mixture, 0.8ml of hot (65ºC) CTAB/NaCl (see appendix C for composition) was added, mixed well and incubated at 65ºC for 10 min.

The solution was extracted with an equal volume of chloroform: isoamyl alcohol (24:1) followed by gentle spinning at 5000Xg for 10min at room temperature. A white interface containing the debris could be visible after centrifugation. The clear upper aqueous layer was transferred to a fresh Oakridge tube.

An equal volume of phenol/ chloroform/ isoamyl alcohol (25:24:1) was added to the solutions, mixed well and centrifuged at 5000Xg for 20 min. The upper clear aqueous layer was transferred to a fresh Oakridge tube.

Approximately 0.6 vol (8 ml) of chilled isopropanol stored at -20ºC was added to precipitate the nucleic acid. The tubes were stored at –20ºC for 2hr. or overnight. Thereafter, the tubes were centrifuged at 5000Xg for 30 min at 4ºC. The pellet thus obtained was washed with chilled 70% ethyl alcohol, air-dried and dissolved in 500µl 1X TE buffer (pH 8.0).

Purification of DNA

Major contaminants found in crude DNA like RNA and protein which may hinder further downstream processing was eliminated in the purification procedure as follows:

RNase A treatment

RNase A (10mg/ml) (refer appendix C for composition) was dissolved in 500µl 1X TE buffer (pH 8.0) containing the crude DNA. The mixture was incubated at 37ºC for 1 hr in a Dry water bath (GeNeiTM make, Cat#107173).

Protein clean up

The protein impurities were removed by adding 500µl of phenol/ chloroform/isoamyl alcohol (25:24:1) which was kept as stock at 4ºC

was added to the crude DNA. Microcentrifugation was done at 14000Xg for 2 min at room temperature. The aqueous phase was then transferred to a fresh microcentrifuge tube (Tarsons, Cat# 500010).

Ethanol precipitation of DNA

The volume of the DNA was measured visually and 0.1 volume of 3M Sodium acetate (pH 5.2) was added and mixed well.

It was followed by the addition of 2 volumes of ice-cold absolute ethyl alcohol and mixed by gentle swirling.

The tubes were placed on ice at −20ºC for 30 mins and centrifuged at 14,000Xg for 10-15 min at 4 ºC.

The DNA pellet was washed with 1 ml of chilled 70% ethanol, air-dried and finally dissolved in 100 µl of 1X TE buffer (pH 8.0).

Quantification of DNA

The assessment of purity and concentration of the isolated crude DNA is necessary for molecular biology experiments. Hence, both the spectrophotometric and agarose gel analysis were done to quantify the DNA.

Spectrophotometric measurement

Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England UK) was calibrated at 260nm and 280nm taking 600µl 1X TE buffer in a cuvette (Photon cell, New Jersey, USA).

DNA (6µl diluted in 594µl of 1XTE) was taken in a cuvette and the optical density (OD) was recorded at both 260nm and 280nm.

The DNA showing A260/A280 ratio of around 1.8 was considered to be of good quality.

The concentration (ng/µl) of isolated DNA was measured by using the following formula:

$DNA = OD_{260} \times 50 \times DF$

where DF stands for 'dilution factor.

DNA analysis by gel electrophoresis

A pure molecular biology grade, DNase free Agarose (0.8%, gelling temperature 36ºC) was used to cast the gel in 0.5X TBE (Tris-Borate-EDTA) buffer containing 7µl of Ethidium bromide (10mg/ml) on gel

platform (100x70mm) (Tarsons, Cat # 7024).

Five (5µl) of DNA samples were mixed with 3 µl of 6X gel loading dye and loaded in the wells carefully.

Ladders like Lambda DNA *Eco*RI-*Hind*III double digest (2 µl) and 100 bp ladder were used as molecular markers to determine the size of genomic DNA.

The gel was run at 50 volts (V) and 100 milli Amperes for 1.5 hr. in the Midi Submarine Electrophoresis Unit (Tarsons, Cat #7050) connected to the Electrophoresis Power Supply Unit (Tarsons, Cat #7090).

The gel was viewed on a UV Transilluminator (GeNeiTM, Cat # SF850). The molecular size of the genomic DNA was detected in the form of bands. The size of the bands was estimated with Photo-Capt Version 12.4, (Vilber Lourmat, USA).

Gel Photography

The gel was photographed by using an indigenously built Gel Documentation System fitted with Canon SLR camera (EOS 350D) bearing Marumi orange filter (58 mm YA2, Marumi, Japan). The software in usage for the purpose was EOS utility software.

16S rDNA –PCR

The purified DNA whose quality assessment by the spectrophotometer was found to be 1.8, was deemed satisfactory for the 16S rDNA PCR amplification. The PCR was performed to amplify a partial region of the conserved gene 16SrRNA. The 16S rDNA sequence was amplified by Polymerase Chain Reaction (PCR) using actino-specific primers: 5'ACA AGC CCT GGA AAC GGG GT 3' Forward and 3'ACG TGT GCA GCC CAA GAC A 5' Reverse primer. In a sterile 0.2 ml thin-walled PCR tube (Tarsons, Cat#500050) following components were added for PCR reaction of 25 µl in the order as given below:

Go Taq Green Master Mix -12.5 µl

Primer- F 1.25 µl

R 1.25 µl

Template DNA-2 µl (25 ng)

Pyrogen free water- to a final volume

of 25µl.

The ingredients were mixed evenly in a SpinWin PCR micro centrifuge (Tarsons, Cat# 1000).

The PCR reactions were performed in (Thermal cycler-Applied biosystems Veriti).

Cycle 1: denaturation at 96ºC for 5 min,

Cycle 2-34: denaturation at 96ºC for 1 min, primer annealing at 57ºC for 1 min, primer extension at 72.0ºC for 1mins. final extension at 72.0ºC for 8 min.

The amplified PCR products were separated on 1.5% (w/v) agarose gel and run in 0.5X TBE buffer (pH 8.0) at 50 volts, 100 amp for 2 hr 30 min.

Lambda DNA/*Eco*RI /*Hind*III double digest (2 µl) and 100 bp ladder were used as molecular markers.

Molecular identification of the isolates was done by the sequencing of these amplified 16SrDNA gene fragments. To confirm the identity of PCR bands generated by 16S rDNA primer pair, the corresponding amplification products were directly sent for sequencing.

A total of 25 µl 16SrDNA PCR product was taken in each PCR tube, labeled properly, sealed with parafilm, packed in an airtight box with icepacks sent for sequencing to Biokart India Pvt. Limited, Bangalore, India.

The raw DNA sequence data samples received from Biokart India, were individually compared to those from GenBank using the Basic Local Alignment SearchTool (BLAST) program (Altschul *et al*. 1990) under NCBI (National Centre for biotechnology information) program online (http:// www.ncbi.nlm.nih.gov/) and similarity-based searches via the ezBioCloud server.

The raw partial 16S rDNA sequences of *the isolates* were submitted online to NCBI nucleotide sequence database with proper annotations and descriptions after registering to the website. The necessary guidelines were followed during the submission process and the sequences were submitted in the

FASTA format.

Sanger sequencing of the partial 16S rDNA helped us in genus-level identification before proceeding with whole-genome sequencing. The 16S ribosomal RNA gene sequences of the isolates were submitted to NCBI. A phylogenetic tree based on 16S rRNA gene sequence was constructed with the neighbor-joining algorithm in MEGA version 5.

3.10 Screening of plant growth-promoting activity

Phosphate solubilization test

The test was performed following the protocol of Pikovskaya (1948). A loopful of freshly grown pure culture was spot inoculated on Pikovskaya's agar medium. The plates were incubated at 30ºC for 3-5 days. The presence or absence of a clear transparent zone around the colonies was recorded. The solubilization zone was calculated by subtracting the diameter of the bacterial colony from the diameter of the total zone.

Indole Acetic Acid production

The IAA production test was followed as per Bric *et al*. (1991).

The nutrient broth was supplemented with a filter- sterilized solution of 0.2% or2 mM L-tryptophan. The broth was inoculated with a loopful of fresh culture and incubated at 200 rpm 30ºC for 5-7 days. The fully grown bacterial cultures were then centrifuged at 7000Xg for 10 min at 4ºC. Two ml of supernatant was then mixed with 4 ml of Salkowski reagent (1 ml of 0.5 M FeCl3 in 50 ml 35% perchloric acid).

The development of pink color indicates the presence of Indole Acetic Acid.

Ammonia production test

Ammonia production was detected by the method of Cappucino and Sherman (1992).

The prepared peptone water was distributed in test tubes and sterilized. Fresh 1 ml culture was inoculated and incubated at 30ºC for 3-5 days. About 0.5 ml of Nessler's reagent was added and the color change was recorded.

Green House Experiment

The experiment was conducted in

the Greenhouse of Molecular Genetics Laboratory, Department of Botany, North Bengal University. Healthy Mung bean (*Vigna radiata*) seeds were soaked in water for around 1 hour to remove the water-soluble toxic substances. Bean seeds were surface-sterilized by washing with 96% ethanol for 30sec and 2.5% sodium hypochlorite for 3 min, and then rinsed several times with sterile, distilled water (Samavat *et al*., 2012). These seeds were allowed to germinate in a Petri dish lined with wet cotton. The soil of the University garden (Shivmandir) was used for the growing of germinated seedlings.

The soil was filled in autoclavable disposable plastic bags and sterilized at 15 lb psi for 1 hr.

The greenhouse experiment was carried out in a randomized block design with three replications to evaluate the PGPR activity of selected isolates. Each pot was filled with sterile soil and kept in the greenhouse.

The germinated seedlings were transferred to the prepared pots.

The plants were allowed to grow under natural conditions of light, temperature and humidity. The daytime temperature ranged from 22ºC to 26ºC and 18ºC to 20ºC during the night.

The pots were irrigated with sterile water as required. The Mung bean being a climber plant, a support system had to be made with the help of ropes, wooden sticks.

The treatment with the isolated culture was started after 4 days of transplantation. the rhizosphere was treated with the culture three times a week. The sterile water was used as a control.

The treatment continued for 4 weeks. The height of the plant, the shoot length and root length was measured.

This experiment was conducted with two isolates separately as well as in a mixture along with a control (sterile water).

3.11 GC-MS analysis

Two of the isolates (VRA 1 and VRA 16) were used for this study. The isolates were grown on Streptomyces media plates at 30°C for 7 days. It

was further grown on sterilized liquid fermentation medium (Peptone, 10.0gm, NaCl-10gm, Glucose-10 gm, $CaCO_3$-1gm, distilled water, 1000 ml and pH adjusted to 7) for the extraction of the bioactive compound at 30°C with 180 rpm for 7 days. After the completion of fermentation, the culture was centrifuged and the supernatant stored at -4°C for further work (Ahsan et al. 2017, Singh et al. 2014).

The culture filtrate extraction was done using the solvents chloroform and methanol. The cell-free supernatant of VRA 1 and VRA 16 was mixed with methanol and chloroform in a 1:1 (v/v) ratio respectively. The mixture was shaken vigorously followed by further mixing on a rotary shaker overnight. The chloroform phase was separated from the aqueous phase. Both the extracts were condensed by evaporating to dryness at a temperature of 50°C. The procedure was repeated several times to collect a sufficient amount of crude extract (Ahsan et al. 2017; Ravi and Kannabiran 2018).

GC-MS analysis of the crude extract carried out by GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) with the headspace sampler (AOC-20s) and an injector (AOC-20i), outfitted with the mass selective detector, having 230°C as ion source temperature and interface temperature of 270°C, a mass range of 40 to 650 m/z, solvent cut time of 3.50 min and threshold of 1,000 eV. Rtx 5 MS capillary column with dimensions of 30 m (length) × 0.25 mm (diameter) × 0.25 µm (film thickness) used for separation of compounds. The split mode was used at the ratio of 10:1. The injector temperature was initialized to 260°C with split injection mode. The temperature was programmed from 60°C (3 min), then increased to 300 at 15°C/min (18 min hold). The carrier gas used was Helium (>99.999%) with 40.1 cm/s as a linear flow velocity. The debit of the gas (helium) vector was fixed to 16.3 mL/ min, with a total flow of 1.21 mL/ min. The 1.0 µL of sample injected and the essential compounds were identified by comparing their retention times relative to homologous alkane series (purchased from Sigma, St. Louis, USA) and their mass spectral fragmentation

patterns with those data in WILEY8.LIB. Identification was assumed on achieving a good match of RI and mass spectrum.

3.12 Molecular docking studies

The physicochemical properties of compounds identified from GC-MS analysis of the isolates (VRA 1 and VRA 16) and the compounds from *Streptomyces actuosus* (Nasr-Eldin et al.2019) were taken as ligands. The properties related to "Lipinski's rule of five" were also determined (https://en.wikipedia.org/wiki/Lipinski%27s_rule_of_five). Compounds obeying the "Lipinski's rule of five" were finally considered as our ligand. The structures of those ligands were obtained from NCBI Pubchem (https:pubchem.ncbi.nlm.nih.gov).Swissdock docking software was used for docking studies. The ligands were uploaded in the MOL2 format. The target proteins of the study were tuberculosis, Enteric Diseases and covid Spike protein. The tertiary structure of the proteins was downloaded from the PDB database (https://www.rcsb.org/) with PDB ID. We followed blind docking to identify the best binding sites with optimum binding affinity. Best ligand -dock complexes were identified based on the binding affinities and their respective images were collected for the best representation of molecular docking.

3.13 Whole-genome sequencing

Whole-genome sequencing was performed by MicrobesNG at the University of Birmingham, the UK using Illumina sequencing technology (Bennett 2004). A paired-end library was constructed using a Nextra DNA library preparation kit (Illumina, San Diego, CA) and sequenced on an Illumina HiSeq 2500 instrument to produce 250- bp paired-end reads. The Illumina sequence data were trimmed by Trimmomatic version 0.36 (Bolger et al. 2014). The closest available reference genome using Kraken (Wood, 2014) to, and map the reads to this using BWA mem (Li,2013) to assess the quality of the data. A *de novo* assembly of the reads using Spades version 3.13 (Nurk et al. 2013) and map the reads back to the resultant contigs, again using BWA mem (Li,2013) to get more quality metrics. Variant calling is performed using VarScan (Koboldt 2012).

Whole-genome annotation was performed using Prokka (Seemann 2014). Whole-genome annotation is the process of identifying features of interest in a set of genomic DNA sequences and labelling them with useful information. Prokka software tool was used to annotate newly sequenced genomes quickly and produced standards-compliant output files. Assembly was done using Spades version 3.13 (Nurk et al. 2013).

3.14 Bioinformatic Analysis

The four whole-genome sequences of *Streptomyces* strains which were isolated and characterized in the lab along with the nearest strains of *Streptomyces* (downloaded from IMG database) and one *Kitasatospora* strains were subjected to Bioinformatic analysis The *Kitasatospora* genome was used as an outgroup. The nucleotide sequences and amino acid sequences of *Streptomyces* were obtained from IMG database (www.img.jgi.doe.gov) (Markowiz et. al, 2012) and the annotated four whole-genome sequences of our strains (NCBI) were retrieved for this study.

Codon Usage variation analysis

The CodonW (ver. 1.4.4) software (Peden 1999) was used to calculate various parameters such as Guanine and cytosine in the nucleotide sequence (G+C content), the effective number of codons (Nc), GC3 (frequency of codons ending with Guanine or Cytosine in the third synonymous position of the codon), frequency of optimal codons (Fop) (Ikemura, 1985), relative synonymous codon usage (RSCU) (Sharp and Li,1986). Amino acid usage indices like relative amino acid usage (RAAU), Grand average of hydropathicity (GRAVY) and aromaticity were also calculated using the same software. DAMBE software (Xia, 2013) was used to calculate the energetic costs of the protein in the selected genomes and the codon adaptation index (CAI).

The effective number of codons (Nc) is a parameter that can measure the codon bias of synonymous codons. It is a quantitative measure reflecting the frequency of a small subset of codons used by a gene (Wright, 1990) and its value ranges from 20 (on the usage of one codon per

amino acid) to 61 (on the usage of all the codons with equal frequency). Nc is computed as in equation (1)

Nc=2+S+{29/[S2+ (1-S)2]} (1)

where *S* represents GC3 values, Frequency of optimal codon (Fop) represents the fraction of synonymous codons that are optimal codons (Ikemura 1985). Its value ranges from 0 (meaning a gene has no optimal codons) to 1 (when a gene is exclusively comprised of optimal codons). Fop is generally determined by equation 2 (a):

$Fop = Noc / Nsc$ (2a)

The original equation is modified in equation (2b), when rare codons are identified, as

$Fop\ (mod) = Noc - Nrc\ /\ Nsc$
(2b)

Where *N* represents the frequency of each codon type used, whereas *Noc*, *Nrc* and *Nsc* stand for optimal codons, rare codons and synonymous codons respectively.

The codon bias index (CBI) is another important estimator of directional bias and determines the level to which a gene uses a subset of optimal codons. The codon bias index value ranges from 0 to 1. It is calculated by following equation (3) as follows:

$CBI = Nopt - Nran / Ntot - Nran$
(3)

where Nopt= number of optimal codons; Ntot = total number of synonymous codons; Nran= expected number of optimal codons in cases where codons are assigned randomly.

Codon Adaptation Index

CAI or Codon Adaptation Index is an excellent tool to assess the probable level of expression of a concerned gene (Naya et al.2001). The CAI value varies between 0 and 1.0 (sharp and Li,1987). The High CAI value suggests that the codon usage pattern of a gene is similar to the gene which is highly expressed (Peden 1999) This can be used for studying the mRNA expression level and is considered as an efficient tool for studying the expression level of proteins (Roymondal et al. 2009, Wu et al. 2005, Jansen et al. 2003, Sharp and Li,1987). To estimate the CAI values of selected strains, CAI calculator 2

was used. The ribosomal proteins which are housekeeping genes that are highly expressed were taken as reference. Of the total, 10% of the genes with high CAI value is taken as potentially highly expressed genes (PHX) and the lower 10% with less CAI value is taken as potentially low expressed genes. The CAI was calculated by the following flrmula:

$$CAI = \exp\left(\frac{1}{L}\sum_{k=1}^{L} \ln \omega k\right)$$

where, ωK signifies the relative adaptivity of the Kth codon and L represents the number of synonymous codons in the gene.

Relative synonymous codon usage

Relative synonymous codon usage is a easy measure of the usage pattern of synonymous codons in the genome (Sharp and Li, 1987). This represent the number of times a particular set of codon is present with respect to the number of times it is expected in case of a uniform synonymous codon usage. It was calculated as follows:

$$RSCU = \frac{Freq_{obs}}{Freq_{exp}}$$

Where $freq_{obs}$ is the frequency of the observed codons and $freq_{exp}$ is the frequency of the expected codons.

If the RSCU value is greater than one means that the observed frequency of synonymous codon is high compared to the expected frequency, while RSCU value less than one shows that frequency of synonymous codon is low. (Reis et al. 2003).

Biosynthetic energy cost

Biosynthetic energy cost is the high energy phosphate bonds required for the synthesis of amino acids and proteins and also for the conversion of precursor molecules to final products (Akashi and Gojobori 2002). This was calculated by Dambe version 6.4.81 (Glass et al. 2008).

Statistical analysis

All the statistical analysis was done via SPSS software version 2.6. ANOVA and Two-tailed t-Tests were done with correlation coefficients at 0.01 and 0.05 levels where applicable.

3.15 Comparative Genomics

BLAST matrix

The selected *Streptomyces* genera along with four isolates from our lab were taken for this study. The genomic and proteomic sequences were downloaded from NCBI database. The sequences with initiation and termination codons with a length of more than 300 nucleotides were chosen for the analysis. BLAST matrix (BM) was constructed using BLAST algorithm with a 50/50 rule (Vesth et al.2013). The blast is significant when the sequences are aligned so that 50% of the alignment is identical with the longest protein in the comparative study. If the sequences have the same cut-off value, it is assigned to the same protein family. The two programs of CMG Biotools namely, "matrix_create config" and "matrix" are used to create the BLAST matrix.

Pan- core-genome plot

The pan-genome is a collection of the core genome, accessory genome, and species- or strain-specific genes. The core genome is the set of genes common by all analyzed microorganisms (Costa et al.2020). These genes are involved in vital roles for the survival of bacteria (Tettelin et al.2008). Accessory or dispensable genome is the set of genes that are present in two or more genomes but not all bacteria (Lapierre & Gogarten, 2009). The species- or strain-specific genes (singleton genes) are those which is limited to only one genome. The accessory and singleton genes are attained by horizontal gene transfer (HGT) or evolved due to mutations in pre-existing genes. They are commonly related to a specific metabolism, virulence, antibiotic resistance mechanism, or other environmental adaptation (Jordan et al. 2001). The Pan-core genome plot was constructed using two programs "pancoreplot_create config" and "pan -core plot" in CMG Bio-tool software.

Average nucleotide identity (ANI) score

ANI score is employed to compare two genomes at the nucleotide level (Yoon et al.2017).ANI Kostas lab was used to calculate the score among the selected genomes. A score above 95% indicates the genomes of the same species and a score value less than 75% was not considered (Rodriguez- & Konstandinis 2016). The matrix was constructed using the All-vs-All

ANI score of all the selected genera for comparative genomic analysis.

Phylogenetic studies

Phylogenetic trees were constructed using Multilocus sequence analysis (MLSA)tree and pan genomic tree.

Multilocus sequence analysis (MLSA)tree is a widely used method to attain a high resolution of phylogenetic relationships of species within a genus or in a family. The genes coding for conserved functions or the housekeeping genes is employed in this method. The five selected genes were Ftsz (tubulin-like GTP-binding protein), SecA (ATPase secretory preprotein translocase), GyrA (DNA Gyrase enzyme), DnaK (Hsp70 chaperone protein), ATP synthase 1 gene. The sequences were concatenated and aligned using ClustalW (Borovska et al. 2014) and Mega version 4 software was used to generate the phylogeny (Tamura et al.2007). The phylogenetic tree was constructed with a neighbor-joining algorithm and bootstrap value of 1000.

Pan genomic tree

The CMG Biotools software was used to construct the phylogenetic tree based on the neighbor-joining algorithm of bootstrap value 1000. "The pan-core_ plot tree" program was used to generate the phylogeny (Vesth et al. 2013). A comparison was made between the two phylogenies constructed by MLSA and Pan core plot.

3.16 antiSMASH studies

The antibiotics and secondary metabolite analysis shell is a software helps in the automated mining of secondary metabolite biosynthetic gene clusters in a particular genome. In order to predict the biosynthetic gene cluster (BGC) antiSMASH version 5.0 was used. The fasta format of the sequenced isolates was uploaded. The enabling of known cluster blast analysis option helps us to identify clusters against MIBiG (Minimum information about a biosynthetic gene cluster)repository.

3.17 Soil Metagenomics

For metagenomics studies the soil samples were collected from various biotopes such as mangroves, forest, paddy fields, vegetable fields, rubber plantation and tea gardens (Figure

Figure 3.3: Collection of soil from the forest of Darjeeling Hills

3.3)

Physical and chemical analysis of soil

Debris like roots, pebbles, etc. from the soil samples were removed by hand and air-dried and gently disaggregated. Soil organic matter (OM), organic carbon, (Walkey and Black, 1974) elemental analysis (N, P, K,) (Jackson, 1973; Baruah & Barthakur 1997; Chapman and Pratt, 1961) and pH were measured.

DNA Extraction

The soil samples (1 g each) were subjected to DNA extraction by using the MoBioPowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA; cat # 12888–100). Briefly, each sample was transferred into separate 2 mL PowerBead tubes and then used for DNA extraction. The purified DNA in triplicate was pooled into a single sample to obtain enough DNA that collectively represented the microbial community composition in the soil samples. The quality and concentration of the pooled DNA from each soil sample were determined by using a Lambda II spectrophotometer (Perkin Elmer,

Norwalk, Conn.) followed by agarose gel electrophoresis (1% w/v agarose in 1XTris-Acetate-EDTA (TAE) buffer, pH 7.8). Then, the purified DNA samples were dried and stored at 4°C until further use. The DNA was subjected to 16s Metagenomics amplicon sequencing (V3-V4) by Genotypic Technology Pvt. Ltd.3. 16s Metagenomics analysis has been performed using the Parallel-META pipeline (version 3.5; Jing, et al. 2017).

PCR Amplification of V3-V4 Region of 16s Gene:

Composition of TAQ Master MIX:

1) High-Fidelity DNA Polymerase

2) 0.5mM dNTPs

3) 3.2mM MgCl2

4) PCR Enzyme Buffer

Primer Details:

V13F: - 5' AGAGTTTGATGMTGGCTCAG3'

V13R: - 5' TTACCGCGGCMGCSGGCAC3'

Conditions Used:

40ng of Extracted DNA is used for amplification along with 10pM of each primer

Initial Denaturation: 95□

25 Cycles of the following condition:

Denaturation - 95□ for 15 sec, Annealing - 60□ for 15 sec, Elongation - 72 □ 2 mins

Final Extension at 72 □ 10 mins and Hold at 4 deg C.

3.18 16S Amplicon sequencing

The 16S rRNA sequencing was done using the Illumina™ Nextseq platform. The raw reads qualities were checked using FastQC. Sequence reads with > 5 low-quality base pairs (< 15 Phred score) were removed. Mean quality score for each base, per sequence quality score, per sequence GC contents and per base, N contents are calculated. Post QC, adapters are identified using the BBmerge function from the BBmap tool (Bushnell 2014). Reads are trimmed with BBDuk tool (Kechin,et al.2017, followed by quality check again. Post trimming, the quality check was done with Fastqc tool for adapter content and read the content of fastq files.

Metagenomic data analysis

MG-RAST server (version) was used to analyse the high-quality reads from each sample for taxonomic profiling and metagenomics data. Metagenome sequences were uploaded to MG-RAST for further processing. For taxonomic annotation of sequence reads, the RefSeq database (Pruitt et al.2006) was used and for functional gene annotation, the SEED database (Overbeek et al.2014) was used. Sequences were annotated using default settings of MG-RAST (maximum e-value cut-off of 10^{-5}, minimum % identity cut-off of 60%, and minimum alignment length cut-

off of 15 bp). Subsystem Function level (the finest level) data was sub-sampled to 963693 bp reads per sample for diversity analysis.

Metagenomic (16S) Analysis workflow

The genes encoding the RNA component of the small subunit of ribosomes, commonly known as the 16S rRNA in bacteria and archaea, are among the most conserved across all kingdoms of life. Nevertheless, they contain regions that are less evolutionarily constrained and whose sequences are indicative of their phylogeny. Amplification of these genomic regions by PCR from an environmental sample and subsequent sequencing of a sufficiently large number of individual amplicons enables the analysis of the diversity of clades in the sample and a rough estimate of their relative abundance. The analytical process is known as "16S rRNA diversity analysis" and is the focus of the present analysis pipeline.

The following steps were performed for 16S rRNA data analysis.

Read quality check

Following quality checks were performed on sequence (fastq) files

- Base quality score distribution
- Sequence quality score distribution
- Average base content per read
- GC distribution in the reads
- PCR amplification issue
- Check for over-represented sequences

Based on the quality check output, sequence reads are trimmed wherever necessary to retain high quality reads for downstream bioinformatics analysis and low-quality sequence reads are excluded, by default. Initial qc was done using FastQC (version 0.11.8) tool (S Andrews). Adapter trimming was performed using BBDuk program (Version 38.67). Post trimming, reads are subjected to QC again using FastQC (version 0.11.8) tool.

OTU picking/Classification

During this phase reads are processed so that comparisons between samples can be made. The first step is to cluster reads based on similarity into OTUs and to select a representative sequence for each OTU. Each OTU is

then classified by comparison to a reference database and a phylogeny inference is made based on sequence alignment and the construction of a phylogenetic tree.

Classification

BLAST against SILVA / greengene/16s database: Reads will be classified against greengene or any reference database for taxonomic profiling/classification.

Phylogenetic analysis/Taxonomic classification

The phylogenetic tree represents the relationship between the sequences in terms of the evolutionary distance from a common ancestor.

Diversity and other statistical analysis

OTU information (number of OTUs, abundance of OTUs) and the phylogenetic tree generated from phase 2 was utilized to estimate diversity within and between samples. Additional statistical analysis to test the significance of the diversities was also done.

Metagenomic analysis

This analysis has been performed using the Parallel-META pipeline (version 3.5; Jing, et al. 2017). Taxonomic and functional profiling by"Parallel-META 3 first constructs Hidden Markov Models using all bacterial 16S rRNA sequences of SILVA (version 123; Pruesse et al. 2007), and predicts the 16S rRNA gene fragments in metagenomic shotgun samples from both the forward sequences and reversed complementary sequences by HMMER (version 3.1, e-value < 1e-5; Eddy, 2011). Then Parallel-META 3 extracts all the 16S rRNA fragments from metagenomic shotgun sequences for profiling. All 16S rRNA gene sequences (either extracted from shotgun sequences or 16S rRNA amplicon reads) are aligned to the Parallel-META 3 reference database by Bowtie2 (Langmead et al 2012) for OTU picking, taxonomical annotation and phylogeny construction. The reference 16S rRNA sequences are from a customized database that integrates GreenGenes (version 13-8, sequence similarity on 97% level; DeSantis et al 2006) with RDP and SILVA consensus taxonomy annotation (assigned by BLASTN with e-value < 1e-30 and similarity

>97%), which raised the proportion of annotated sequences at the genus level from 35.8% to 81.5%. The phylogenetic architecture of all reference sequences is built by FastTree (Price et al. 2010). Since 16S rRNA gene copy number varies greatly among different bacterial species, Parallel-META 3 also calculates the precise relative abundance of each organism by 16S rRNA copy number calibration using IMG database (Markowitz et al. 2012). In addition, considering that the uneven sequencing depth (number of sequences) among multiple samples may introduce bias in detecting diversity patterns (Koren et al. 2013), an optional sequence rarefaction for sequencing depth normalization at the OTU level is provided after the taxonomic profiling." (Jing, et al. 2017) "For prediction and annotation of functional profile, Parallel-META 3 re-implements the PICRUSt (Langille et al. 2013) algorithm using KEGG database (Kanehisa et al. 2012) to estimate all the functional genes harboured in a microbiota using 16S rRNA gene OTUs. The functional genes are annotated by KO (KEGG Ontology) and KEGG pathway. Parallel-META 3 also measures the prediction accuracy by the NSTI (Nearest Sequenced Taxon Index) value (Langille et al. 2013), which is calculated by the sum of distances between OTUs and their nearest individually sequenced relatives in the phylogenetic architecture." (Jing, et al. 2017) "After taxonomical and functional profiling, Parallel-META 3 parses out the sequence counts and relative abundances (normalized into 0–100%) for all OTUs, and estimates the same information for annotated taxa from the phylum level to the genus level, as well as the genes and the pathways. Such data is framed into tables that are compatible for further analysis in Parallel-META 3 and also suitable for manual examination by users." (Jing, et al. 2017)

"Parallel-META 3 evaluates α diversity that describes the inner complexity of each individual microbiota sample. This process generates rarefaction curves of α diversity based on observed OTU number and Shannon index to

determine the adequacy of the sequencing depth. The rarefaction performs a series of random sequence selection on different sequencing depths with bootstrap (default is 20, and can also be defined by users), and the α diversity in the curves is calculated by the mean sequence count of each OTU among the bootstrapping procedures. Then the influence of each environmental factor on α diversity is quantitatively evaluated by multivariate statistical analysis with Shannon index, Simpson index and CHAO1 index." (Jing, et al. 2017) The β diversity evaluation and statistics "Parallel-META 3 examines β diversity of multiple microbiota samples based on their pair-wise similarity matrix to discover the patterns of organism/gene sharing and variation among samples. The quantitative similarity between each sample pair is computed by Meta-Storms (Su et al. 2012) algorithm, which considers both the relative abundance of OTUs existent in two samples and the distances among OTUs in the phylogenetic architecture. The β diversity evaluation includes unsupervised hierarchical clustering, supervises clustering using PCA (Principal Component Analysis), PCoA (Principal Co-ordinate Analysis) and multivariate statistical analysis that quantitatively evaluates the correlation between environmental factors and the sample similarities." (Jing, et al. 2017) Biomarker discovery "Parallel-META 3 can also identify key organisms or functional genes that are highly correlated with the variations of the habitats or other types of metadata. Organisms or genes with significant differences among microbial community samples were firstly chosen using Kruskal-Wallis or Wilcoxon rank-sum test as candidate makers, and these candidate makers were then ranked based on their contribution to the differentiation among samples using the Random Forest algorithm." (Jing, et al. 2017).

Construction of microbial interaction network

The microbial interaction network was constructed to explore co-occurrence and co-exclusion patterns of organisms or functional genes

across microbial community samples. In the interaction network, each node represents a single organism (or gene), and nodes were connected by links that represent their correlation coefficient of abundance variation among multiple samples (Faust, et al. 2012). Then Parallel-META 3 illustrates the global pattern among multiple samples by the network's topological characters such as node number, isolated island number, density, diameter, radius and centralization" (Jing, et al. 2017).■

Chapter 4
Results & Discussion

The important thing is not to stop questioning. Curiosity has its own reason for existing.

Albert Einstein

4.1 The Health Survey

The survey conducted among the various tea gardens showed the prevalence of non-communicable diseases rather than communicable diseases. Though the living conditions are improved nowadays, there are still some cases of tuberculosis among the workers. The most common ailments were skin diseases and allergies. The resident doctors and government officials are actively involved in the welfare programs of the workers. Diarrhoea and worm infections are also present in spite of having safe drinking water. Addictions such as smoking, alcohol, non-smoked tobacco are also common among the workers, irrespective of any gender differences. The prevalence of the covid pandemic remained moderate in tea gardens

Table 4.1: Common ailments and addictions of Tea garden workers

Common Ailments	
Communicable disease	16%
Non-communicable disease	41%
Cough & cold	59%
Tuberculosis (in men)	3%
Tuberculosis (in women)	14%
Skin diseases	64%
Gastrointestinal disorders	19%
Smoking	16%
Common Addictions	
Non-smoked tobacco	60%
Alcohol consumption	40%

without many devastating effects. The results of the survey are summarised in the Table. 4.1 . The sample survey sheet is enclosed (Figure 4.1)

4.2 Collection of soil samples

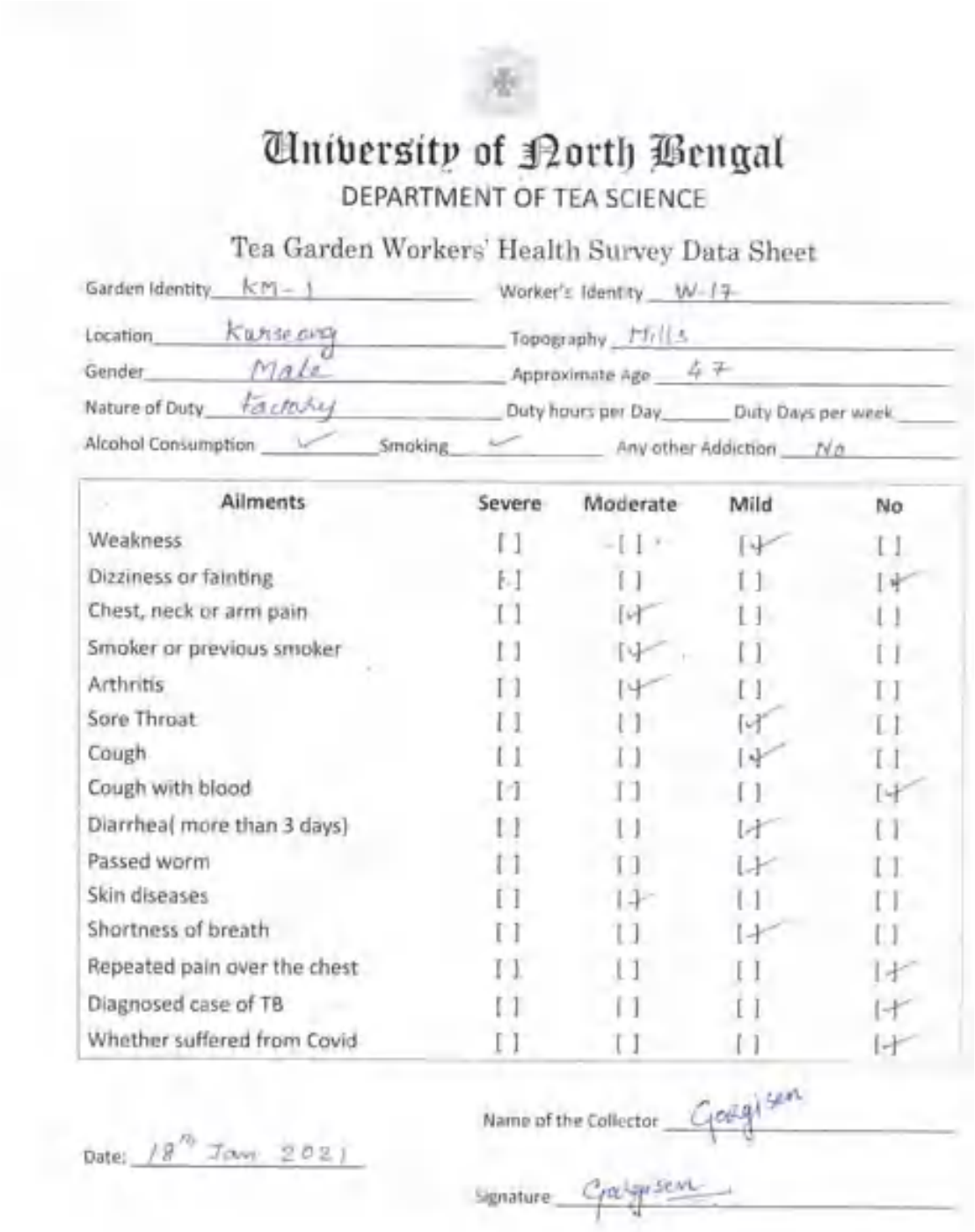
The soil samples were collected from mangroves of Sunderban (West Bengal), Vyppin, Kochi (Kerala), Tea gardens of North Bengal, Vegetable fields (Rangapani), Paddy field of Balurghat, North Dinajpur (West Bengal), forest soils of Darjeeling hills, Rubber estate of the University of North Bengal. The soil samples collected at 5-25 cm depth helped to get a maximum number of aerobic actinobacteria. The samples in sterile bags were stored at 4°C till further processing.

4.3 Physico-chemical analysis of soil samples

The debris like roots, pebbles, etc. from the soil samples was removed by hand, clumps of the soil were separated and air-dried**.** The qualitative analysis of soil for Soil organic matter (OM), organic carbon, (Walkley & Black 1934) **e**lemental analysis (N, P, K,) **(**Jackson 2005), and pH were measured. The soil samples had a pH ranging from 3. 08 to 7. 9. Soil texture varies from clay to sandy loam. The electro-conductivity of soil samples was ranging from 0. 1-0. 5. Further soil analysis revealed slight variation in macro and micronutrients. Available Nitrogen and P2O5 were different for various biotopes which had shown alteration with varying geographical conditions. Iron was medium to high and the other micro and macro elements fluctuated (Table 4.2).

4.4 Isolation and characterization:

The air-dried soil samples for 7-10 days were dried at 50ºC in an oven helped to eliminate the vegetative bacteria and fungi. One gram of dried soil sample was suspended in 9ml of sterile saline solution (0. 85% NaCl in



University of North Bengal

DEPARTMENT OF TEA SCIENCE

Tea Garden Workers' Health Survey Data Sheet

Garden Identity KM-1 Worker's Identity W-17

Location Kurseong Topography Hills

Gender Male Approximate Age 47

Nature of Duty Factory Duty hours per Day _____ Duty Days per week _____

Alcohol Consumption ✓ Smoking ✓ Any other Addiction No

Ailments	Severe	Moderate	Mild	No
Weakness	[ ]	[ ]	[✓]	[ ]
Dizziness or fainting	[ ]	[ ]	[ ]	[✓]
Chest, neck or arm pain	[ ]	[✓]	[ ]	[ ]
Smoker or previous smoker	[ ]	[✓]	[ ]	[ ]
Arthritis	[ ]	[✓]	[ ]	[ ]
Sore Throat	[ ]	[ ]	[✓]	[ ]
Cough	[ ]	[ ]	[✓]	[ ]
Cough with blood	[ ]	[ ]	[ ]	[✓]
Diarrhea(more than 3 days)	[ ]	[ ]	[✓]	[ ]
Passed worm	[ ]	[ ]	[✓]	[ ]
Skin diseases	[ ]	[✓]	[ ]	[ ]
Shortness of breath	[ ]	[ ]	[✓]	[ ]
Repeated pain over the chest	[ ]	[ ]	[ ]	[✓]
Diagnosed case of TB	[ ]	[ ]	[ ]	[✓]
Whether suffered from Covid	[ ]	[ ]	[ ]	[✓]

Name of the Collector Gargi Sen

Date: 18th Jan 2021

Signature Gargi Sen

Figure: 4.1 Survey data sheet

distilled water) and serially diluted in saline solution up to 106dilution and 0. 1 ml each dilution inoculated by standard spread plate technique. The pre-treatments of soil and the different enrichment media (Nutrient agar, Actinobacteria isolation agar, Bennet agar) allowed the isolation of a

Table 4.2: Details of analysis of collected soil samples

Biotopes	**pH**	**EC** (µS/ cm)	**OC** (%)	**OM** (%)	**N** (%)	**K_2O** (ppm)	**P_2O_5** (ppm)	**SO_4** (ppm)
Mangrove 1	3. 34	6569	4.421	7. 605	0. 380	68	20	528
Mangrove 2	4.84	994	1. 825	3. 139	0. 157	42	150	23. 3
Rubber field	4.44	206	4.562	7. 846	0. 392	11	15	6. 5
Paddy field 1	5. 27	163	0. 491	0. 845	0. 042	15	14	19
Paddy field 2	5. 15	162	0. 561	0. 966	0. 048	25	5	2. 7
Vegetable field 1	5. 15	225	2. 176	3. 742	0. 187	47	110	14
Tea garden	4.88	421	2. 807	4.829	0. 241	96	60	12. 7
Vegetable field 2	7. 65	5982	0. 421	0. 724	0. 036	195	8	88. 5

EC=Electric conductivity; OC= Organic Carbon; OM= Organic matter.
Mangrove 1= Kochi ; Mangrove 2= Sundarban; Paddy field 1= Balurghat; Paddy field 2=Atharokhai, Darjeeling Dist. ; Vegetable field 1=Rangapani 1; Vegetable field 2=Rangapani 2

maximum number of morphologically different actinobacteria from various biotopes. Supplementation of antibiotics like Rifampicin (5µg/ml), Nalidixic acid (75µg/ml) and Fluconazole (80µg/ml) also influenced the suppression of non-actinobacterial and fungal pathogens. The well-grown conspicuous and morphologically different colonies of actinobacteria were selected. Out of these, the characteristic chalky white, grey, brown spore-producing colonies (typical of streptomyces) were selected for further studies. After several rounds of purification, the purified isolates were sub-cultured on ISP4 slants and incubated for 7 days at 30°C then preserved at 4°C (Shepherd et al. 2010).

4.4.1 Subculture of Actinobacteria

Different media such as nutrient agar, Streptomyces media and the International Streptomyces Project (ISP 4) were used for subculturing of isolates (Figure 4.2). It was noted that the same isolate produced different colored pigments in different media. To avoid color confusion in the soluble pigment production in different media, only ISP4 (all the isolates grew well in ISP4) was used as the standard media for soluble pigment production. Different colored metabolites were produced by the actinobacteria in the

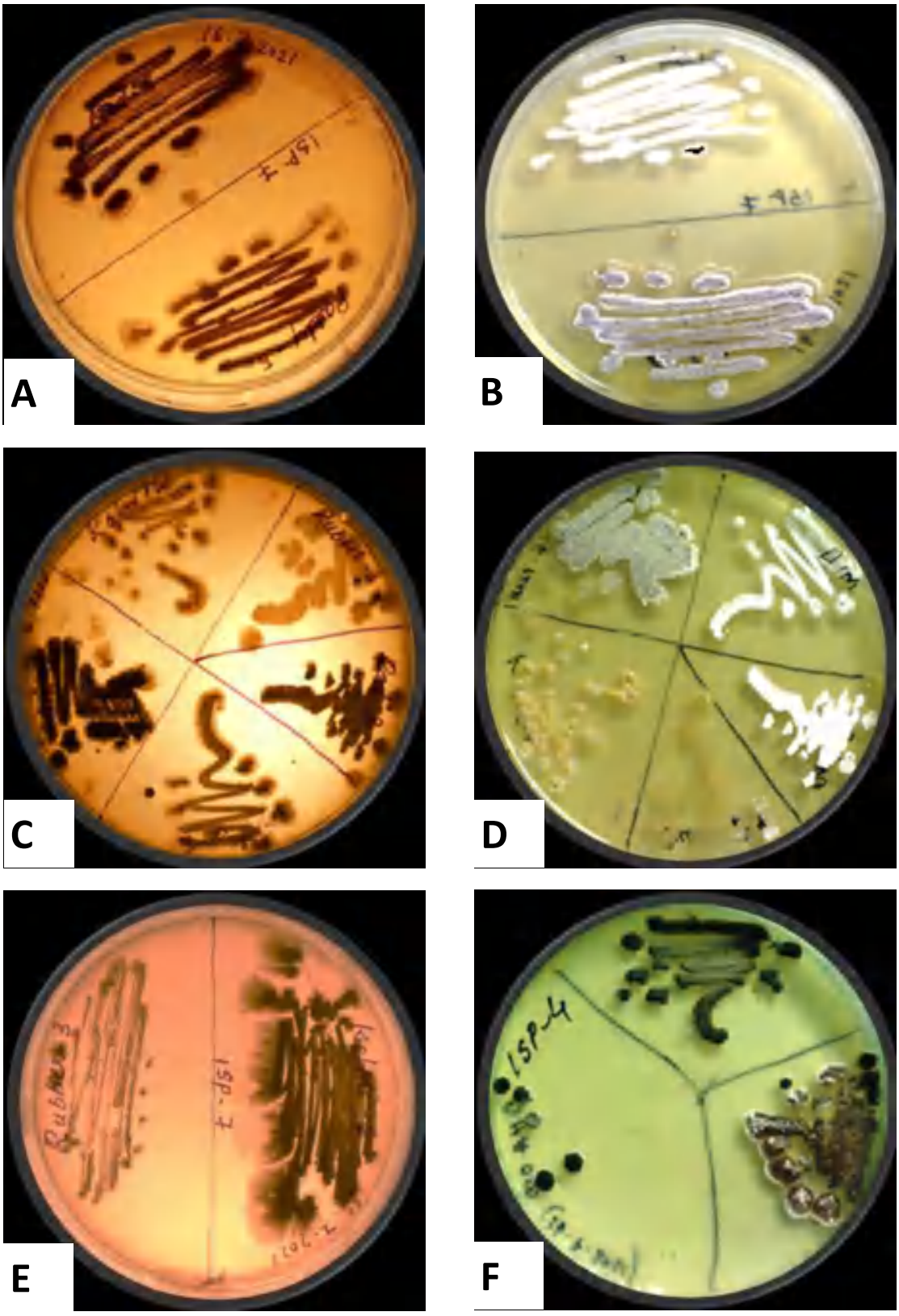


Figure 4.2: Growth of isolates in different media: A=isolates RAD3 and PAD5 in ISP 7 medium; B= VRA 1 and VRA 16 in ISP 7 medium; C=RUB1, RUB3, RAD3, KOL5 and KOL6 in ISP4 medium; D=VRA 1, VRA16, VRA17, TEA10 and TEA2 in AIM medium; E=rub3 and KOL6 in ISP 7 medium; F=KOl3, PAD5, and tea10 in ISP4 medium

Table 4.3: Morphological characteristics of selected isolates

Isolate	ISP4 medium	Mycelium color		Soluble pigment
		Aerial	Substrate	
VRA1	+++	White	Cream white to yellow	
VRA16	+++	White with yellow tinge	Straw golden yellow	Brown
VRA17	+++	White	Purple brown	-
RUB1	++	White	Creamy grey to purple	-
RUB3	++	White	Cheese white	
PAD4	+++	White with yellow tinge	Straw yellow	-
PAD5	++	White	Creamy white	-
KOL3	++	White	Creamy white to purple	-
KOL5	+++	White	Yellow	-
KOL6	+++	White to grey tinge	Cheese white	-
KOL8	+++	White	Creamy white	-
KOL9	+++	White	Light yellow	-
RAD3	+++	White	Creamy white	Yellow
TEA2	++	White	Cream white	-
TEA10	++	White	Light yellow	-
MTA1	++	Grey	Greyish Brown	Purple
MTA2	++	Yellow	White	-
NPA1	++	Light Yellow	Yellowish white	-
NPA2	++	White	Dark Yellow	-
ARA1	+++	Grey	Brown	-
ARA2	++	Brown	Brownish black	-
ARA3	+++	Greyish blue	Grey	-
ARA4	+++	Brown	Grey	-
VRA14	+++	White	Creamy white	-
VRA12	+++	Green	Bluish green	Brown
VRA3	+++	White	Yellowish white	-
VRA9	+++	Brown	Greyish Brown	-
VRA19	+++	Grey	Brown	-

+++=Very good growth; ++=Moderate growth

form of diffused soluble pigments and insoluble pigments in the solid media. There were 25 Streptomyces isolates from soil samples of mangroves, 10 from tea garden,8 from the vegetable field, and 2 each from Rubber estate and forest soil which was isolated on selective media- Streptomyces media

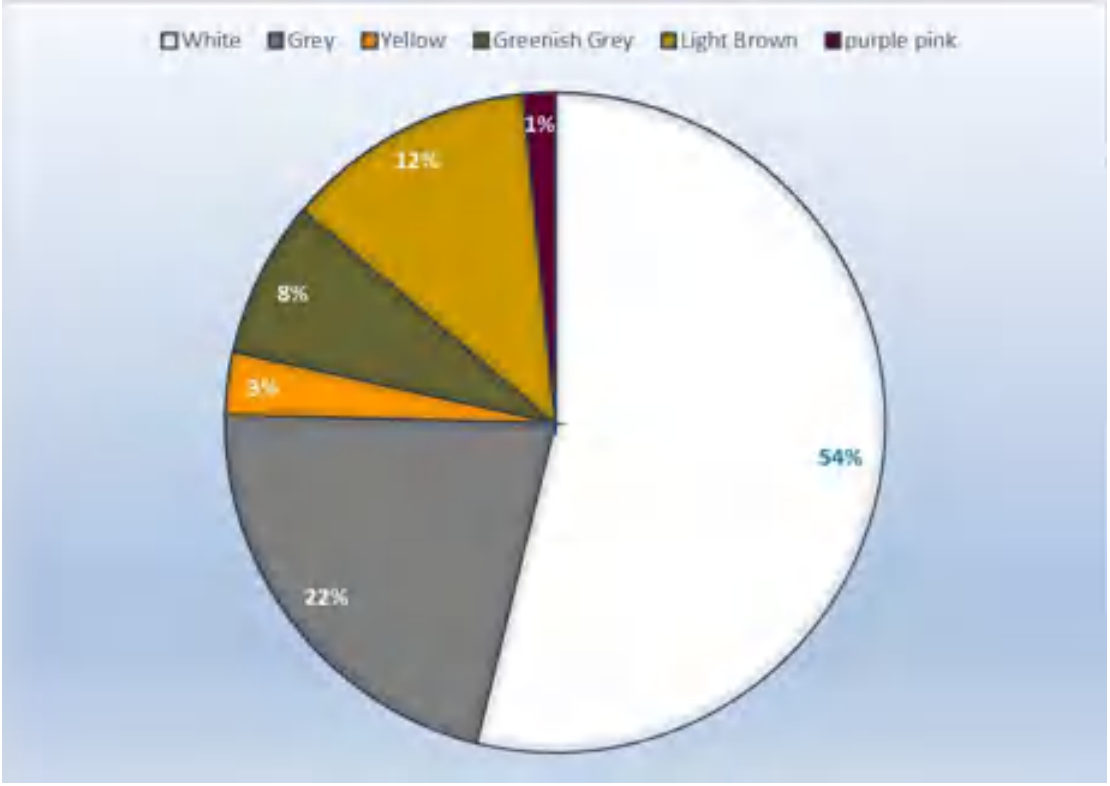


Figure 4.3: Morphological characteristics of selected isolates: based on the color of the mycelium

and International Streptomyces Project (ISP 4). These total of 65 isolates were further characterized. The isolates produced aerial spores with different color morphology- 35 were white (53. 84%), 14 were grey (21. 53%), yellow 2 (3. 07%), greenish-grey 5 (7. 69 %), light Brown 8 (12. 30%), and purple-pink 1 (1. 53%) (Figure 4.3).

4.5 Microscopy

The dominant colonies were confirmed as gram-positive by gram staining reaction (Hucker & Conn 1923). Cellular morphology was determined using light microscopy (Figure 4.4 a) and scanning electron microscopy (SEM). For SEM, a standardized protocol (Saha et al. 2019) was used and the isolates from a log phase culture (after 4 days of incubation in Bennet Broth) were observed. The strains were gram+ve and filamentous. In the SEM micrographs, the filamentous structure could be clearly visible (Figure 4.4 b, c, d) which confirmed the actinobacterial morphology.

4.6 Biochemical characterization

The isolates were biochemically characterized by several bioassays. Most of the isolates exhibited the production of various enzymes which

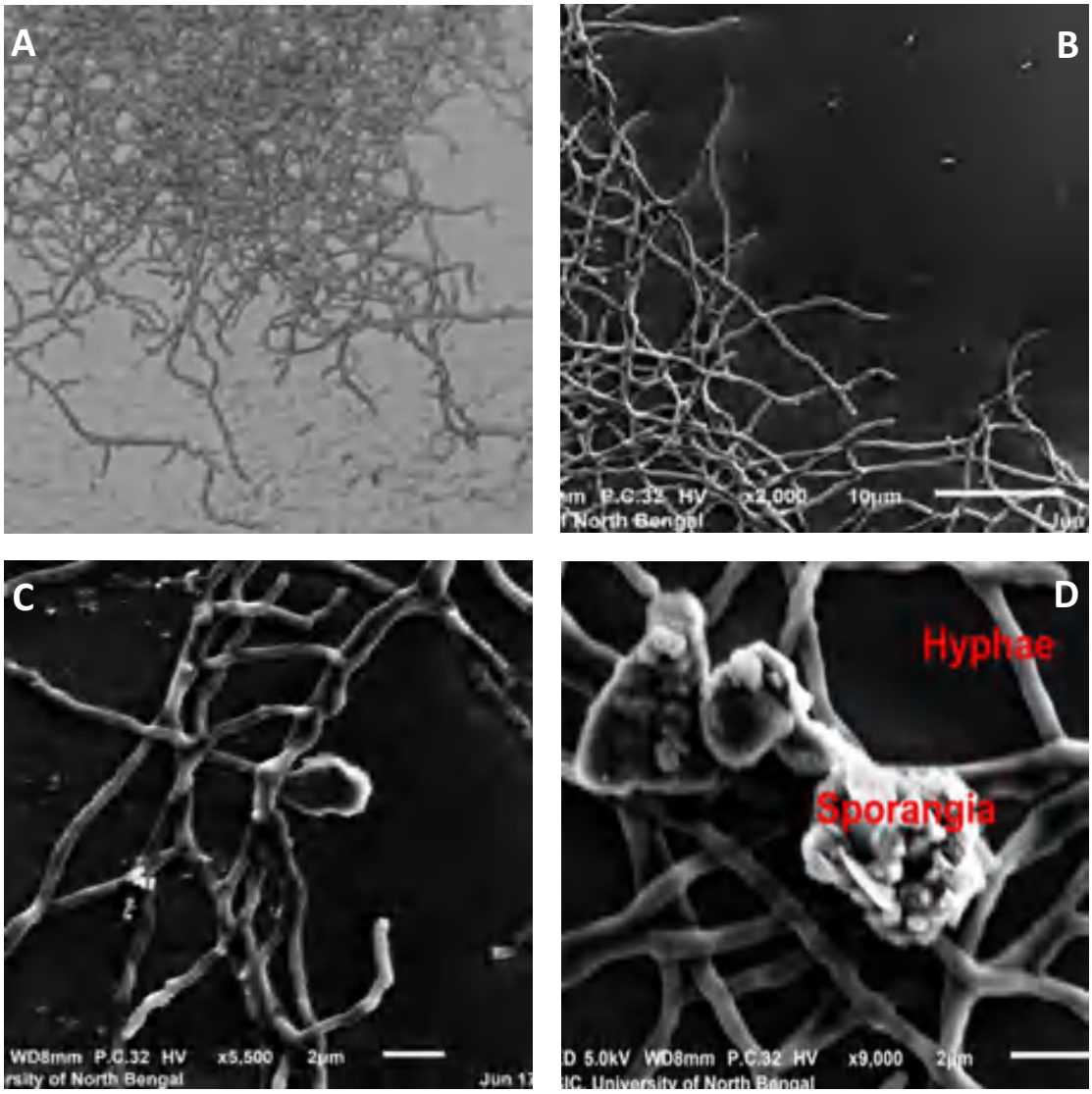


Figure 4.4: Micrograph of the isolates: A=Light micrograph of VR5; SEM of VRA17 showing young Sporangia; SEM of VRA17 showing Hyphae and mature sporangia

is in concordance with the properties of the *Streptomyces* genus. Hydrolysis of starch and its conversion to amylase and liquefaction of gelatin were considered as important characters of actinobacteria (Waksman, 1961). Similarly, the production of hydrogen sulphide and melanin were utilized as a taxonomic tool for the identification of *Streptomyces* (Gordon and Smith, 1955; Waksman 1957, 1961; Kuster and Williams, 1964).the microbial lipases are widely used in paper, detergents, food industry etc. for the trans esterification and hydrolysis of fats.most of our isolates exhibited the potential for hydrolysis of fats. The details of the observation of the studied

Table 4.4: Biochemical characterization of the selected isolates; '+' indicates positive result and '-' indicates negative result

Reaction	**Response**	**VRA1**	**VRA16**	**VRA17**	**RUB1**	**RUB3**	**PAD4**	**PAD5**	**KOL3**	**KOL5**	**KOL6**	**KOL8**	**KOL9**	**RAD3**	**TEA2**	**TEA10**
Amylase	Clear zone	+	+	+	+	-	-	+	+	-	+	-	+	-	+	-
Catalase	Brisk effervescence	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Gelatin hydrolysis	Clear zone	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+
Xanthine decomposition	Clear zone	+	+	-	-	-	-	+	+	+	-	-	+	-	+	+
Esculin hydrolysis	Media blackening	+	+	+	-	+	-	+	-	+	+	-	-	-	+	-
Melanin formation	Media blackening	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Nitrate reduction	Red color Formation	+	+	+	-	-	-	+	-	+	+	+	-	+	+	+
Tween 80 hydrolysis	Clear zone	+	+	-	-	-	-	+	-	-	-	-	-	-	+	+

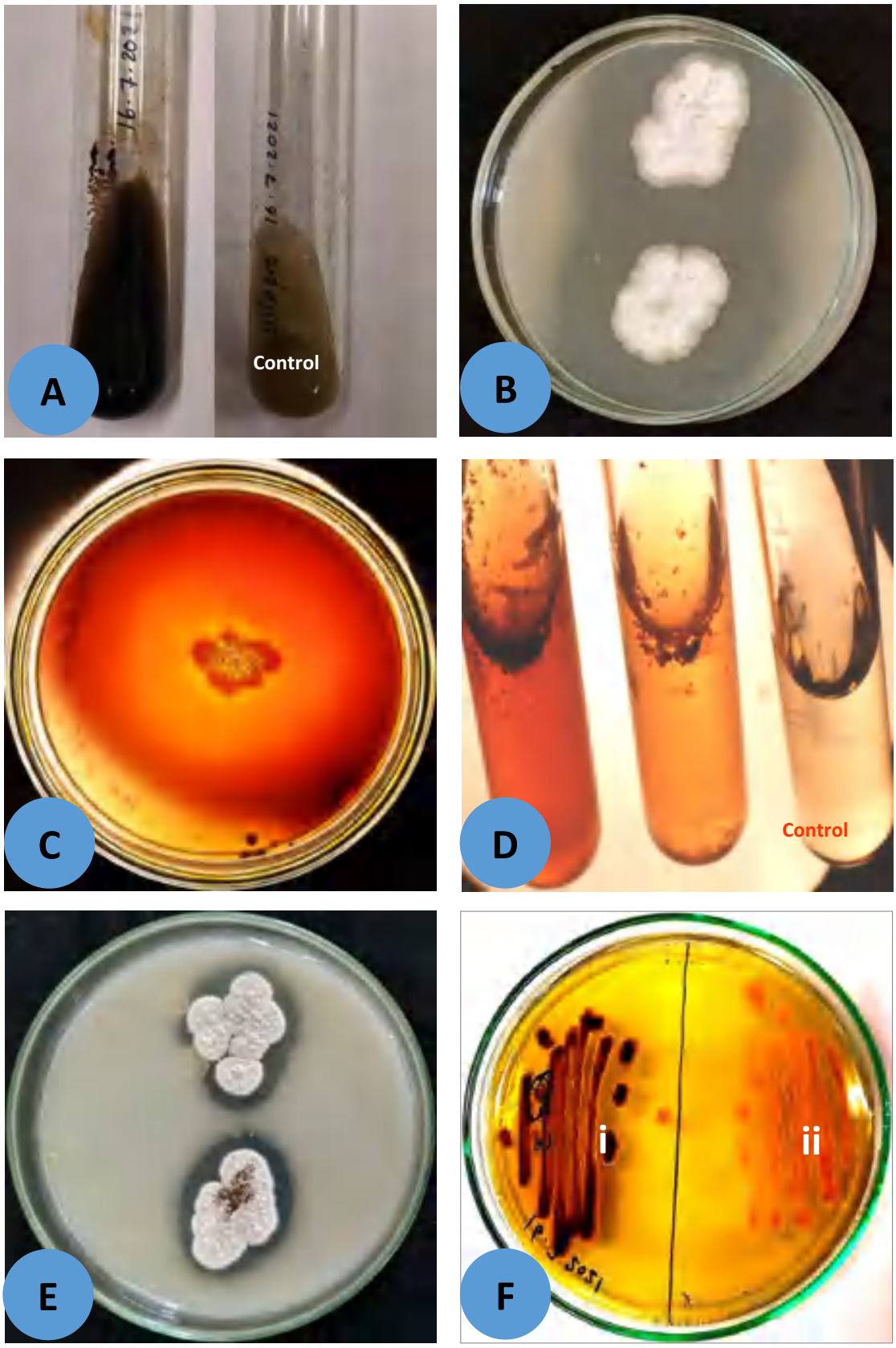


Figure 4.5: Biochemical characterization A= Esculin hydrolysis; B= Gelatin hydrolysis; C= Starch hydrolysis; D= Nitrate reduction; E= Xanthine hydrolysis; F= Melanin producing (i) and non-producing (ii) strains

Figure 4.6: Intrinsic antibiotic resistance test (IAR) of the isolates, A=KOL5; B=RAD3

isolates for different biochemical tests in the form of presence or absence of growth, color change, etc. have been depicted in Figure 4.5. and Table 4.4.

4.7 Intrinsic antibiotic resistance (IAR) test

The spread of resistant microbes initiated the search for new antibiotics

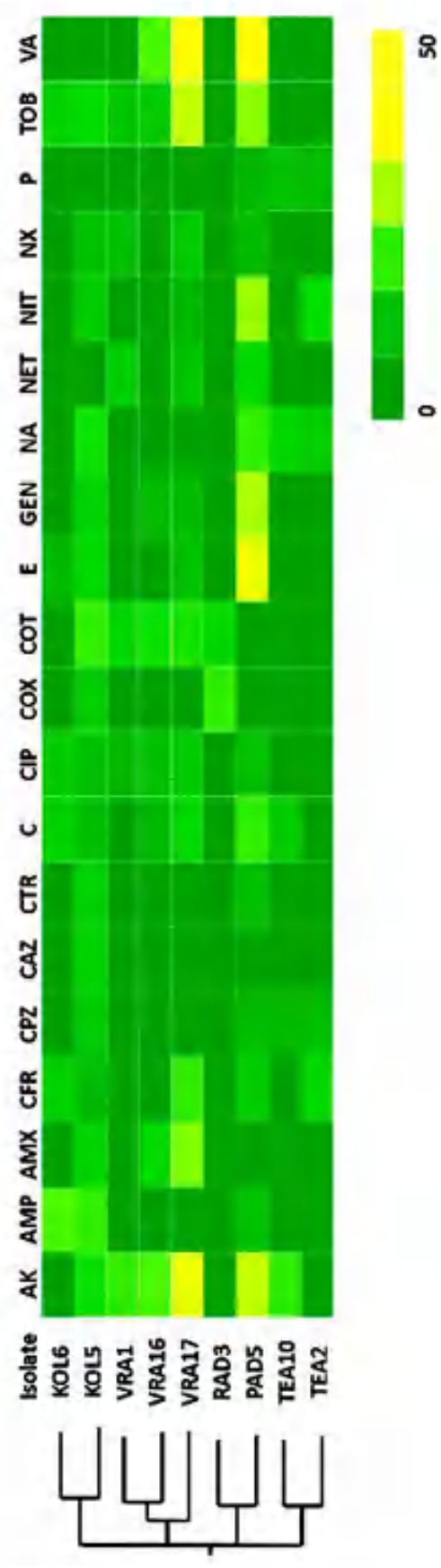


Figure 4.7 Heat-map showing antibiotic profiling of selected isolates

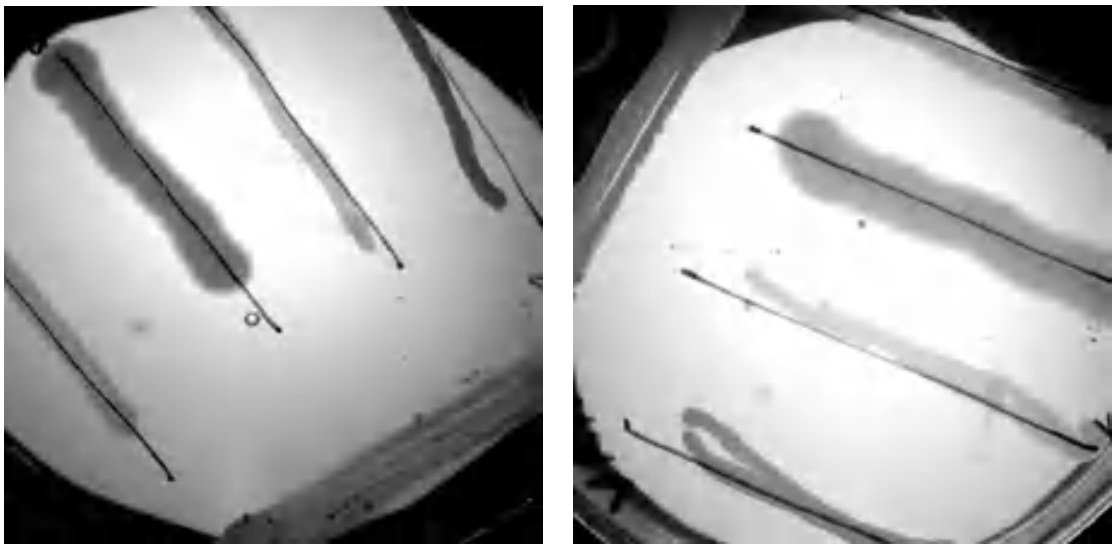


Figure 4.8: Antimicrobial tests for the isolates, A=isolate RAD3 and B=VRA1

to control the pathogens. In nature, antagonistic microbes play a significant part by suppressing the activity and spread of plant and human pathogens. Actinobacteria are known for their antagonistic property among microbes, producing effective and safe antibiotics. In this study, the agar disc diffusion test of Bauer et al. (1966) was followed for the IAR test, as it is regarded to be the most convenient and widely used method for routine antimicrobial susceptibility testing. Each of the strains showed a wide variation in their result in response to different concentrations of twenty antibiotics (Figure 4.6.). Most of the studied isolates showed antibiotic resistance, this may be due to their exposure to the wide use of antibiotics in nature. This might have triggered them to evolve into resistant microbes. A heat map was produced based on the results. The color ranged from green (very resistant) to red (sensitive). From the heat-map, it was found that the studied isolates were divided into clusters depending upon the sensitivity against their antibiotics. Interestingly the strains have clustered as per their geographical distribution (Figure 4.7). The isolates from mangroves (VRA), Paddy field (PAD), vegetable field (KOL), tea gardens (TEA) were clustered together.

4.8 Antimicrobial test of the selected isolates

The Cross streak method was used for confirmation of antibacterial activity for the active isolates. The active isolates which were grown on Muller-

Hinton agar plates were subjected to the cross-streak method. The isolate VRA 1 showed maximum inhibition to all of the test pathogens except *Klebsiella.* The isolate KOL6 inhibited the growth of *Bacillus,* RUB 3 inhibited the growth of *E. coli* and KOL 5 exhibited inhibition against *Staphylococcus.* The other tested isolates did not exhibit any significant results. The antagonistic activity of the newly isolated strains proved their potential to be utilized as antimicrobial agents in the future (Figure 4.8).

Growth of isolate in different pH

The hydrogen ion concentration is an important criterion for determining the growth of microorganisms. The isolates from various biotopes showed profuse growth on the media of pH ranging from 7 to 9 and moderate growth was recorded at pH 10 and 11. There was negligible or nil growth was observed at pH 4 and 5. This suggests that the isolates are alkaliphilic rather than acidophilic (Table 4.5).

Growth of isolates in different temperatures

The *Streptomyces* genera from the terrestrial field normally show optimum growth at 30ºC. Our isolates also grew well at temperatures 25ºC, 28ºC and 30ºC. Poor growth of isolates was noticed at 45ºC though moderate

Table 4.5: Growth of isolates in different Physicochemical conditions

Condition	Growth
Different pH	
5	-
6	-
7	++
7. 5	+++
8	+++
9	+++
10	++
11	++
12	++
Different Temperature	
20ºC	++
25ºC	+++
28ºC	+++
30ºC	+++
37ºC	++
40ºC	++
45ºC	+
Different Salinity	
0	++
2	+++
4	+++
6	+++
8	+++
10	++
12	++
14	-

Table 4.6: PGPR studies of selected isolates

Isolate	Ammonia production	Phosphate solubilization	IAA production
VRA 1	+	+	+
VRA 16	+++	+++	+++
VRA 17	+++	+++	+++
KOL5	++	+	++
KOL 6	+	+	+
RUB 3	++	-	+++
RAD 3	+	-	-
PAD 5	+++	+++	+++
TEA 2	+	++	+
TEA 10	-	++	+

+++ high; ++ medium; + low; - negative

growth appeared for the isolates between 20ºC, 37ºC and 40ºC (Table 4.5).

Growth of isolates in different salt concentrations

The growth of isolates in different salinity was studied using the various concentrations of NaCl. The isolates from mangroves showed good growth ranging from 7%-12% of salinity in nutrient broth. The growth of the isolates from other biotopes ranges from 6%-8%. No growth was observed in 14% of sodium chloride for all isolates (Table: 4.5)

4.9 Plant growth-promoting traits studies

Phosphate solubilization activity

The appearance of a transparent halo zone around the colony grown in Pikovskaya's agar medium indicated the phosphate solubilizing activity of the strains (Pikovskaya 1948). The clear halo zone was observed in studied isolates such as PAD 05, VRA 16, VRA 17. These isolates actively

Table 4.7: PGPR Activities of the isolates on Mung bean plant

Treatment with isolates	Length (cm/plant)		Total length	Fresh weight (mg/plant)		Total weight
	Shoot	Root		Shoot	Root	
Control	14.5	6. 5	21	502	86	588
VRA 1	14.5	10	24.5	595. 3	91. 3	686. 6
VRA 16	21. 2	10	31. 2	624.6	92. 3	716. 9
VRA1+VRA16	16	13	29	575. 2	91. 6	666. 8

utilize the phosphate salts by phosphatase, which may be responsible for supporting plant growth. This property will be highly beneficial in the case of the isolates that may be used as the biocontrol agent for suppressing the disease potential of pathogens. The halo-zone diameter measured from the center of the colony and the Phosphate solubilizing efficiency (PSE) of PAD 05 was found to be the highest among the studied isolates (Figure 4.9 and Table: 4.6).

IAA production

The appearance of pink color produced after exposure of broth to Salkowski reagent confirmed the isolates as IAA producers. The following isolates were positive for IAA production. The isolates VRA 17, PAD05 and RUB 3 showed the highest intensity for IAA production (Figure 4.9 and Table: 4.6).

Ammonia production test

Another important trait of PGPR is the production of ammonia that indirectly influences plant growth. The incubation of the isolates in peptone

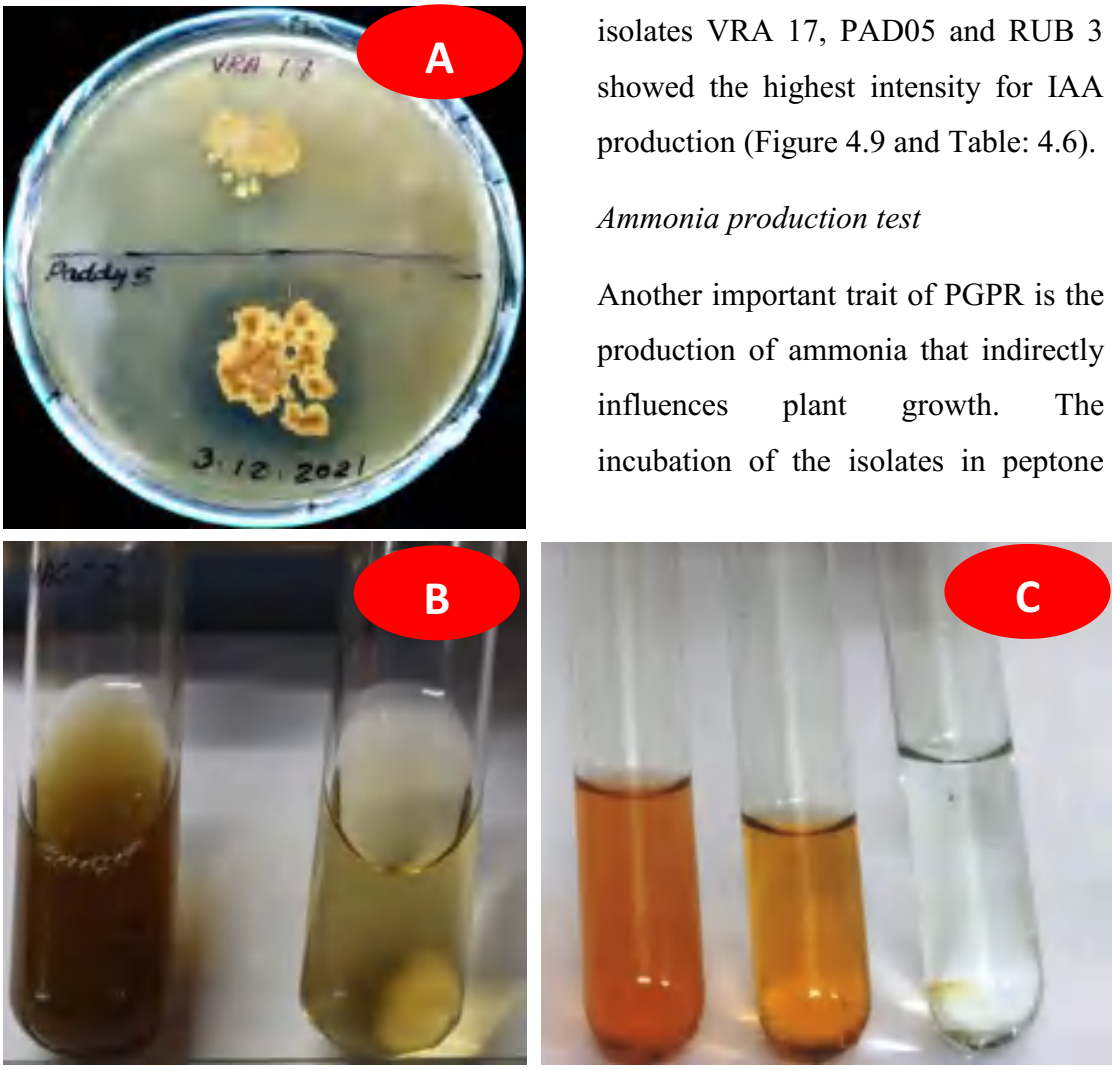


Figure 4.9: PGPR studies of the isolates A=Phosphate solubilization; B= Ammonia production; C= IAA production

Figure 4.10: PGPR activities on Mung bean (*Vigna radiata*)A= with VRA1 and B=VRA16

water broth for 3-5 days was followed by the addition of a few drops of Nessler's reagent. A change from transparent to yellow coloration is indicative of ammonia production. In this study, most of the studied isolates showed the property of Ammonia production (Figure 4.9 and Table: 4.6).

PGPR studies on Mung bean

The treated seedlings produced young tertiary leaves and very long main roots with 20 secondary roots with the formation of tertiary roots. The fresh weight of seedlings from the treatment of VRA 16 was higher than compared to seedlings in other treatments. It was found that VRA 16 influenced the growth of seedlings higher than in the control. But it was observed that the combined effect of the isolates (VRA 1 and VRA 16) on growth was less in comparison to VRA 16. Fig 4.10 and Table 4.7

4.10 Molecular characterization

DNA isolation

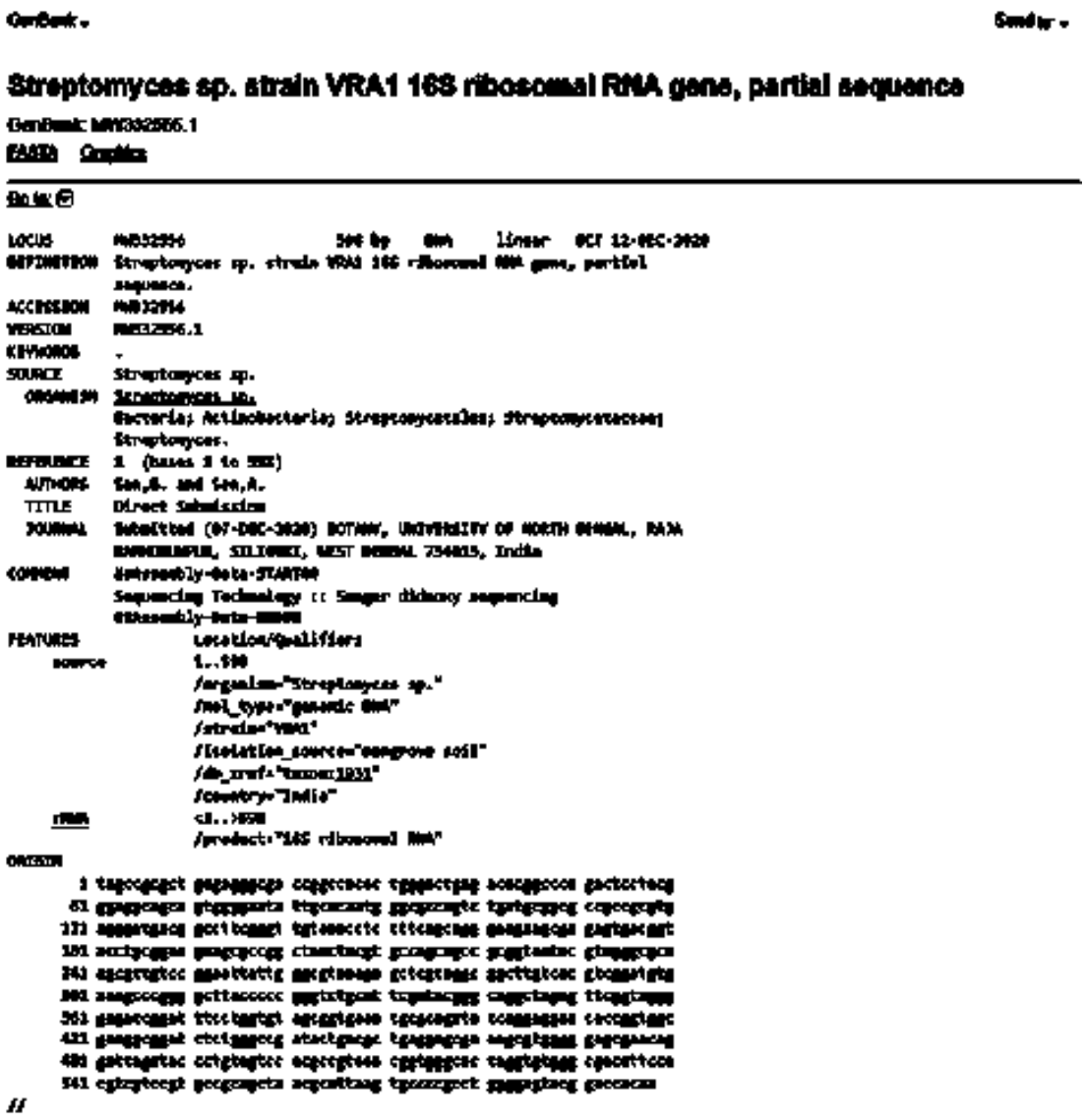
The genomic DNA was isolated using the standard protocol of William & Feil (2012) with minor modifications.

Chemical dissolution by hot lauryl sulfate (SDS) and enzymes like lysozyme and proteinase K employed better lysis of the cell wall for the good yield of total genomic DNA. The presence of several impurities such as cell wall debris, proteins and RNA acts

Figure 4.11: PCR amplification of 16S rDNA. L= 100 bp Ladder (Promega); S1 to S19 amplification of different isolates

Table 4.8: Partial 16S sequences of the isolates submitted to NCBI and their accession numbers Lane number corresponds to the Figure 4.11

Lane No.*	Strain	Accession number	Possible genus
S1	VRA-1	MW332556.1	*Streptomyces actuosus*
S2	VRA-3	MW585686.1	*Streptomyces s*p. CT9210B3
S3	VRA-9	MW585687.1	*Streptomyces tanashiensis*
S4	VRA-10		*Streptomyces*
S5	VRA-12	MW332566.1	*Streptomyces*
S6	VRA-14	MW332567.1	*Streptomyces*
S7	VRA-16	MW332568.1	*Streptomyces* sp.
S8	VRA-17	MW332569.1	*Streptomyces* sp.
S9	VRA-19	OL 851820.1	*Streptomyces*
S10	VR-05		*Streptomyces*
S11	MTA.1	MW332570.1	*Streptomyces*
S12	MTA.13	MW332571.1	*Streptomyces*
S13	Tea02	MK299993.1	*Streptomyces*
S14	TEA10	MK290326.1	*Streptomyces*
S15	ARA.1	MW585688.1	*Streptomyces fimbriatus*
S16	ARA.2		*Streptomyces microflavus*
S17	ARA.3	MW585689.1	*Streptomyces s*p. PDP
S18	ARA.4	MW585690.1	*Streptomyces viridifaciens*
S19	KL02		*Streptomyces*



Streptomyces sp. strain VRA1 16S ribosomal RNA gene, partial sequence

Figure 4.12A: NCBI accession for the partial 16S rDNA sequence of the isolate VRA1

as interference for the downstream processing and reactions of DNA and therefore needed to be purified. The protein impurities were removed by Phenol: chloroform: isoamyl alcohol (25:24:1). TE buffer (1X) was used as DNA storage solution, as EDTA may block the activity of residual nucleases. The ratios of A260 and A280 values obtained spectrophotometric readings were calculated for each sample. The ratio of all the DNA samples was found to be around 1. 8. This showed that the isolated DNA samples were rationally pure. The concentration of the isolated DNA samples varied from 300-485 ng /µl. The intactness of the DNA was checked by Agarose gel

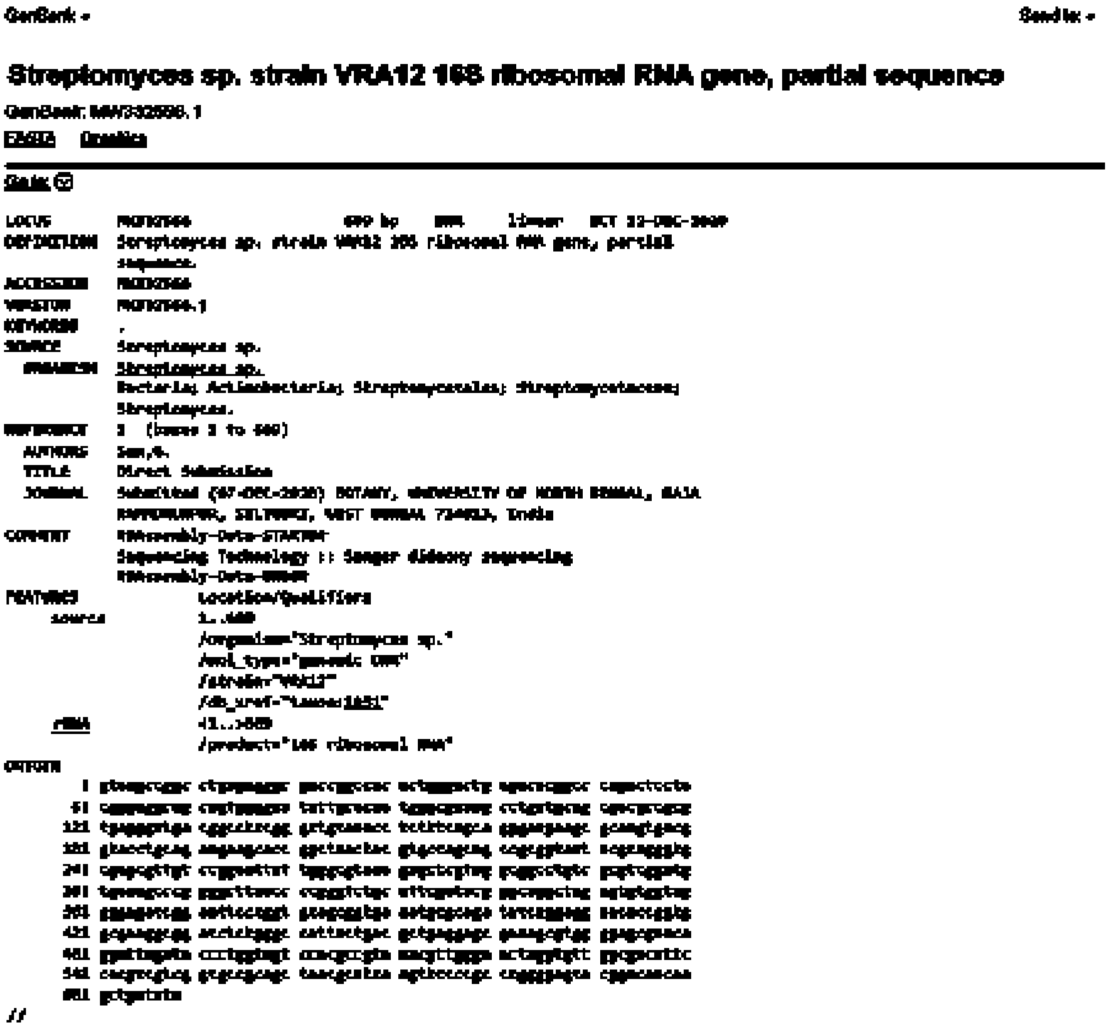


Figure 4.12B: NCBI accession for the partial 16S rDNA sequence of the isolate VRA12

electrophoresis. The DNA samples of the isolates were also purified to remove the RNA by RNAase ttreatment.

4.11 16S rDNA PCR amplification.

The partially conserved 16S rRNA genes of all isolates were subjected to amplification with Actinospecific primers The primer pair successfully amplified the conserved region of the genome in all the isolates. The size of the amplified products was determined through Gel electrophoresis using PhotoCapt software and was found to be 874 bp (Fig 4.11).

Out of the 65 studied isolates, 20 were chosen for partial sequencing of the 16S rRNA gene. Emphasis was given on the diversity of strains while

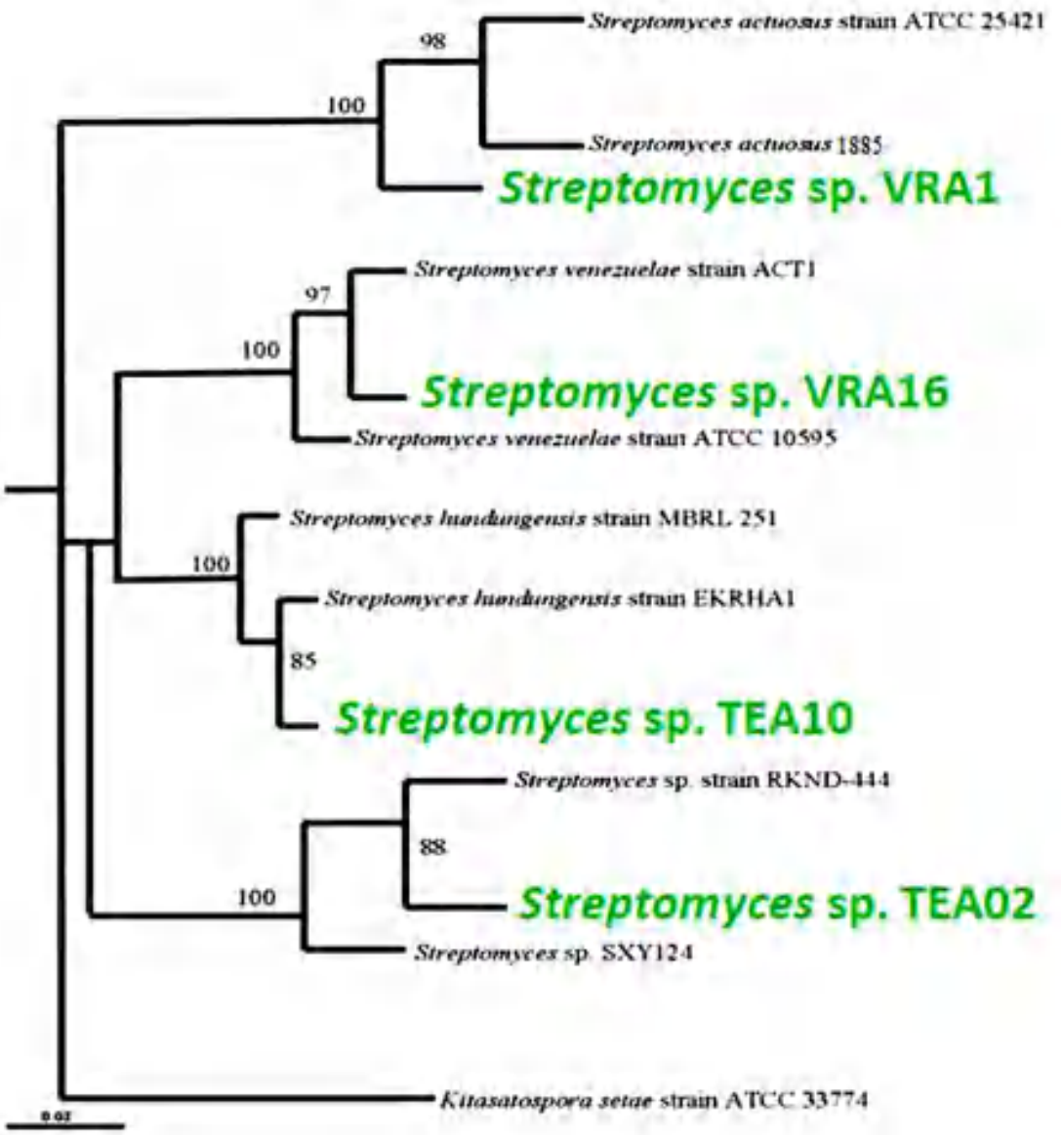


Figure 4.13: Phylogeny based on 16S rDNA sequences from isolates along with *Streptomyces* strains downloaded from NCBI. *Ketasatospora setae* ATCC 33774 was taken as out-group (strains in green were isolated for the present study).

choosing for sequencing. All the products were sequenced properly and the sizes of the sequences were around 874 bp. The sequences were subjected to alignment with the global partial 16S rRNA gene database from GenBank through the BLAST program. The result showed that the isolates showed maximum resemblance with various strains of *Streptomyces* (Table 4.9). The partial sequences were submitted to NCBI.

Table 4.9:Information on the whole genomes of the *Streptomyces* strains sequenced for the present study

Isolates	Nearest strain	NCBI Accession No.	ANI score
VRA1	*S. actuosus*	JAFFZS000000000	87.59%
VRA16	*S. venezuelae*	JAFVLN000000000	82.04%
VRA17	*Streptomyces* sp.	-	
TEA10	*Streptomyces* sp.	JAIGNV000000000	89.47%
TEA02	*S. hundengensis*	JAIGNW000000000	81.09%

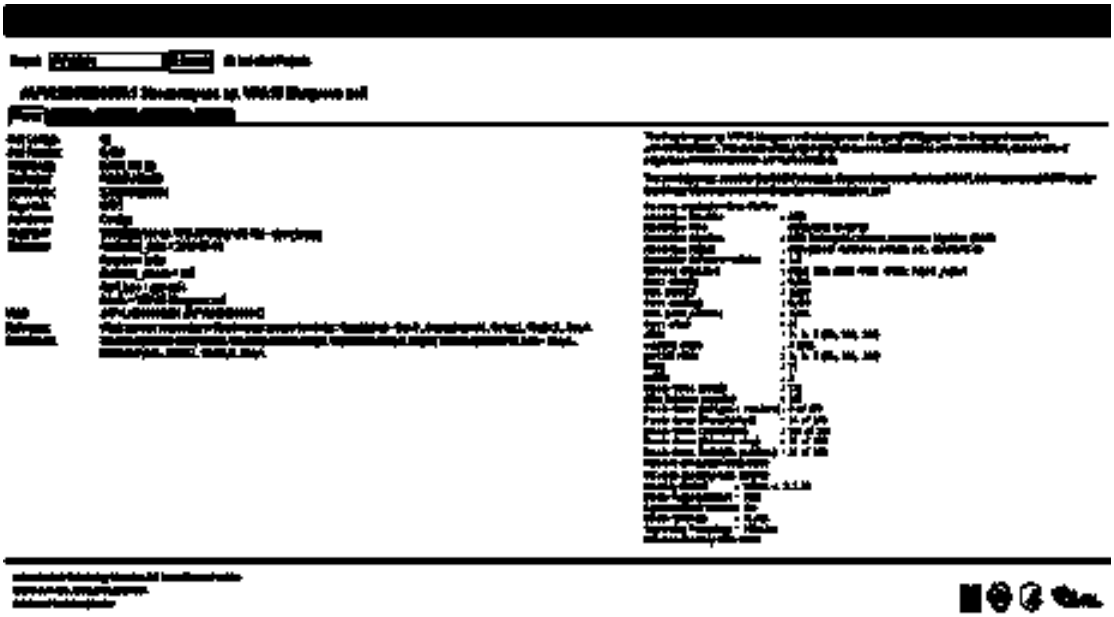


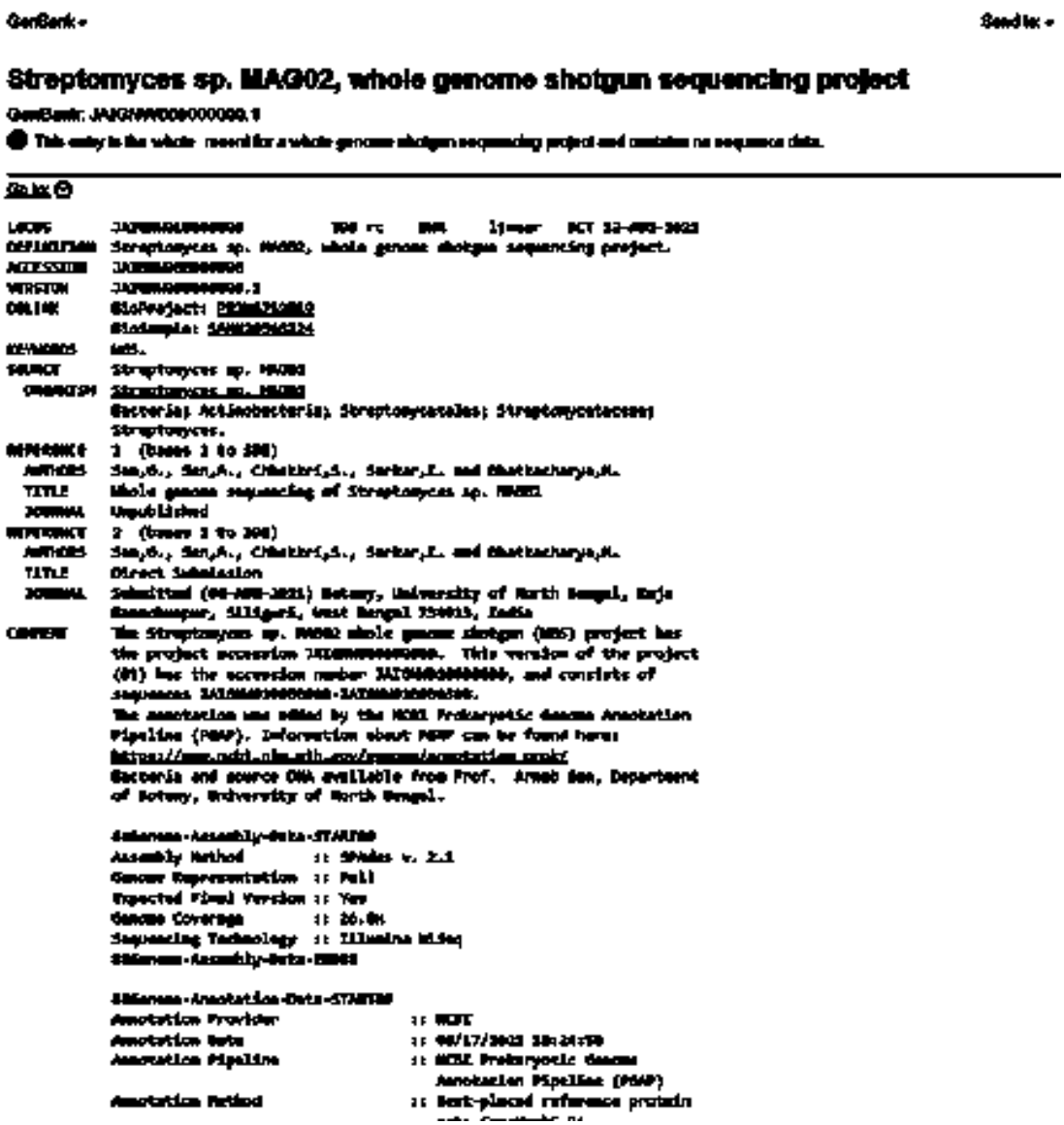
Figure 4.14A: NCBI accession page of the whole genome submission of Streptomyces isolate VRA16

The accession numbers are provided in the above table along with their possible genus. The NCBI accession page of two representative isolates are given for reference (Figure 4.12A & B). The accession numbers for the partial sequence of the isolates submitted to the NCBI are provided in the Appendix B.

4.12 Phylogeny studies with 16S

The phylogeny analysis helps to understand the evolutionary relationship of the isolate with the known strains. For this study,

16S \rDNA was used for the construction of the phylogenetic tree. *Ketasatospora setae* was taken as the outgroup for this analysis. A dendrogram was generated by MEGA ver 10 based on maximum likelihood built with 1000 bootstrap values. From



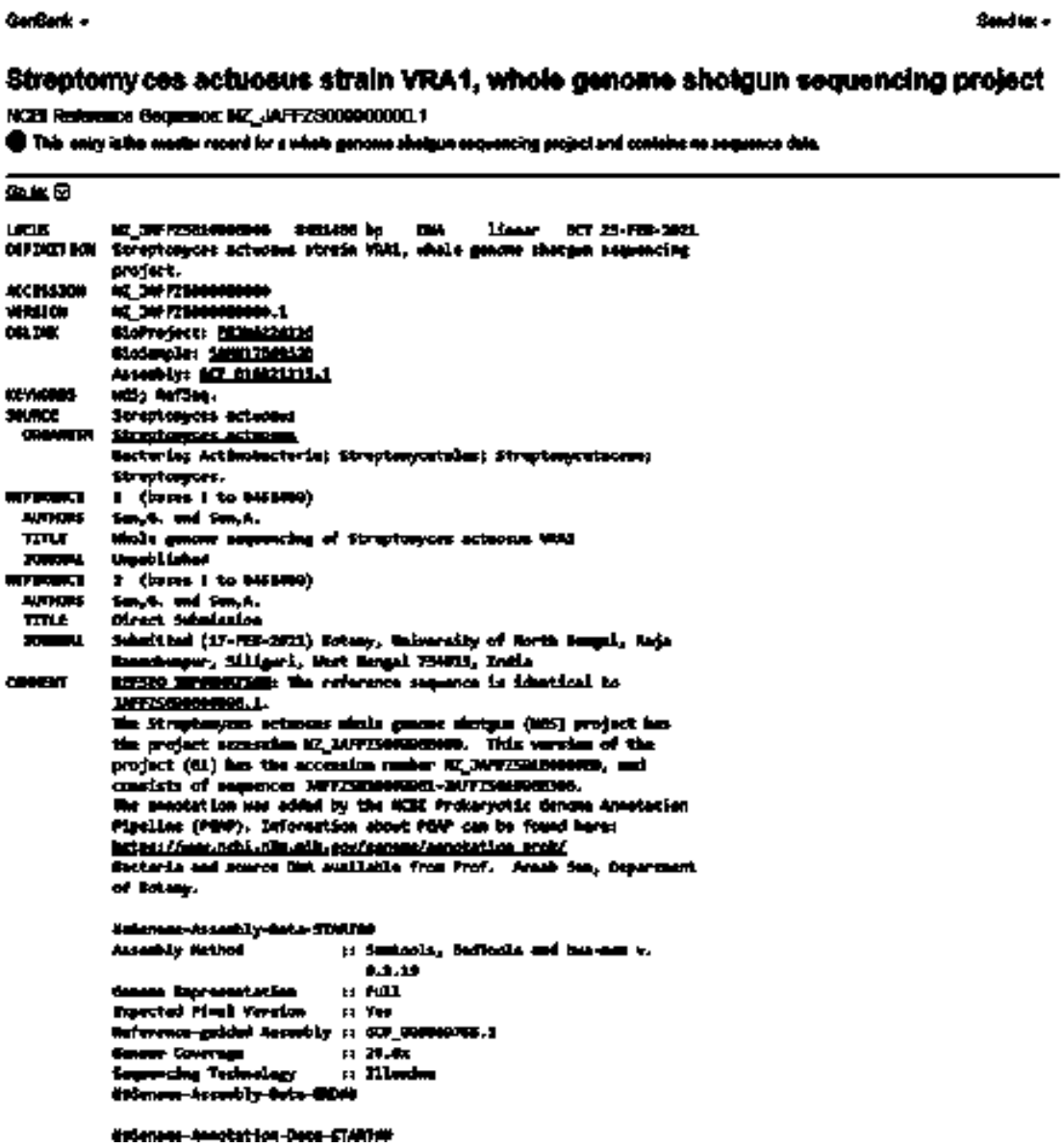
Streptomyces sp. MAG02, whole genome shotgun sequencing project

Figure 4.14B: NCBI accession page of the whole genome submission of Streptomyces isolate MAG02

the tree, it was observed that the isolate VRA 1 clustered with *Streptomyces actuosus* (ATCC25421), VRA 16 with *Streptomyces Venezuela* (ATCC 10595), Tea02 isolate with *Streptomyces hundungensis* EKRH1), Tea10 isolate with *Streptomyces* sp. RKND-444.(Figure 4.13).

4.13 Whole-genome sequencing and assembly

The five isolates were sent for the whole genome sequencing. The sequencing was done by MicrobesNG at the University of Birmingham, the UK using Illumina sequencing technology (Bennett 2004). The reads



GenBank ▾ Send to ▾

Streptomyces actuosus strain VRA1, whole genome shotgun sequencing project

NCBI Reference Sequence: NZ_JAFFZS000000000.1

● This entry is the master record for a whole genome shotgun sequencing project and contains no sequence data.

Go to: ⊡

LOCUS NZ_JAFFZS010000000 0481488 bp DNA linear SCT 25-FEB-2021
DEFINITION Streptomyces actuosus strain VRA1, whole genome shotgun sequencing project.
ACCESSION NZ_JAFFZS000000000
VERSION NZ_JAFFZS000000000.1
DBLINK BioProject: PRJNA624716
BioSample: SAMN17560520
Assembly: GCF_018821715.1
KEYWORDS WGS; RefSeq.
SOURCE Streptomyces actuosus
ORGANISM Streptomyces actuosus
Bacteria; Actinobacteria; Streptomycetales; Streptomycetaceae; Streptomyces.
REFERENCE 1 (bases 1 to 8481488)
AUTHORS Sen,G. and Sen,A.
TITLE Whole genome sequencing of Streptomyces actuosus VRA1
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 8481488)
AUTHORS Sen,G. and Sen,A.
TITLE Direct Submission
JOURNAL Submitted (17-FEB-2021) Botany, University of North Bengal, Raja Rammohunpur, Siliguri, West Bengal 734013, India
COMMENT REFSEQ INFORMATION: The reference sequence is identical to JAFFZS000000000.1.
The Streptomyces actuosus whole genome shotgun (WGS) project has the project accession NZ_JAFFZS000000000. This version of the project (01) has the accession number NZ_JAFFZS010000000, and consists of sequences JAFFZS010000001-JAFFZS010001586.
The annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Information about PGAP can be found here: https://www.ncbi.nlm.nih.gov/genome/annotation_prok/
Bacteria and source DNA available from Prof. Arnab Sen, Department of Botany.

##Genome-Assembly-Data-START##
Assembly Method :: Samtools, BedTools and bwa-mem v. 0.7.19
Genome Representation :: Full
Expected Final Version :: Yes
Reference-guided Assembly :: GCF_000960765.1
Genome Coverage :: 29.0x
Sequencing Technology :: Illumina
##Genome-Assembly-Data-END##

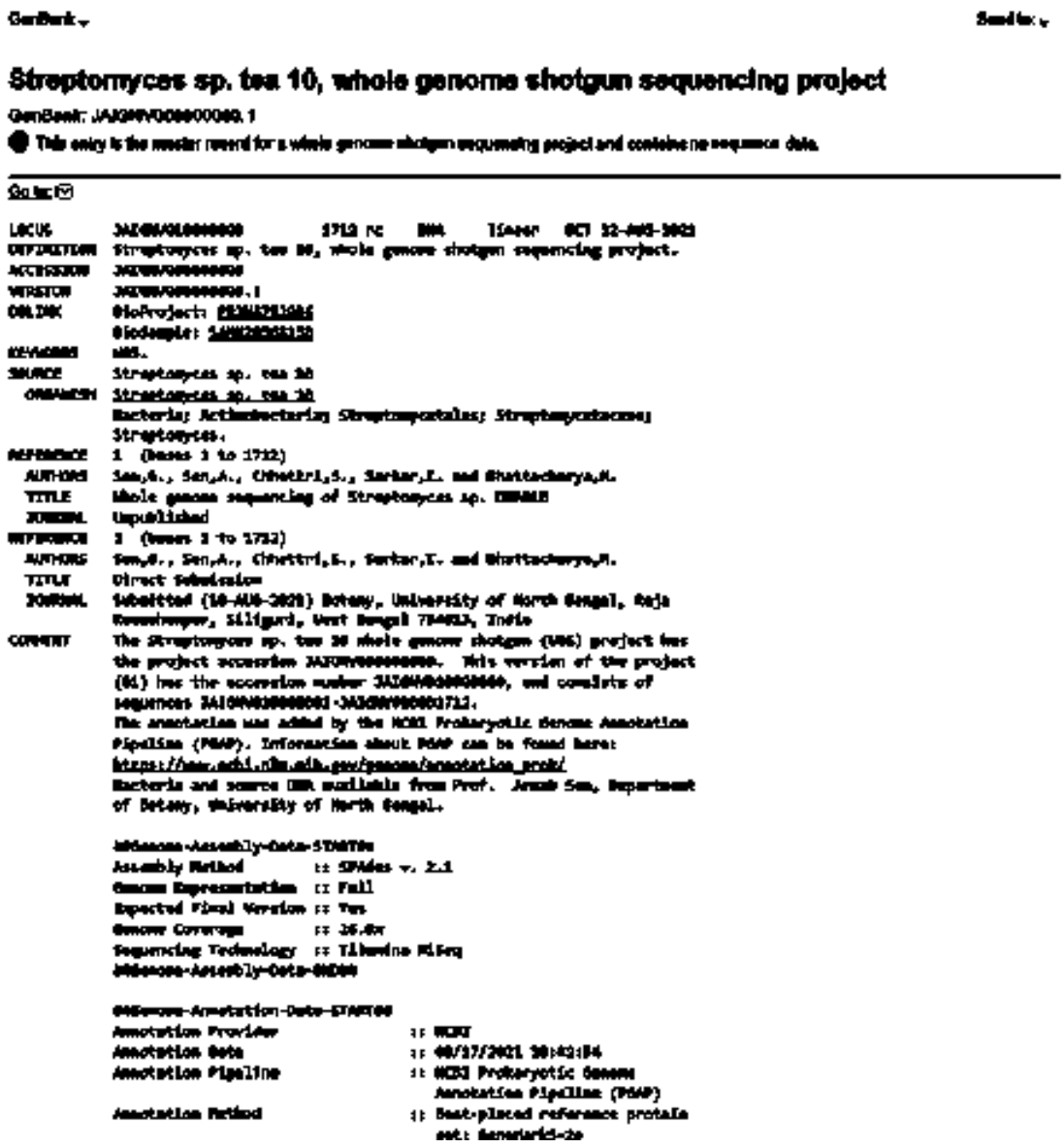
##Genome-Annotation-Data-START##

Figure 4.14C: NCBI accession page of the whole genome submission of Streptomyces isolate VRA1

were trimmed using Trimmomatic and the quality was assessed using in-house scripts combined with the following software: Samtools(Li et al. 2009), BedTools and Bwa-mem (Li et al. 2013)

The BLASTN program of the whole genome sequence of five isolates revealed similarity with the *Streptomyces* species. Out of the five isolates, one isolate showed slight contamination, so opted out from the further analysis.

The number of reads, contigs, GC %, mean reads are given in Table 4.10. The sequence similarity studies to the



Streptomyces sp. tea 10, whole genome shotgun sequencing project

Figure 4.14D: NCBI accession page of the whole genome submission of Streptomyces isolate Tea10

genus and species are also depicted in table 4.11. The assembly has been submitted to NCBI. The accession number, annotation details are provided in the table. Also refer to the annotation page of the genomes, NCBI, (Figure 4.14). The complete list of the accession numbers for the four isolates are provided in the AppendixB

ANI Score

The average nucleotide identity score was calculated using ANI Kostas lab. ANI average was 81. 69% and ranged from 76. 02 to 100%. At 100% 16S nucleotide identity, *Streptomyces* strains can have as low as 84.42% ANI, and across *Streptomyces* ANI and

Cod	VRA1	VRA16	TEA10	TEA02	S.HUN	S.VENEZ	s.SP444	s.HNb38	S.act	Kita	AA
GCU	187	164	55	183	82	80	175	152	99	36	Ala
GCC	10680	12431	1007	15449	13090	13666	11350	10590	12424	15003	Ala
GCA	340	284	127	464	256	290	316	253	226	125	Ala
GCG	4893	6852	1232	9677	3582	3522	4788	5489	4148	3037	Ala
CGU	474	625	102	508	275	269	321	469	307	45	Arg
CGC	5802	6944	742	8709	6052	6442	6349	5671	6776	6848	Arg
CGA	69	42	51	90	63	78	45	66	45	63	Arg
CGG	2336	1838	546	3893	2081	2217	1650	1633	1795	2677	Arg
AGA	7	9	71	18	20	26	5	10	28	8	Arg
AGG	253	130	522	227	126	144	52	121	143	56	Arg
AAU	23	38	20	77	13	14	26	36	9	0.5	Asn
AAC	3101	4022	444	4728	2988	2950	3162	3317	2848	2157	Asn
GAU	138	150	49	334	55	58	107	173	67	13	Asp
GAC	8282	9957	581	12409	8072	8253	7921	8253	8454	7853	Asp
UGU	16	40	47	52	19	15	7	45	23	2	Cys
UGC	1015	1106	427	1469	905	913	912	984	983	920	Cys
CAA	31	44	15	109	29	39	13	72	24	121	Gln
CAG	3826	4513	320	5957	3510	3475	3687	3846	3720	3034	Gln
GAA	645	733	94	1257	1053	1191	678	818	727	1411	Glu
GAG	7862	9081	671	11448	6873	6702	7689	7511	7434	5073	Glu
GGU	1029	1263	172	1161	507	453	706	937	540	125	Gly
GGC	9849	12352	540	15526	10525	10753	10039	10229	10604	9968	Gly
GGA	376	379	76	579	661	771	330	371	326	314	Gly
GGG	1032	1024	534	2033	753	793	958	970	724	497	Gly
CAU	68	54	26	126	35	40	21	69	45	3	His
CAC	2859	3347	254	4192	3025	3100	2768	2600	3034	3198	His
AUU	11	34	16	51	9	10	15	32	6	2	Ile
AUC	5368	6791	643	8399	4922	4784	5393	5787	5020	3524	Ile
AUA	18	47	5	77	28	34	28	43	30	7	Ile
UUA	0.5	0.5	12	0.5	0.5	2	0.5	0.5	1	0.5	Leu
UUG	44	69	551	172	16	22	26	104	29	35	Leu
CUU	48	87	7	205	38	42	52	129	27	10	Leu
CUC	4603	6777	268	8541	8053	8630	5544	5681	5468	6611	Leu
CUA	5	5	3	13	3	4	2	9	4	4	Leu
CUG	7371	7681	423	10910	4261	4188	6486	6140	6889	6663	Leu
AAA	35	42	34	39	58	77	35	42	43	34	Lys
AAG	4417	5909	379	7071	3978	3767	4463	5162	4298	2176	Lys
AUG	2626	3299	557	4048	2291	2227	2513	2656	2359	1565	Met
UUU	3	13	12	13	0.5	2	7	13	5	1	Phe
UUC	3924	5000	448	6451	3820	3863	3906	4089	3923	3022	Phe
CCU	51	47	26	55	29	27	47	47	22	5	Pro
CCC	2819	2989	626	4573	3848	4281	2692	2933	3484	4323	Pro
CCA	17	7	44	39	22	33	11	26	19	15	Pro
CCG	3736	4919	814	6300	3063	2989	3935	3585	3522	3364	Pro
UCU	4	19	26	13	8	8	7	15	2	1	Ser
UCC	3468	3599	1043	4195	3632	3776	3422	3258	3548	2691	Ser
UCA	24	11	85	40	22	23	9	12	26	5	Ser
UCG	1577	2699	929	3479	1107	1036	1698	2019	1094	834	Ser
AGU	28	41	87	81	16	24	22	42	22	7	Ser
AGC	1131	1424	645	2218	1261	1281	1156	1168	1233	1382	Ser
ACU	39	53	24	78	22	28	39	57	27	15	Thr
ACC	5790	6724	627	8921	7201	7526	6180	6195	6796	7559	Thr
ACA	56	53	51	69	36	55	20	35	48	16	Thr
ACG	2112	3368	846	3226	1351	1266	1787	1843	1558	357	Thr
UGG	1639	2065	425	2713	1743	1829	1685	1588	1874	1714	Trp
UAU	34	60	16	149	13	17	17	100	26	0.5	Tyr
UAC	3161	3941	268	4833	3094	3108	3136	3085	3244	2547	Tyr
GUU	44	42	33	112	35	35	51	70	29	2	Val
GUC	7759	9435	468	10725	8718	8859	7860	7427	8010	7530	Val
GUA	97	137	25	310	148	172	211	172	158	25	Val
GUG	3389	3648	903	6332	1874	1802	2727	3393	2762	1784	Val

Figure 4.15: Heat map of Codon Usage of studied strains of *Streptomyces*

Table: 4.10 Variations in the codon usage indices of the studied isolates along with the reference strains of *Streptomyces*

Strains	C3s	G3s	CBI	Fop	Nc	GC3s	GC	Gravy	Aromo	CAI
Streptomyces VRA1	0.60	0.46	0.27	0.26	0.56	32.18	0.91	328.10	-0.12	0.06
Streptomyces VRA16	0.61	0.47	0.27	0.26	0.56	31.91	0.92	331.05	-0.07	0.06
Streptomyces Tea10	0.38	0.38	0.18	0.12	0.48	45.40	0.68	2501.36	-0.70	0.04
Streptomyces Tea2	0.65	0.44	0.27	0.57	30.46	0.93	0.71	-0.06	0.06	0.58
S. actuosus ATCC 25421	0.62	0.48	0.28	0.28	0.57	30.43	0.94	324.78	-0.09	0.06
S. venenzuela Shinobu 719, ATCC 10712	0.65	0.44	0.25	0.24	0.55	30.86	0.93	332.12	-0.08	0.06
S. hundungensis BH38	0.61	0.45	0.27	0.25	0.56	33.22	0.91	318.58	-0.08	0.06
Streptomyces sp. NRRL S-444	0.61	0.46	0.27	0.26	0.56	31.75	0.92	262.20	-0.08	0.06

16S identity do not exhibit a linear relationship (Chevrette et al. 2019) The similarity score and the closest genera have been provided in Table 4.12

4.14 Bioinformatic studies.

Codon usage heterogeneity analysis

A synonymous codon usage pattern and heterogeneity study was done to estimate the level of heterogeneity in codon use. The selected genomes were analyzed for codon heterogeneity using the parameters like GC content, GC3 content and Nc. The codon usage of the four sequenced isolates (VRA 1, VRA 16, TEA 02 and TEA 10) and the selected *Streptomyces* strains along with an out-group *Kitasatospora* indicated towards the biased use of GC rich codon. Proportions of GC, GC3, C3, G3 varied between (57. 75 to 76. 6%, 81. 3 to 92. 5%, 42. 3 to 65. 15% and 42. 6 to 46. 3% respectively. Nc was varied between 20-61 and Fop was ranging between 0. 217-0. 795. The observed lower Nc value indicates higher GC, GC3 and Fop. This shows that GC richness is an important factor that determines codon usage. The effective number of codon (Nc) of the genomes suggests that these high GC-

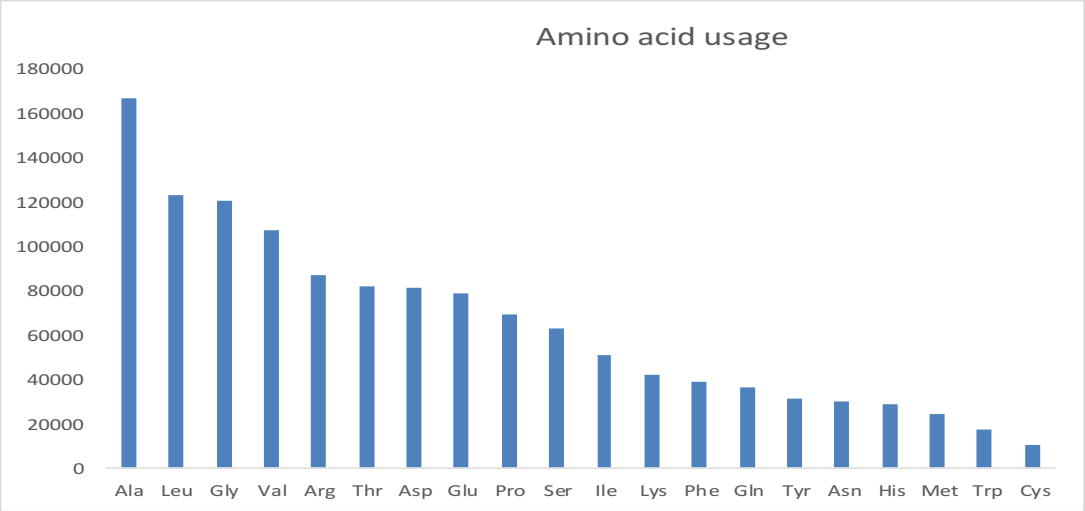


Figure: 4.16 Amino acid usage analysis among the studied strains

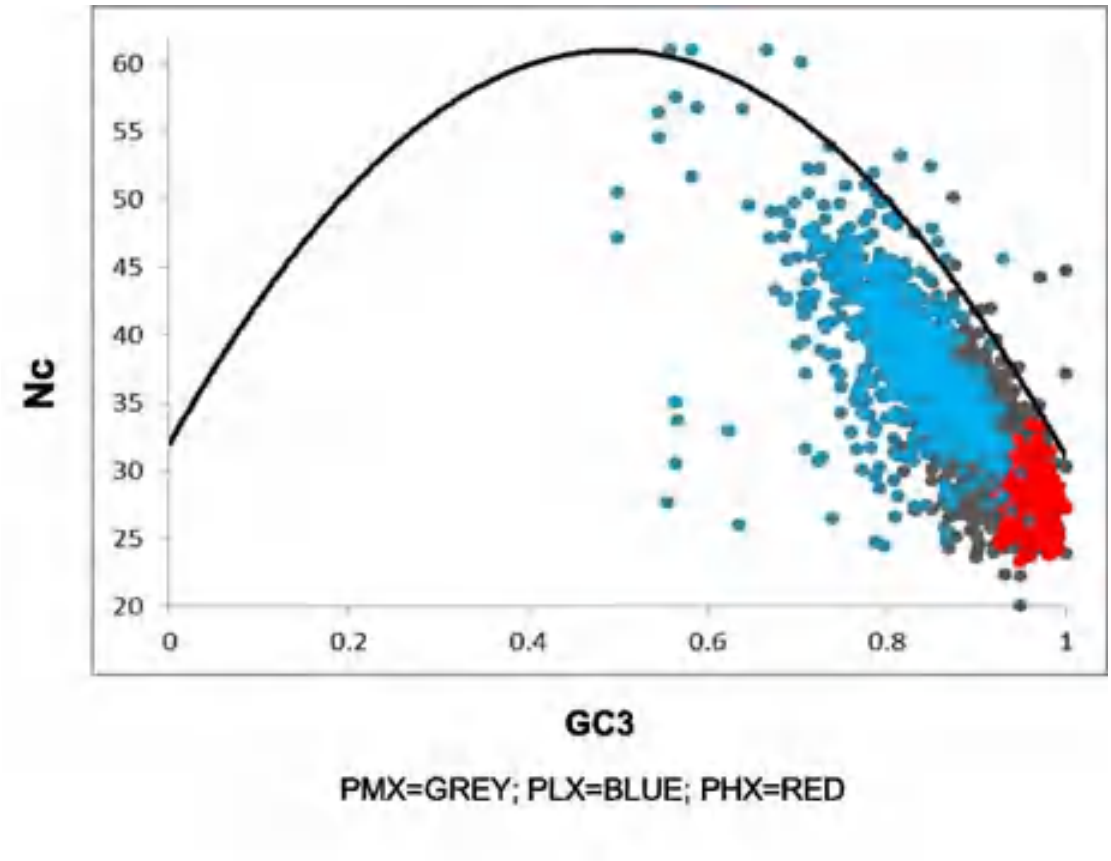


Figure: 4.17 GC_3-Nc plot of the studied isolate VRA 16

rich genomes exhibited considerable heterogeneity in codon usage.

The codon usage heat map showed that GC-rich codons like GCC, GAC, GGC were highly used. This result supports the effect of compositional strains on

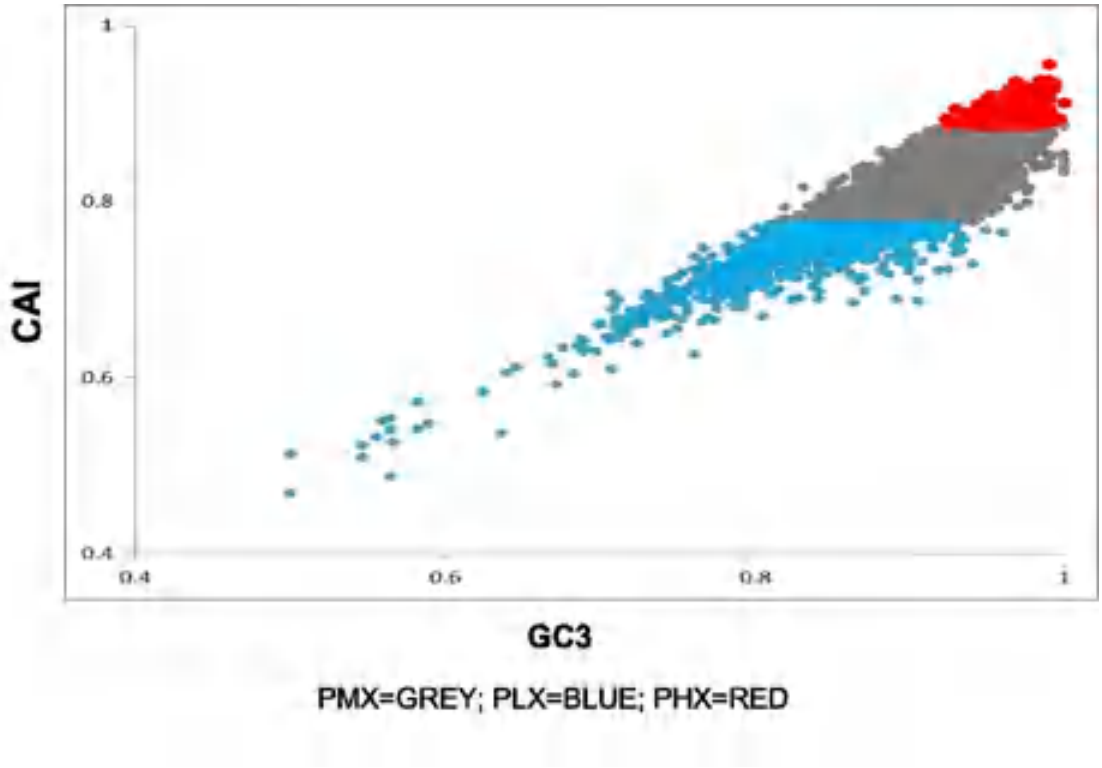


Figure: 4.18 CAI/GC3 plot of the studied isolate VRA16

these genera. Amino acid usage analysis of the above-mentioned strains showed that Alanine, Leucine, Glycine, Valine were the most preferred amino acids.

GC3 vs. Nc plots were generated by the protein-coding genes of the selected genera and it is shown in Figure 4.15. This can be effectively employed to investigate the factors underlying variations in codon usage patterns among genes and genomes. Wright (Wright 1990) has suggested that all the genes concerned would fall on the 'continuous Nc plot curve' or popularly the 'rainbow curve' if the codon usage of a genome is completely governed by only GC compositional constraint. It was found that the majority of the genes were lying below the expected curve towards the GC3 region with lower Nc. This indicated that there might be other crucial factors apart from the influence of compositional constraint which is governing the codon usage patterns of these strains. It has been proposed by (Comeron & Aguade 1998) that genes with low Nc values (<40) are under the influence of a strong codon bias and

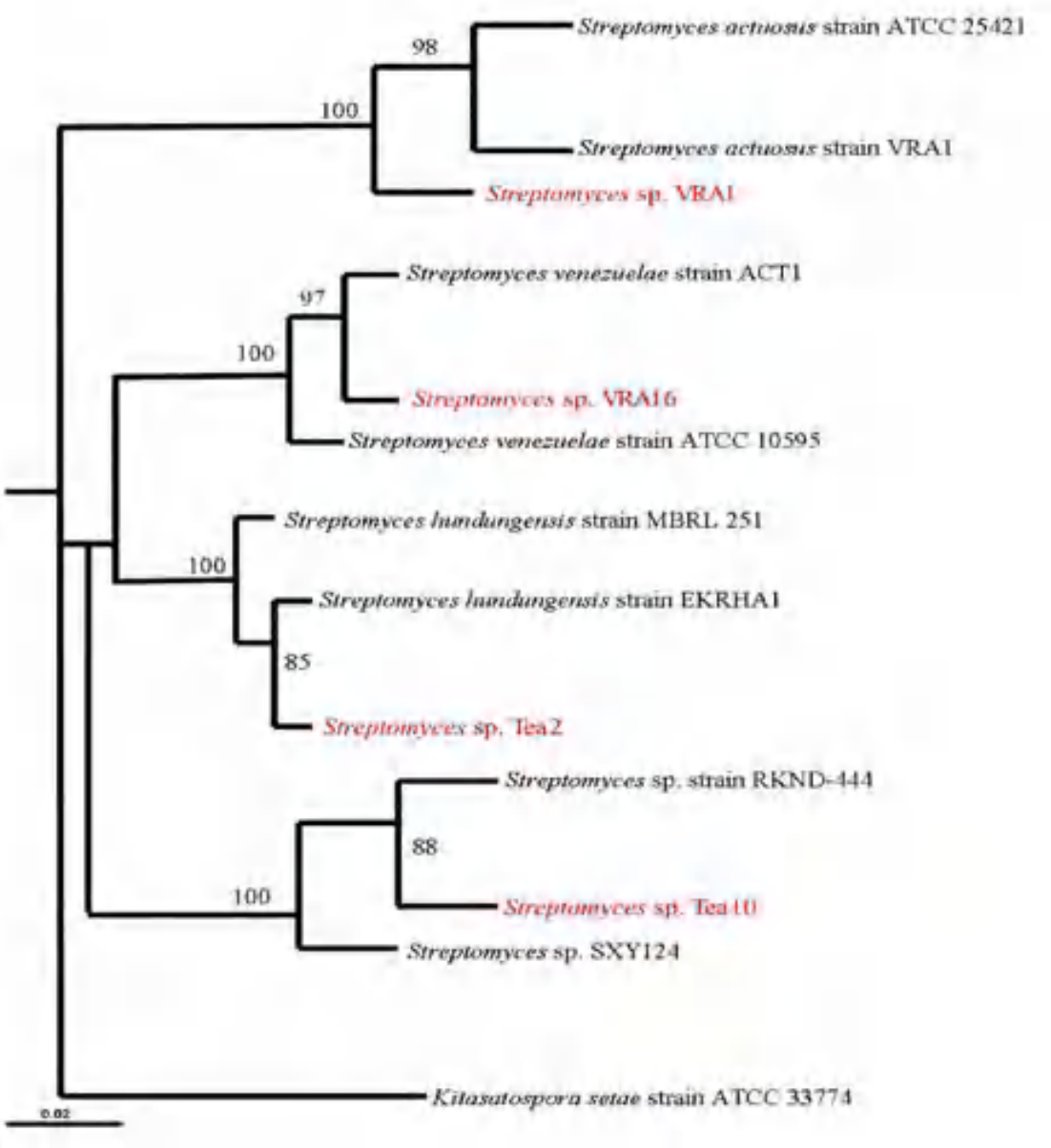


Figure: 4.19: MLSA Phylogeny of the studied strains of Streptomyces along with Kitasatospora as out group (strains in red were isolated in the present study)

would cluster at the lower part of the plot and indicate a strong compositional strain.

Codon adaptation index is one of the major preferred techniques for analyzing the codon usage bias of an organism. CAI measures the deviation of a gene from a reference set of a gene. The ribosomal protein genes were used as the reference set for calculating the codon adaptation index (CAI) and have plotted the CAI values against the GC3 values. (Figure:4.17)

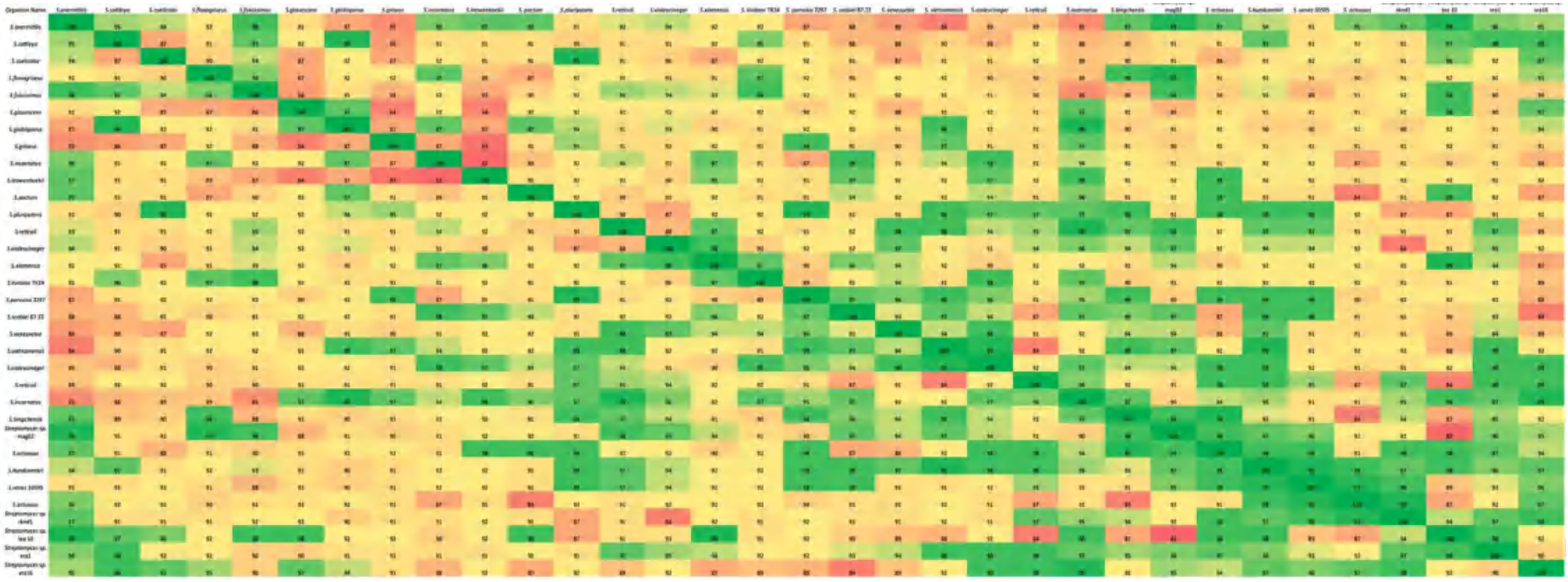


Figure 4.20: Blast-matrix analysis of Streptomyces genomes along with the isolates used in the present study

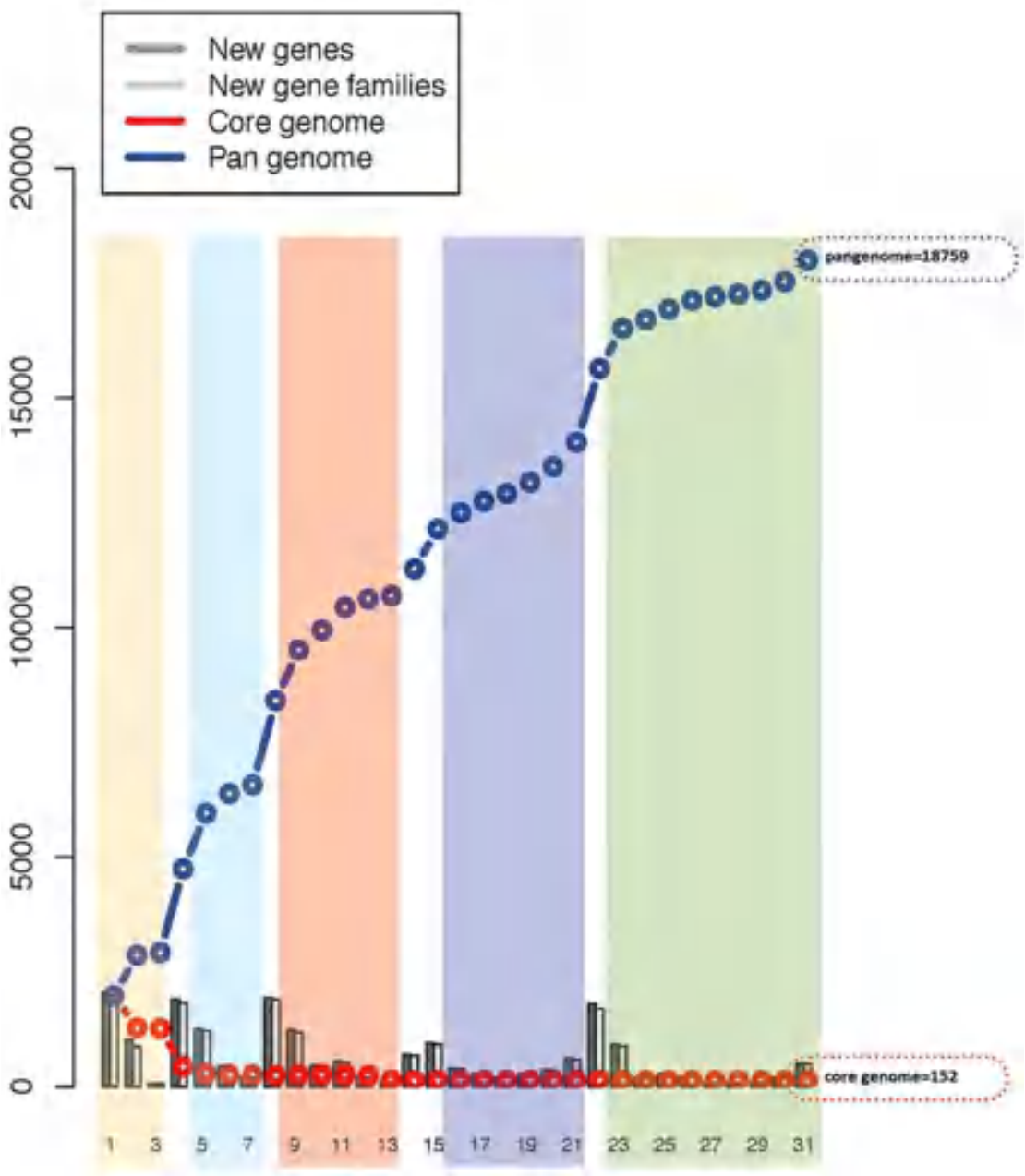


Figure: 4.21: Pan-Core Genome plot of *Streptomyces* genomes along with isolated strains

For PHX of all 4 strains CAI was negatively correlated with ENc and for PLX the correlation was positive ($p<0.01$).

A strong positive correlation between CAI and Fop was observed in all the cases revealing the utilization of the majority of optimal codons by PHX rather than PLX ($p<0.01$). CAI values also showed a significant positive correlation with GC3 and Nc values attributed to the strong compositional

constraints among the genes. Biosynthetic energy cost and aromaticity were correlated with CAI for select genomes. This analysis explored the fact that PHX was using amino acids with low biosynthetic cost than PLX ($p<0.01$). This result completely supports an earlier finding of actinobacterial biosynthetic energy cost (Sarkar et al. 2018)

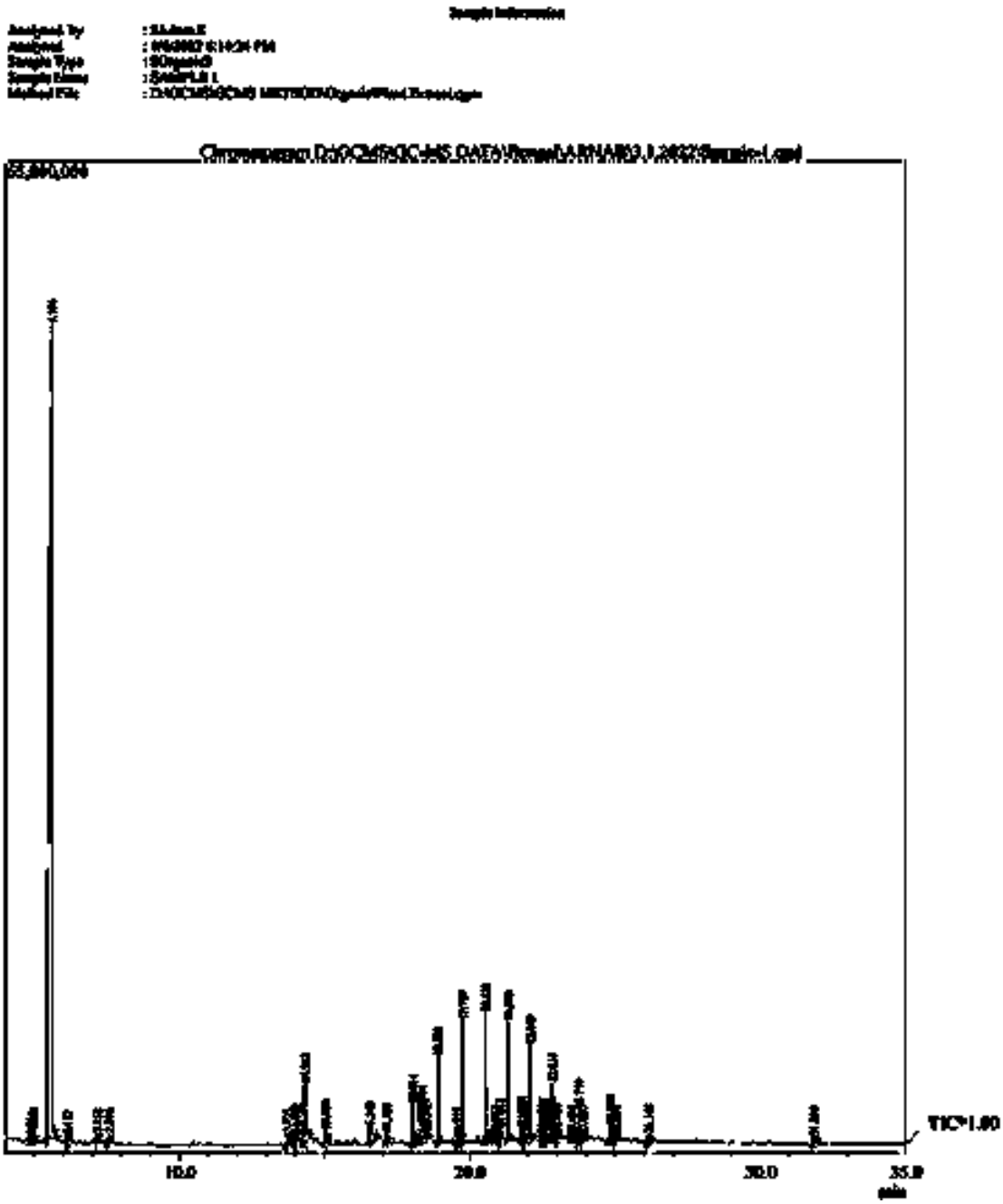


Figure 4.22: GC-MS Chromatogram of the chloroform extract of *Streptomyces* sp. VRA16

Table 4.11: List of Compounds detected through GC-MS analysis in *Streptomyces* sp. VRA16

Peak#	R.Time	Area	Area%	Name
1	4.854	857511	0.16	3-(4-AMINOBUTYL)PIPERIDINE
2	4.953	1405203	0.27	2-CYCLOHEXEN-1-ONE, 6-[(DIMETHYLAMINO)MET
3	5.595	386037567	72.91	4-Piperidinone, 2,2,6,6-tetramethyl-
4	6.132	1366839	0.26	Benzedrex
5	7.152	1077869	0.20	2-BUTENAL, 3-METHYL-, DIMETHYLHYDRAZONE
6	7.576	2406755	0.45	Tricyclo[4.4.0.0(2,7)]decane, 1-methyl-3-methylene-8-(1-m
7	13.723	1710521	0.32	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl
8	14.002	3968605	0.75	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl
9	14.312	17241097	3.26	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
10	15.006	3289521	0.62	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpr
11	16.540	5842717	1.10	N.omega.-Nitro-L-arginine
12	17.105	2186828	0.41	Heneicosane
13	18.014	3856909	0.73	Docosane
14	18.280	3674814	0.69	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-
15	18.344	7435601	1.40	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-
16	18.503	1547475	0.29	Cyclopropanecarboxamide, N-cycloheptyl
17	18.886	8606324	1.63	PENTACOSANE
18	19.577	519009	0.10	9-Octadecenamide, (Z)-
19	19.725	12109819	2.29	PENTACOSANE
20	20.529	14091138	2.66	PENTACOSANE
21	20.837	613299	0.12	Bis(2-ethylhexyl) phthalate
22	21.012	1291692	0.24	CELIDONIOL, DEOXY-
23	21.303	12418870	2.35	PENTACOSANE
24	21.768	1397559	0.26	PENTACOSANE
25	21.846	786162	0.15	3-Methylhexacosane
26	22.049	9870315	1.86	PENTACOSANE
27	22.480	302905	0.06	TRICOSANE
28	22.527	935385	0.18	Tetratetracontane
29	22.613	864129	0.16	3-Methylheptacosane
30	22.837	6874157	1.30	PENTACOSANE
31	22.965	600392	0.11	Squalene
32	23.492	475659	0.09	3-Methyloctacosane
33	23.750	4806056	0.91	PENTACOSANE
34	23.865	722375	0.14	n-Heptadecylcyclohexane
35	24.834	2924658	0.55	Tetratetracontane
36	25.006	878632	0.17	n-Heptadecylcyclohexane
37	26.148	1594442	0.30	Tetracontane
38	31.859	2898369	0.55	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)

RSCU values obtained from Codon W were subjected to multivariate statistical analysis (correspondence analysis) for investigating the source of the complex codon usage. Axis 1 of RSCU was also found to correlate strongly with Fop, GC3 and CAI This correlation pointed towards the preferred usage of GC3 rich optimal codons and a certain level of translational selection on these genes. Hence, we may hypothesize that GC compositional constraint along with the translational selection influence the codon usage as well as expression pattern.

4.15 MLSA Phylogeny

The five housekeeping proteins (atp1,

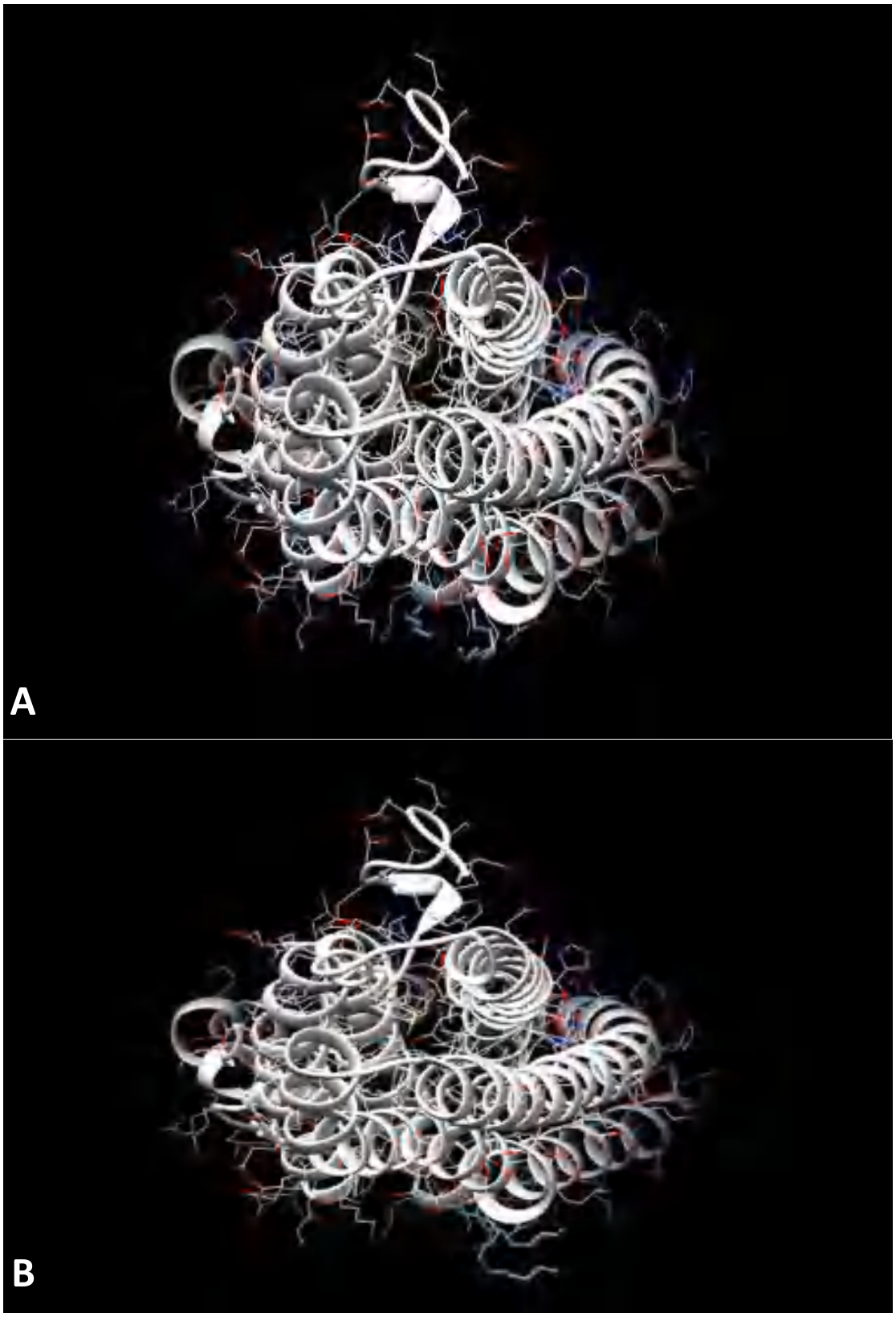


Figure 4.23: Molecular docking: Interaction between 2NRJ protein with A– Pentacosane; B-Celidoniol

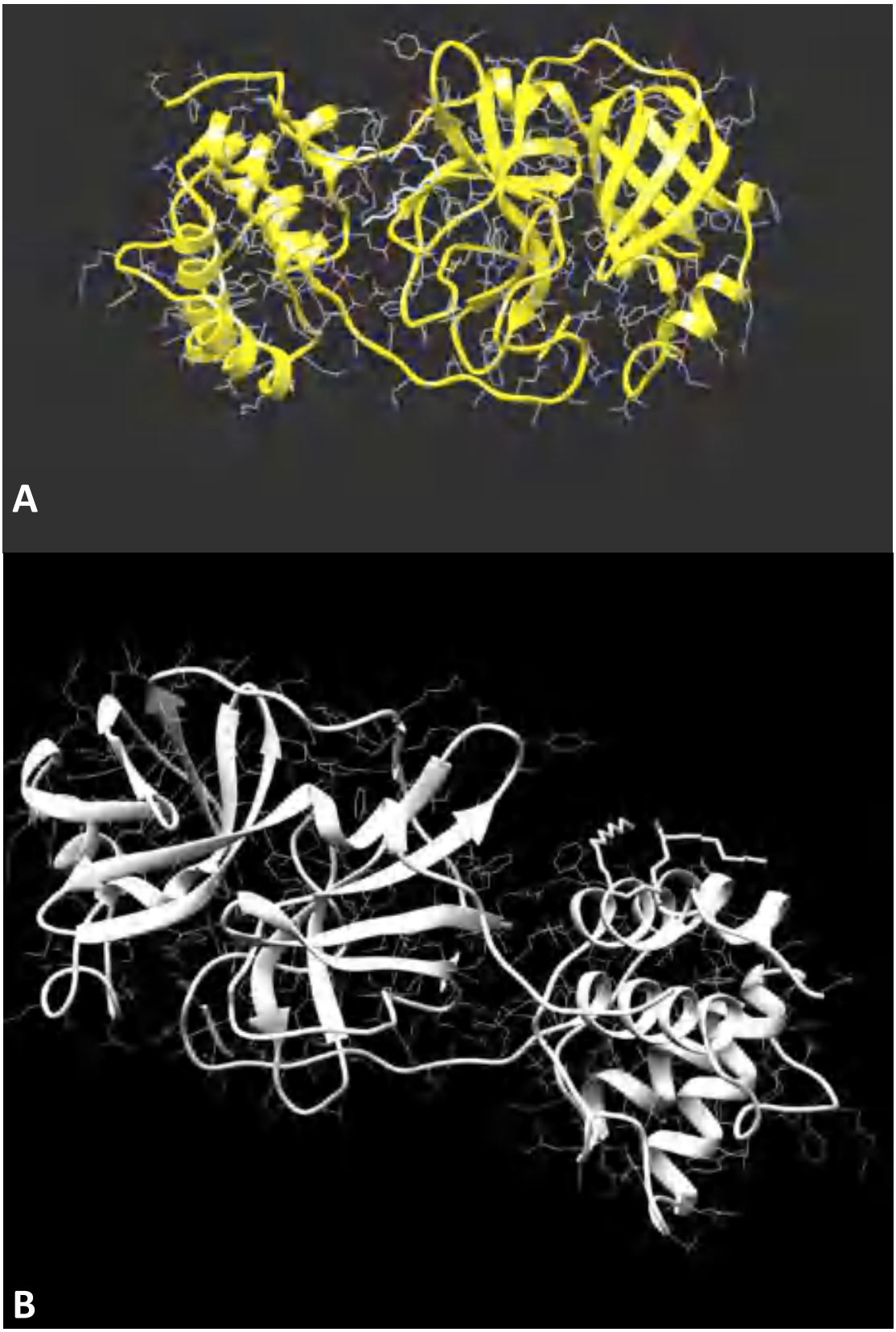


Figure 4.24: Molecular docking: Interaction between 6LU7 protein with A– Pentacosane; B-Celidoniol

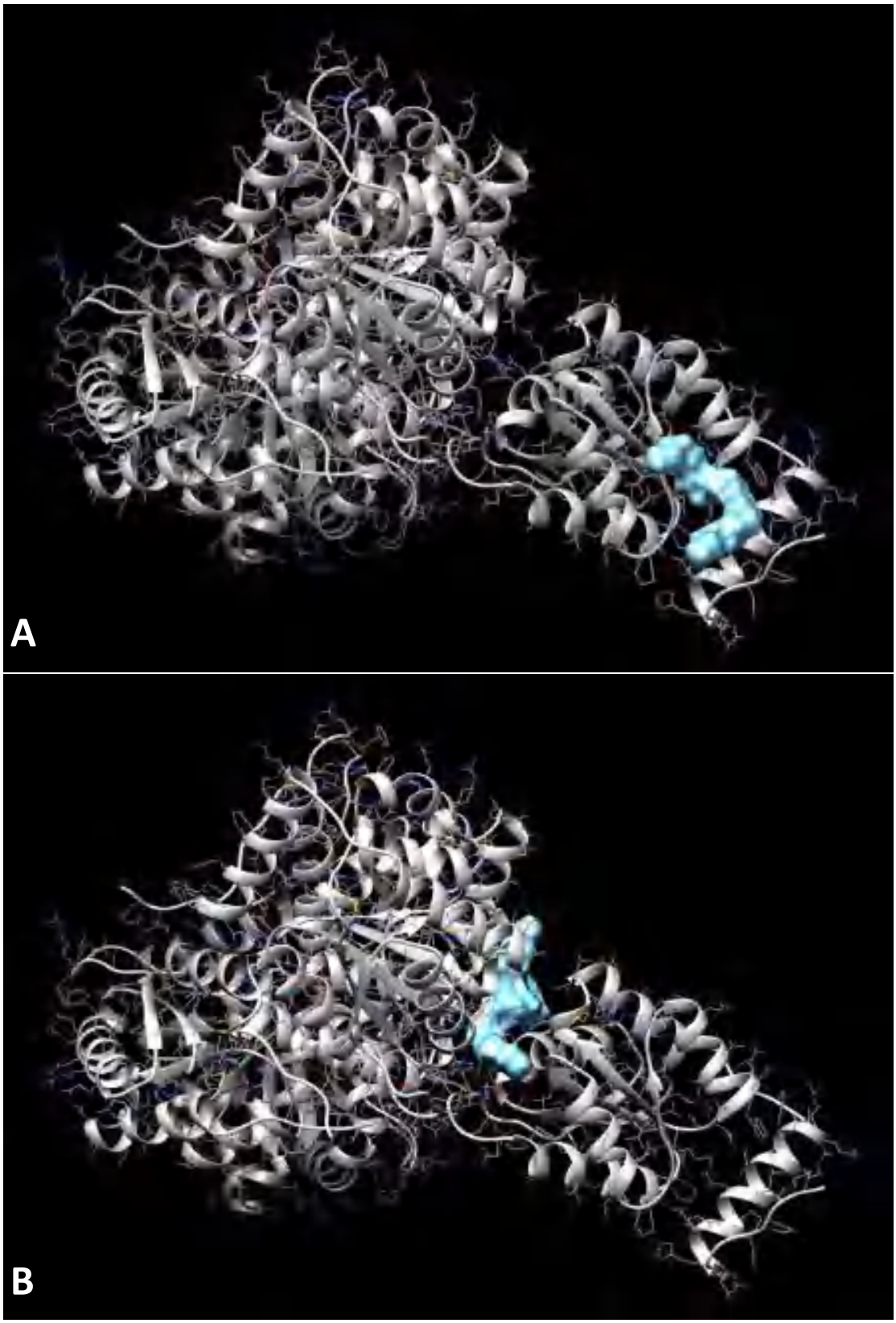


Figure 4.25: Molecular docking: Interaction between 1USL protein with A– Pentacosane; B-Celidoniol

Table 4.12: Docking results showing the binding energy score of the receptor with the ligand

PDB ID	Sourse of the ligand	Ligand	Affinity (Kcal/mol)
1USL	*Streptomyces actuosus*	Nonalactone	-6.45
1USL	*S. actuosus SCF 20*	Deoxyspergualin	-8.34
2BES	*S. actuosus SCF20*	Deoxyspergualin	-7.60
2VIVO	*S. actuosus SCF 20*	Deoxyspergualin	-8.11
5V8	*S. actuosus SCF 20*	Deoxyspergualin	-9.00
7NMQ	*S. actuosus SCF20*	Deoxyspergualin	-7.61
3PYD	*S. actuosus SCF20*	Deoxyspergualin	-8.21
2NRJ	*S. actuosus SCF20*	Deoxyspergualin	-7.70
5V8	*S. actuosus SCF20*	Nonalactone	-6.32
2VIVO	*S. actuosus SCF20*	Nonalactone	-6.47
2BES	*S. actuosus SCF20*	Nonalactone	-6.18
3BZQ	*S. actuosus SCF20*	Nonalactone	-6.26
7NMQ	*S. actuosus SCF20*	Nonalactone	-5.60
3PYD	*S. actuosus SCF20*	Nonalactone	-6.33
2NRJ	*S. actuosus SCF20*	Nonalactone	-6.36
3BZQ	*S. actuosus SCF 20*	Nonalactone	-6.26
2NRJ	*Streptomyces* sp. VRA16	Celidoniol,	-7.34
2NRJ	*Streptomyces* sp. VRA16	Pentacosane	-7.37
2NRJ	*Streptomyces* sp. VRA16	Benzenedrex	-6.52
2NRJ	*Streptomyces* sp. VRA16	3,4 aminobutylpiperidine	-6.68
2NRJ	*Streptomyces* sp. VRA16	Celidoniol,	-7.34
2NRJ	*Streptomyces* sp. VRA16	Benzenedrex	-6.52
2NRJ	*Streptomyces* sp. VRA16	3,4Aminobutylpiperidine	-6.52
1USL	*Streptomyces* sp. VRA16	Celidoniol	-8.88
1USL	*Streptomyces* sp. VRA16	Pentacosane	-8.05
1USL	*Streptomyces* sp. VRA16	Benzenedrex	-6.63
1USL	*Streptomyces* sp. VRA16	2,5, Piperazine	-6.44
6LU7	*Streptomyces* sp. VRA16	Benzenedrex	-5.87
6LU7	*Streptomyces* sp. VRA16	2,5, Piperazine	-6.68
6LU7	*Streptomyces* sp. VRA16	Pentacosane	-7.48
6LU7	*Streptomyces* sp. VRA16	Celidoniol	-7.83
6LU7	*Streptomyces* sp. VRA16	Pyrrolo[1,2-a]pyrazine-1,4-dione,	-6.23

Figure 4.26: Screenshot of antiSMASH result of studied isolate *Streptomyces* sp. VRA1

dnaK, gyrB, ftsZ and secA) were considered for creating MLSA phylogeny. The amino acid sequences of these protein-coding genes of the selected genomes were downloaded for this purpose. It has been observed that the isolate VRA1 clustered with *Streptomyces actuosus* ATCC 25421, VRA 16 clustered with *Streptomyces venezuelae* ACT1, TEA02 clustered with *Streptomyces hudungenesis* EKRHA1 and TEA 10 clustered with *Streptomyces sp.* RKND-444.Both 16S and MLSA phylogeny produced similar results (Figure: 4.18).

4.16 Blast Matrix

The blast matrix showed that newly sequenced genomes showed maximum similarity to Streptomyces. VRA16 has shown 95%, VRA1-96%, TEA10-99%, TEA02-99% similarity with *Streptomyces avermitilis*. The isolates had 97- 98% similarity with *Streptomyces cattleya* except TEA02. It showed 99% similarity with *S. fulvissimus*. The isolates TEA10 and Tea 02 had a similarity index of 98% with *S. glauscens*. The isolates from mangroves exhibited similarities with *S. violeucineger, S. reticuli, S. incarnatus*. The isolate VRA1, VRA16, TEA 10 has a similarity index of 96 -98% with *S. hudungensis*. The other isolate TEA02 showed 98% similarity with *S. venezuelae* (Figure 4.19)

4.17 Pan and Core genome analysis

All the proteins present in the studied genomes constituted the pan pool and the set of proteins common among the strains form the core pool. The pan core plot has been given in Figure 4.21. A total of 18759 genes were accumulated in the pan-genome. However, the core gene count was reduced to 152. The core genes were found to be the housekeeping genes involved in some major metabolic and signal transduction pathways. This wide variation indicates the diversity among the genomes.

4.18 GC -MS analysis

The extracts of the isolates VRA1 and VRA16 were prepared using the organic solvent methanol and Chloroform respectively. The organic phase of the culture extract were subjected to GC-MS analysis. Several bioactive compounds were detected and the chromatogram is provided in

the Figure 4.22. Compounds on further studies depicted antimicrobial, antifungal and antioxidant activities Thus, the isolate with the potential to produce antimicrobial compounds along with the selected compounds of *Streptomyces actuosus* has been further explored for its bioactivity against pathogens that cause various diseases that are common in tea gardens. The common diseases chosen for the present study were tuberculosis, gastrointestinal disorders, Covid - 19. Among the studied compounds, Pentacosane, Celidoniol, Benzedrex, 3,4,aminobutylpiperidine, Deoxyspergualin, Nonalactone, Piperazine etc. produced promising results. In this context, selected compounds were then assessed by *in silico* molecular docking approach against tuberculosis proteins, enteric toxins and Covid Spike protein.

The ADMET score and Lipinski's rule of five were assessed for all major compounds obtained from GC-MS analysis. From this analysis, it was evident that listed compounds have the potency to become drug molecules so they were considered for docking and

Table 4.13: Details of the sampling sites along with the Physiochemical characteristics of the soil samples collected

Sample name	Place	Altitude (in Meters)	Latitude	Longitude	Soil Temp.	pH	OC (%)	OM (%)	N (%)	P (ppm)	K (ppm)
R1	Ghoom	(2282)	27°0'11" N	88°0'13" E	18°C	3.62	3.28	5.64	0.28	16.1	214
NR1	Ghoom	(2282)	27°1'15" N	88°0'9" E	18°C	3.68	3.26	5.6	0.26	16	194
R2	Pashupatinagar	(1877)	26°0'55" N	88°8'48" E	20°C	3.71	2.35	4.04	0.21	15.5	156
NR2	Pashupatinagar	(1877)	26°1'25" N	88°7'68" E	20°C	3.73	2.26	3.88	0.18	15.3	145
R3	Mirik	(1650)	26°52'54" N	88°11'19" E	21°C	3.74	2.74	4.71	0.25	15.3	85.4
NR3	Mirik	(1650)	26°72'34" N	88°15'29" E	21°C	3.92	2.52	4.33	0.22	15.3	87.3

R=Rhizosphere; NR=Non-rhizosphere; OC=organic carbon; OM=Organic matter; N=Nitrogen; P=Phosphorous; K=Potassium

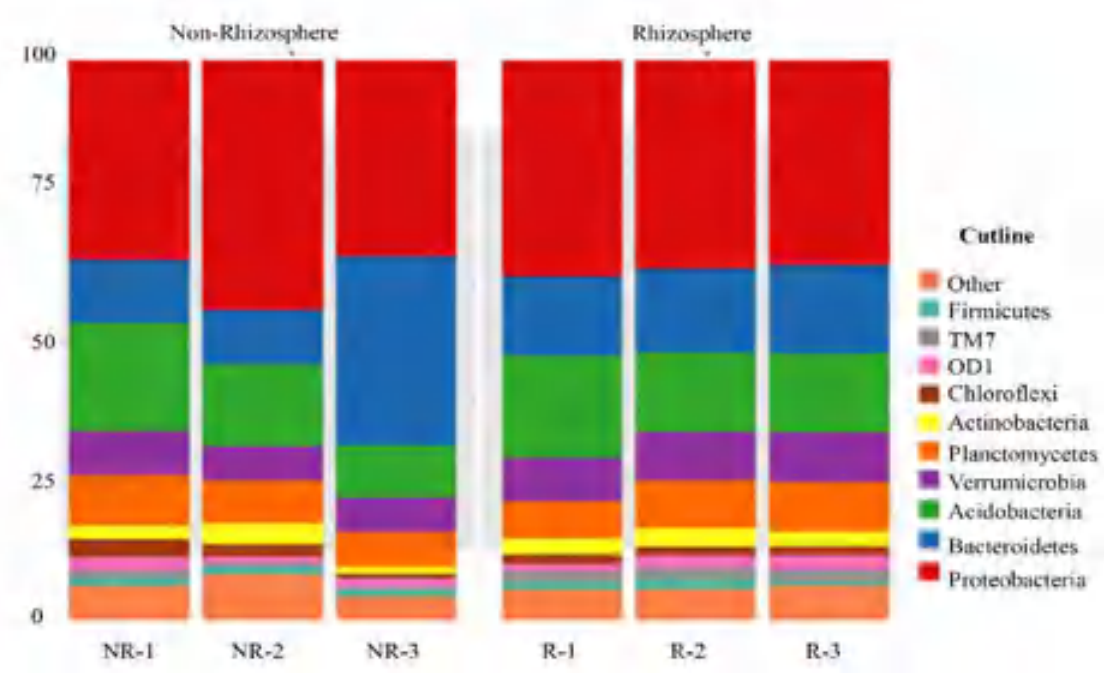


Figure 4.27: Taxonomic abundance analysis based on phylum

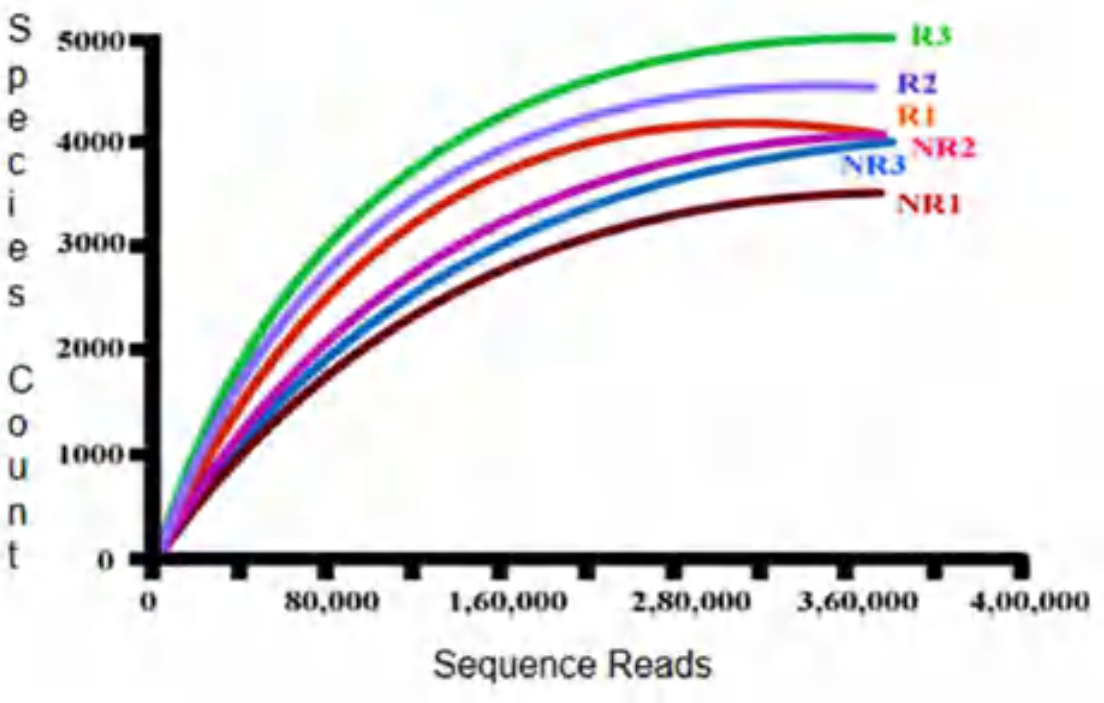


Figure 4.28: Rarefaction curve of studied samples

as probable ligands.

4.19 Molecular Docking

Molecular docking was done in SwissDock software. Blind docking was performed to identify the best

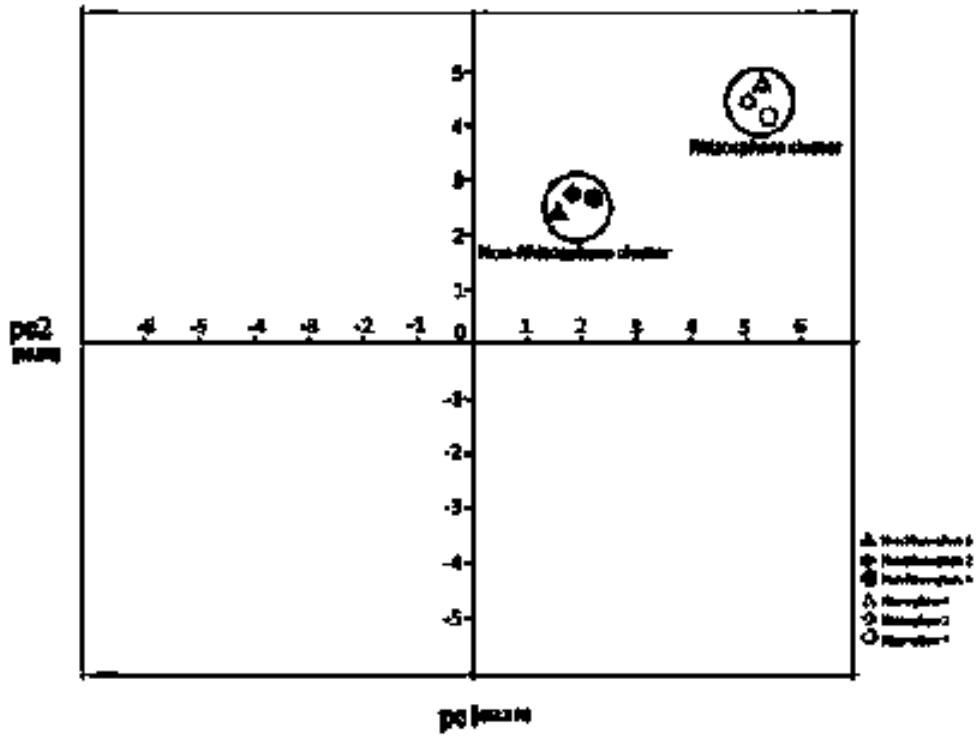


Figure 4.29: PCA plot for rhizospheric and non rhizospheric sample

binding sites using proteins for tuberculosis, Covid spike proteins and enteric toxins. The target proteins for tuberculosis were 1USl, 2VVO,3BSQ,1BES. The enteric toxins of *Bacillus cereus* were 2 NRJ,3PYD,5V8E and the Covid spike protein 6LU7 were selected for the docking studies. Thus, we identified the exact pocket of the protein where the ligands bind with an optimum binding affinity (Table 4.14). After molecular docking analysis, compounds with a binding affinity of more than -7. 0 kcal/mol were only considered. Best ligand-dock complexes were identified based on the binding affinities and their respective images were collected for the best representation of molecular docking (Figure 4.22).

4.20 AntiSMASH analysis:

The bioactive metabolites of the isolates were further studied by *antiSMASH* software. This has further validated our earlier findings. The isolates are indeed enriched with several antibiotics, industrially important enzymes, pigments, etc. The compounds with70% or more identity

scores were considered. These include non-ribosomal peptide synthetase (NRPs), terpenes, lanthipeptides, polyketide synthase (PKS), butyrolactone, saccharide, bacteriocin, hybrids gene clusters such as NRP + Polyketide, Polyketide + Saccharide: Hybrid/tailoring and others. These findings may help to explore and utilize the secondary metabolites in agriculture, the pharmaceutical industry, etc. The known cluster obtained from the isolates revealed similarity with several antibiotics, pigments with similarity percentage of more than 70%. This emphasizes the potential nature of the *Streptomyces* and also proved the efficacy of the studied isolates.

4.21 Metabarcoding of soil samples from selected biotopes

The metagenomics analysis of soil samples from different biotopes was studied with special reference to actinobacteria. The diversity studies of soil microbiome from different biotopes are presented in three different sections for ease of understanding. Details of the sampling are provided in the material & methods section.

4.21.1 Alnus rhizosphere versus non-rhizosphere

The study focused on the 16S metabarcoding of soil samples from the *Alnus* rhizosphere and non-rhizosphere of natural forest areas of different altitudes of Darjeeling hills, India. Physical and chemical analysis of soil Analysis of the soil revealed it to be acidic (pH 3. 62-3. 92), with organic Carbon (3. 28-2. 52%) and organic matter (5. 64-4.04%). The Nitrogen content varies from (0. 28 -0. 18 ppm); Phosphorus (16. 1-15. 3 ppm) and Potassium (214-85. 4 ppm). The rhizosphere soil of the highest altitude showed high organic carbon and organic matter. Though nitrogen and potassium did not show significant variation, potassium content was declining steadily with a decrease in altitude. Details of the sampling sites along with the Physiochemical characteristics of the soil samples collected is provided in the Table 4.13

Sequence processing

FastQC report revealed good quality reads indicating successful metagenomic sequencing**.** Read adapters were trimmed and post

Table 4.14: Major microbial communities in the Rhizosphere and Non-Rhizosphere

R-1	Count	R-2	Count	R-3	Count	NR-1	Count	NR-2	Count	NR-3	Count
Nocardia	3413	***Nocardia***	3420	***Nocardia***	3416	*Xanthobacter*	2879	*Xanthomonas*	2222	*Xanthomonas*	2225
Isosphaera	2223	*Isosphaera*	2230	*Isosphaera*	2226	*Streptomyces*	1849	*Rubrobacter*	2122	*Rubrobacter*	2125
Gemmata	2133	*Gemmata*	2140	*Gemmata*	2136	*Rubrobacter*	1780	*Streptomyces*	1888	*Gemmata*	1848
Cyanobacteria	1780	***Cyanobacteria***	1787	***Cyanobacteria***	1783	*Polaromonas*	1580	*Ureaplasma*	1561	*Streptomyces*	1824
Gemmatimonas	1580	***Gemmatimonas***	1587	***Gemmatimonas***	1583	*Sideroxydans*	1561	*Thermodesulfobacterium*	1511	*Borrelia*	1564
Clostridium	1561	*Clostridium*	1568	*Clostridium*	1564	*Thiobacillus*	1511	*Bilophila*	1457	***Cyanobacteria***	1514
Corynebacterium	1511	*Corynebacterium*	1518	*Corynebacterium*	1514	*Curvibacter*	1411	*Anaeroplasma*	1205	***Gemmatimonas***	1208
Burkholderia	1050	*Burkholderia*	1057	*Burkholderia*	1053	*Methylobacillus*	1050	*Kosmotoga*	1050	*Anaeroplasma*	1053
Rhodopirellula	798	*Rhodopirellula*	805	*Rhodopirellula*	801	*Candidatus* Accumulibacter	798	*Thermosipho*	879	*Clostridium*	882
Frankia	637	***Frankia***	644	***Frankia***	640	*Desulfatibacillum*	617	*Petrotoga*	798	*Ureaplasma*	801
Acidobacterium	631	*Acidobacterium*	638	*Acidobacterium*	634	*Bilophila*	614	*Rubritalea*	621	*Corynebacterium*	624
Staphylococcus	614	*Staphylococcus*	621	*Staphylococcus*	617	*Desulfotalea*	611	*Aspergillus*	526	*Thermodesulfobact.*	617
Saccharopolyspora	536	*Saccharopolyspora*	543	*Candidatus* Cloacamonas	539	*Desulfuromonas*	516	*Paracoccidioides*	460	*Burkholderia*	529
Pseudomonas	460	*Pseudomonas*	467	*Vaccinium*	463	*Pelobacter*	460	*Nectria*	426	*Kosmotoga*	463
Ktedonobacter	426	*Ktedonobacter*	433	*Bacteroides*	429	*Corallococcus*	426	*Trachelomonas*	282	*Rhodopirellula*	429

R: Rhizosphere, NR: Non-rhizosphere; (Diazotroph taxa are in bold)

trimming adapter content fell between 0% - 1% for all samples. The paired-end reads from soil samples gave 54% to 56% average GC content and reads were 0. 3M to 0. 4M sequences with duplication values ranging from 47. 30 % to 82. 90%. These meta sequences are deposited to NCBI SRA and Accession numbers are given in Appendix B.

Taxonomic abundance analysis

Out of the six samples under study, the major phyla present in the rhizosphere (R1, R2, R3) and bulk soil or non-rhizosphere (NR1, NR2, NR3) are Proteobacteria, Bacteriodotes, Acidobacteria, Verrumicrobia, Planctomycetes, Actinobacteria, Chloroflexi, Firmicutes, OD1, TM7 and others. Proteobacteria was the most abundant phylum (37. 25%) with no significant difference in the distribution in the rhizosphere and non-rhizosphere region of different altitudes. This phylum is an important component of the soil microbiome which is involved in biogeochemical cycles (Hunter et al. 2006). Bacteriodetes was the second abundant phylum with a distribution of 10 to 12% in all samples but for the non-rhizosphere of Mirik (NR3), its population was exceptionally high (35%) ($p<0.001$). It is a known fact that this phylum is adapted to different ecological niches and is a part of the gut microbiome (Leng et al. 2010, Moore et al. 1974). Interestingly, the increase in the Bacteroidetes population in NR 3 area drastically reduced the presence of other phyla. It is also observed that the population of Acidobacteria, Verrumicrobia, Planctomycetes were reducing with a decrease in altitudes in the non-rhizosphere region but it remained more or less the same in the rhizosphere region of different altitudes. The Actinobacteria phylum represented a uniform distribution in the rhizospheric region irrespectve of the different altitudes (Figure 4.27).

At the genus level, there were a total of 2728 genera (apart from the unclassified taxa) from the six samples (R1, R2, R3, NR1, NR2, NR3). The genera *Nocardia, Isosphaera, Gemmatimonas, Clostridium, Corynebacterium, Burkholderia, Rhodopirellula, Frankia* were the most abundant common genera with uniform

distribution in the rhizosphere region of *Alnus* irrespective of their difference in altitudes. This indicates that a specific microbial community is being attracted towards the rhizospheric region and also found that the majority of the core microbial community in the soil shows nitrogen-fixing properties. Nevertheless, the non-rhizosphere region exhibited no specific choice in microbial population and had a high diversity index

Rhizospheric effects on microbiome diversity.

Based on the OTU number, Chao1 bacterial species abundance index, and Shannon microbial diversity index, α-diversity analysis was conducted on the microbial diversity of various samples. Results indicate that indigenous microbial community diversity was significantly higher in non-rhizospheric soil than, *Alnus* rhizosphere which holds good for all three altitudes. This validates the previous report which shows rhizospheric diversity is lower than bulk soil (Hein et al. 2008, Costa et al. 2006). A significant rhizosphere effect was reflected by reduced microbiome diversity in the *Alnus* rhizosphere compared with that of the non-rhizosphere. The Simpson index of alpha diversity for the rhizosphere ranges from 76. 31 to 125 and in the non-rhizosphere it varies from 25. 38 to 69. 45. The higher value for Simpson index indicates lower diversity. The microbial diversity of the rhizosphere decreases from high to low altitudes but intriguingly, diversity in non-rhizosphere regions decreases from low to high altitudes.

The assessment of the Beta diversity of microbial communities across different rhizosphere and bulk soil indicates the effect of altitudes among the microbial population of the rhizosphere and non-rhizosphere. On the functional front, both the altitudes and soil conditions such as temperature and pH play an equal role in microbial diversity. PCA analysis was used to study the similarity among various rhizosphere and bulk soil samples in the structures of bacterial communities exploring the main influencing factors driving the differences in micro-community compositions. The PCA axis in the PCA plot indicated the total sequence reads for each soil sample. The

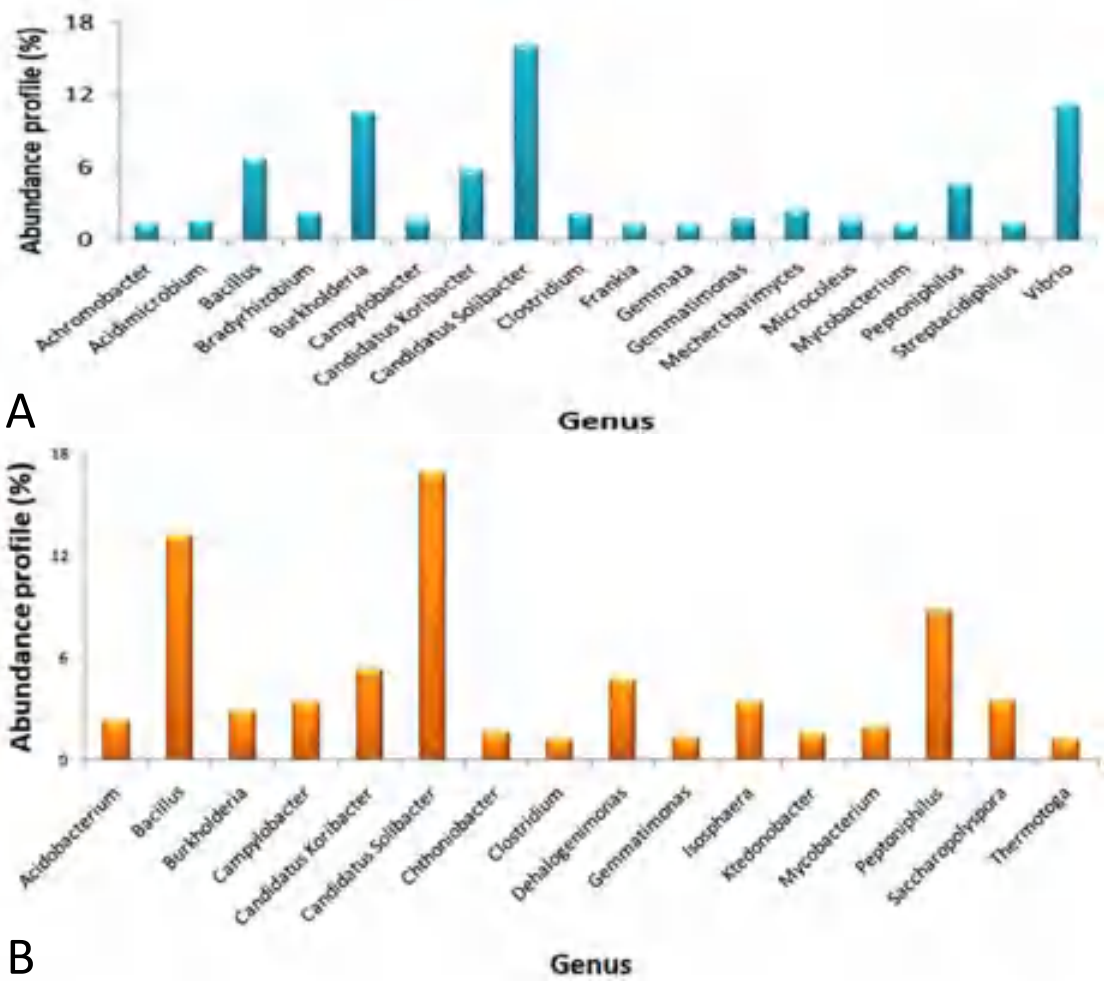


Figure 4.30: Taxonomic abundance analysis at genus level A = Mak; B = Cas

C= Diversity index for pathogenic microbial genera in Cas & Mak

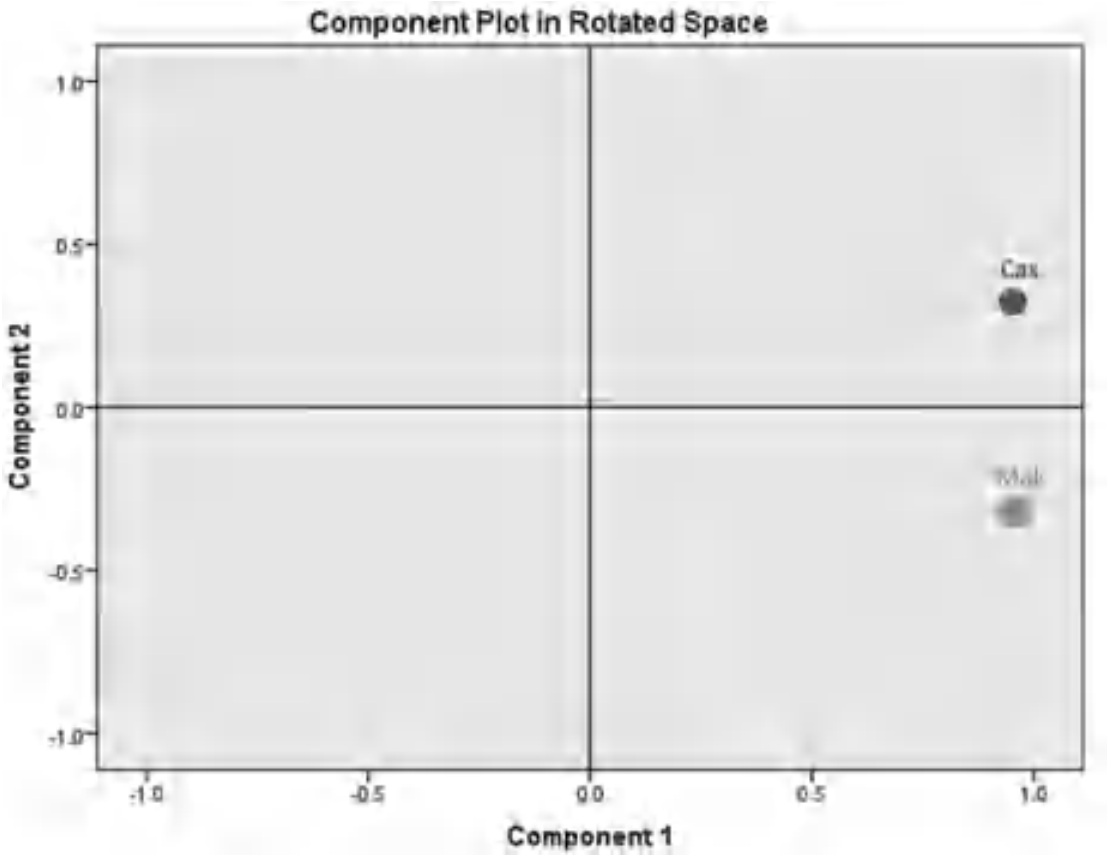


Figure 4.31 : Principal Component Analysis showing two distinct cluster in two different quadrant for Cas & Mak

subsequent multivariate analysis shows the clustering of non-rhizosphere and rhizosphere on the same axis indicating similar physical environmental conditions of the soil. But interestingly, the rhizosphere and non-rhizosphere formed a distinct and separated cluster on the same axis demonstrating the specific choice of the microbial community in these regions (Figure 4.28). The rarefaction curve for the rhizosphere and non-rhizosphere shows that the microbial population of rhizosphere soil is getting saturated at a higher level (5000 sequence reads) compared to the non-rhizosphere (3500 sequence reads). This is indicative of the volatile microbiome of the *Alnus* rhizosphere due to the host plant's interaction with the microbial population. Fascinatingly, it is observed that the non-rhizosphere presents a stable scenario where it gets saturated at a lower level (Figure: 29).

The functional profiling analysis

showed that KEGG Ontology (KOs) were mainly involved in 23 KEGG level 2 pathways and 25 KEGG level 3 pathways for both rhizosphere and non -rhizosphere. This may be due to the fact that microbes are evolved in the same physical environment which has resulted in the lack of variation in metabolic pathways.

The microbial interaction network is constructed to explore co-occurrence and co-exclusion patterns of organisms or functional genes across microbial communities. In the interaction network, each node represents a single organism (or gene), and nodes are connected by links that represent their correlation coefficient of abundance variation among multiple samples (Faust, et al. 2012). The network analysis shows that the major phyla were represented as 23 nodes belonging to Proteobacteria, Cyanobacteria, Nitrospirae, Firmicutes, Acidobacteria, Planctomycetes, Actinobacteria, Verrucomicrobia, Choloroflexi, Bacteroidetes, etc. Further, when we analyzed the bacterial count of rhizosphere versus non-rhizosphere (bulk soil), we found that approximately 50% of the microbial population was associated with nitrogen metabolism in rhizosphere (Table 4.14). This substantiates our findings that the *Alnus* rhizosphere is enriched with a specific microbial community that exhibits nitrogen fixation properties. Contrary to that, the non-rhizospheric soil exhibit rather neutral behaviour in case of microbial populations. This corroborates with the earlier findings where allelochemical inhibition of nitrification in nondiazotrophic plants was reported (Rice & Pancholy (1972), and Gokçeoglu (1988).

4.21.2 *Comparative metagenomics of tea garden soil*

We studied two very popular tea gardens of Darjeeling hills- Makaibari (Mak) and Casselton (Cas). The main difference between them is the type of manure they use. Mak is exclusively an organic tea garden using all organic manure and fertilizers whereas Cas uses inorganic pesticides and fertilizers. The Cas soil was clay type whereas the Mak soil was light, friable loam with porous subsoil. This soil type is preferred for tea cultivation due to the free percolation of water. The

low pH of both the soils indicated the acidic nature of the soil which is good for tea. The paired-end reads from Mak and Cas soils gave 56% and 55% average GC respectively.

Major microbial phyla identified in both of the soil samples. were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Choloflexi*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes* etc. *Cyanobacteria* and *Gemmatimonadetes* were present in Mak constituting 1. 32% and 1. 24 % of the total microbial population respectively whereas; they constituted less than 0. 5% in Cas soil. The relative abundance of the pathogenic microbial population was found to be more in Cas than in Mak (Figure 4.30). The pathogenic microbes like *Burkholderia, Campylobacter*, and *Bacillus* were much higher in Cas than in Mak. Moreover, the abundance of *Mycobacterium* was also more in Cas than in Mak. Along with those mentioned genera, the presence of *Candidatus Solibacter, Candidatus Koribacter, Peptoniphilus, Peptoniphilus, Clostridium, Gemmatimonas* were found in both Cas and Mak. *Bradyrhizobium, Microcoleus, Acidimicrobium, Streptacidiphilus, Achromobacter, Gemmata and Frankia* were solely present in Mak but not in Cas however, *Dehalogenimonas, Saccharopolyspora, Isosphaera, Acidobacterium, Chthoniobacter, Ktedonobacter, Thermotoga* were exclusively present in Cas but not in Mak. This indicated the differential bacterial population among Cas and Mak. A PCA plot based on the pathogenic microbial population also supported the ANOVA results where Mak and Cas were placed in two different quadrants of the PCA plot (Figure 4.31).

Alpha (α) diversity is a direct measure of mean species diversity of habitat and a higher α diversity value indicates more diversity. The α diversity value o based on Shannon index of Cas was 48. 69 and for Mak, it was 56. 62 pointing to more species richness in Mak. A Rare-fraction curve that allows us to calculate the species richness from a given number of individual samples was further implemented to support our aforementioned hypothesis. A common pattern of this curve is, it grows rapidly at first due to the most common species present in

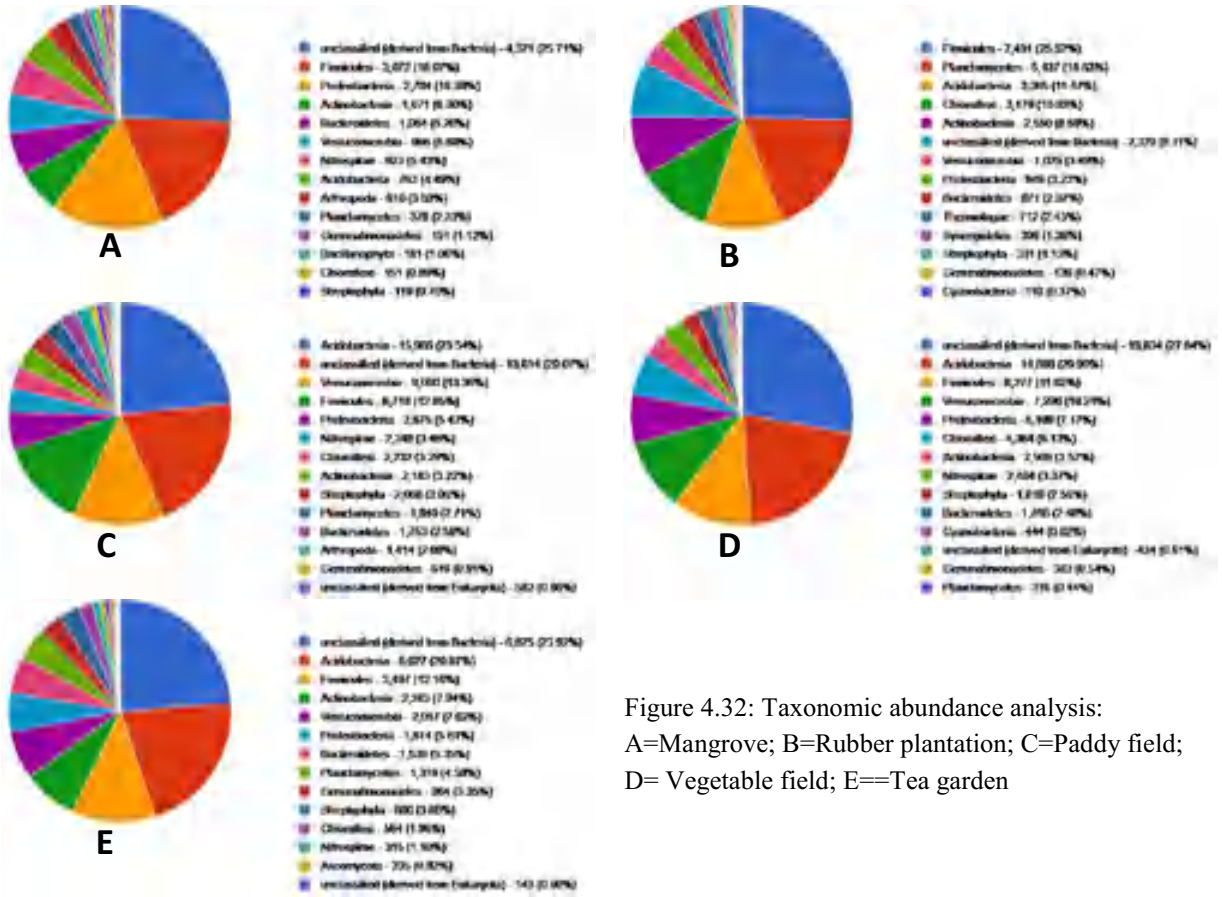


Figure 4.32: Taxonomic abundance analysis: A=Mangrove; B=Rubber plantation; C=Paddy field; D= Vegetable field; E==Tea garden

the samples and gradually becomes a plateau as the rarest species remain to be sampled. In CAS, the curve started to get a plateau state at species count 1400 wherein MAK the stage came at species count 3000. Hence, it is evident from taxonomical abundance profiling, α diversity and rare-fraction curve analysis that, Mak is more ecologically diverse with a higher microbial population rather than Cas.

The health survey conducted among the tea garden workers of various tea gardens of North Eastern India has established the fact that workers are suffering from various ailments such as gastrointestinal disorders, respiratory disorders, skin diseases due to the unhealthy environmental condition (Rajput et al. 2021, Ahmmed and Hossain,2016, http://www. tezu. ernet. in › project reports). Cas microflora shows the predominance of pathogenic flora, *Bacillus*, which includes the food -borne pathogenic species *Bacillus cereus.* This is known to cause mild gastroenteritis to severe and sometimes the source of hepatitis, fatal diarrhoea, typhoid fever and dysentery (Bottone,

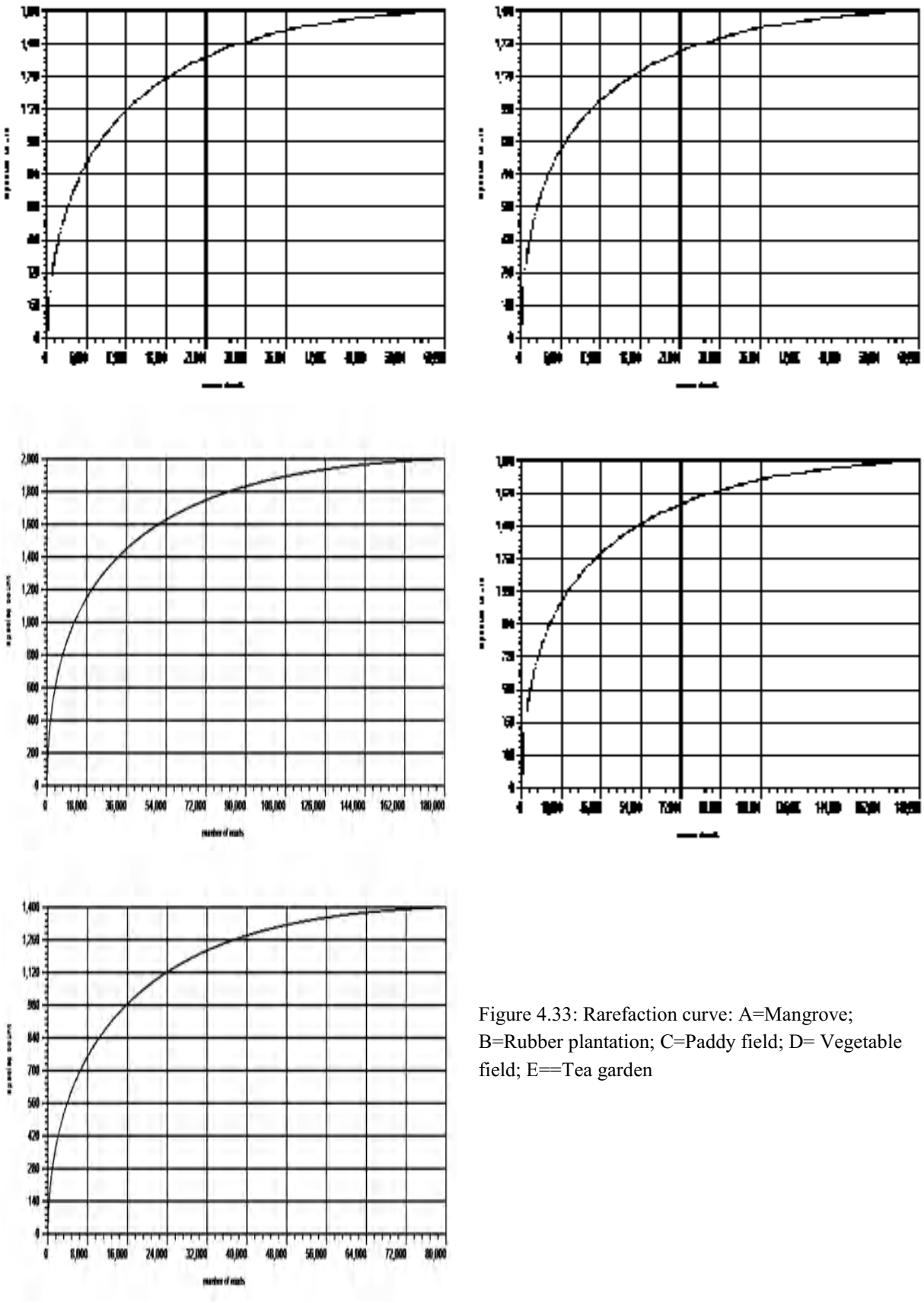


Figure 4.33: Rarefaction curve: A=Mangrove; B=Rubber plantation; C=Paddy field; D= Vegetable field; E==Tea garden

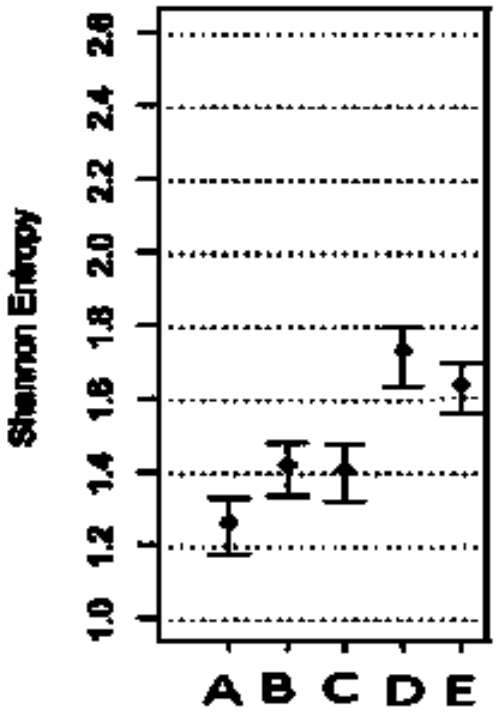


Figure 4.34: Shannon Entropy: A=Mangrove; B=Rubber plantation; C=Paddy field; D= Vegetable field; E==Tea garden

2010). Tea garden workers are also prone to infections of the respiratory tract (e. g. *Legionella*, typical mycobacteria). The other pathogenic microbes include *Naegleria fowleri, Burkholderia pseudomallei* which cause infection in the skin and brain (Inglis & Sagripanti 2006). The microaerophilic and capnophilic *Campylobacter spp*. are some of the most important causes of acute gastroenteritis worldwide (Frost 2009) which shows prevalence in Cas field. On the other hand, the frequency of beneficial soil microflora such as *Bifidobacterium* in Mak indicates the positive impact of organic farming. The probiotic agent *Bifidobacteria* has been commercially exploited due to their associated health benefits and has GRAS (Generally Recognized As Safe) status (Picard et al. 2005). Apart from these, the use of *Bifidobacteria* to treat various gastrointestinal disorders has also been reported. *B. longum* sub-sp. *infantis* CECT 7210 and *B. breve* K -110 were found to inhibit *Rotavirus*, which causes sporadic diarrhea in infants (Bae et al. 2002; Chenoll et al. 2015).

When we considered another tea garden (Salkavita, Darjeeling) that uses chemical fertilizers, the prominent microbial population were Acidobacteria, Firmicutes, Actinobacteria, Verrumicrobia, Proteobacteria, Bacteroidetes, Planctomycetes, Gemmatimonadete etc. Though the phyla present in all the tea gardens were similar, there is variation in their distribution. Acidobacteria was the prominent phylum in this tea garden whereas Proteobacteria was the dominant

phylum in Mak and Cas. The major genera were *Candidatu*s Solibacter, *Rhodanobacter, Clostridium, Acidobacterium, Isosphaera, Candidatus* Koribacter*, Bacillus, Dehalogenimonas, Dyella etc.* (Figure 4.32). The Rare-fraction curve of this tea garden gets stabilized at 1800 species count.indicating alower species richness value. These findings again underline the fact that organic manure is having a positive impact on the species richness of the region Figure 4.33.

Comparative analysis of five different biotopes

The third part of the metabarcoding or 16S amplicon sequencing study involved the comparative analysis of five different biotopes namely, tea garden, vegetable field, paddy field, rubber plantation and mangroves. The taxonomic abundance analysis of the studied biotopes showed a characteristic pattern of distribution. The phylum based study showed that the phylum Firmicutes were abundant in mangroves and rubber plantation, while the phylum Acidobacteria exhibited more or less uniform distribution in paddy field, vegetable field and tea garden. This particular phylum take part in nitrogen fixation, (Eichorst et al. 2018) and their richness is regulated by pH, soil moisture, temperature, ammonia concentration etc. (Fanani et al. 2018). The metagenomic analysis of paddy fields, tea gardens, and other selected biotopes showed the abundance of *Candidatus* Solibacter of Acidobacterium.

The actinobacterial population was high in rubber plantations (8. 69%) followed by tea gardens (7. 94%), mangroves (6. 3%), paddy fields (3. 22%), vegetable fields (3. 52%) respectively. The mean relative abundance for the Actinobacteria class was 23. 81% in all biotopes, except in mangroves (10.71%). When we analyzed the presence of super phylum PVC, we could detect Planctomycetes and Verrumicrobia but not Chlamidae. Earlier reports suggest that 20% of the bacterial community is represented by Verrumicrobia (Bergmann et al. 2011). They are known as methanotrophs (Van Teeseling et al. 2014) and are also involved in nitrogen fixation

(Erikstad & Birkeland 2015). Though the distribution of class Verrumicrobia was found to be high in paddy and vegetable fields whereas its presence was much reduced in mangroves and tea gardens (Figure.4.32) Interestingly, their absence was conspicuous in the rubber plantation. The Planctomycetes population was much high in rubber plantations with *Isosphaera* genera representing the most abundant genera. The Planctomycetes are known ammonia oxidizers and prefer an anoxic environment (Broeckling et al. 2008) Similarly, the Ktedonobacteria a gram-positive bacteria that thrive in the acidic environment were of higher abundance (1054) in rubber plantations compared to other biotopes. These bacteria are capable to hydrolyze starch, casein and produce the enzyme catalase. This corroborates with the earlier reports by Kim et al. (2015), Effendi et al. (2020). The class Nitrospira were of uniform density in paddy and vegetable fields and intriguingly this particular class was absent in rubber plantations and exhibited less density in mangroves and tea gardens. This may be due to the extreme pH and salinity conditions prevailing in these regions (tea garden-acidic and mangroves-alkaline). The presence of phylum Proteobacteria in mangroves was found to be high in relation to other biotopes. Among the Actinobacterial phylum, the genera *Bifidobacterium, Streptomyces, Mycobacterium, Atopobium, Saccharopolyspora* were present in all studied biotopes and the genera *Frankia* was uniquely present in the paddy field.

The diversity of the microbiome is an important parameter to predict the status of an ecosystem. It can be studied by alpha and beta diversity analysis. The alpha diversity measures the number of different species in the environment. The diversity analysis of the various biotopes in the present study disclosed a higher diversity index for rubber plantations (74.91). The rare fraction studies of this biotope get stabilized at 1400 reads indicating the less volatile microbial biome. Contrastingly, the rarefaction curve of paddy biotope was reaching the plateau at 2000 reads displaying high volatile ecosystem. The rare faction curve for rubber plantations and tea garden reached the plateau at 1400 reads and

the vegetable field and the mangrove showed the plateau at 1800 and 1600 respectively. The alpha diversity values were 34.1,30.51,29.11,25.20 for vegetable field, paddy field, mangroves, tea respectively. The rarefaction curve for all biotopes are depicted in the Figure 4.33. The metabarcoding studies suggest that rubber plantation is displaying a unique microbial population, whereas paddy fields and vegetable fields were exhibiting more or less similar microbiome. The tea garden and mangroves were showing analogous behavior in microbial distribution. The reduced diversity index also indicates that mangroves which were considered as the pristine environment is influenced by manmade activities and it is having a negative influence on the microbial population. The microbial population was moreover similar in the paddy and vegetable fields but the individual count of microbes was more in the vegetable field in relation with the paddy field. In this case, also, the studied vegetable field uses only organic manure but the paddy field uses inorganic fertilizers. These results again underline the importance of organic manure in cultivation.■

Conclusion

Live your life as an exclamation rather than explanation.

Sir Issac Newton

The present work was initiated in the year 2018 with a focus to study the diversity of the actinobacterial population from selected biotopes of our country. We envisaged both *in vivo* and *in silico* approaches to tap the unexploited microflora of these biotopes and the identification of bioactive metabolites which can be further explored for novel drugs, bio-fertilizers, etc. From the social perspective, we also documented the health status of tea garden workers of the Darjeeling and Dooars regions. The main aim was to understand the correlation between microbial flora and its impact on the health of the workers. The soil microbiome from biotopes such as tea gardens, paddy fields, vegetable gardens, rubber plantations and mangroves were considered for the diversity studies using metabarcoding studies. The isolation and characterisation of actinobacteria was mainly focused on *Streptomyces.* It was isolated and characterized by morphological and biochemical methods. Selected isolates were characterized up to molecular level by 16S amplification. Out of the total 65 isolates, five of the isolates were sequenced to the genome level.

The biochemical studies of these strains revealed several potential properties such as melanin production, cellulase production, lipase production, etc. Apart from this, the selected isolates were employed for PGPR studies in the mung bean plant. Among these, one of the isolates VRA-16 significantly exhibited PGPR properties. This particular isolate could be a good agent for biofertilizer production.

Furthermore, the culture extracts of two strains were sent for GC-MS analysis which had helped us to detect several bioactive compounds. Some of the selected metabolites were subjected to molecular docking with tuberculosis proteins 1USL,1BES,2VVO,3BSQ and 2NRJ,3PYD.5V8E (enteric toxins of *Bacillus cereus*). The compounds Celidoniol, Pentacosane showed a docking score of more than -7 can be explored further for novel drugs. The study of the bacterial gene clusters by antiSMASH software further elucidated the potential of the newly isolated strains and showed the presence of various antibiotics, pigments etc.

Bioinformatic tools have become indispensable nowadays because of the surge in genomic data. We have done the whole genome sequencing of five of the characterized isolates. Codon usage analysis of the selected genomes along with the sequenced isolates has been done. It has revealed the preference of GC-rich codons which is the characteristic attribute of the actinobacteria. The lower effective number of codons showed higher GC, GC_3 as well as Fop and it is evident that GC richness was influencing codon usage in *Streptomyces*. RSCU analysis showed the optimal use of G/C ending codons and C ending codons were more frequent than G ending codons. Codon usage heat map displayed the use of GC rich codon GCC, GAC, GGC. The amino acid usage analysis showcased the use of Alanine as most-used amino acid followed by Leucine, Glycine, Valine.GC3-NC plot of the protein-coding genes of the strains displayed the deposition of PHX and PLX separately and the PHX genes were more towards the extreme GC-rich region as expected. The correlation between CAI and GC_3 as well as Fop underlines the fact that gene expression

was affecting codon usage variation and its frequencies in the genomes.

Phylogenetic analysis by 16S and MLSA tree exhibited similar grouping of the isolates in both cases. BLAST matrix is used to compare the similarities and differences among the genomes. The Pan Core genome plot studies revealed vast differences between Pan-genome and Core-genome which underlines the diversity among the genomes.

The 16S amplicon sequencing-based on V3-V4 region was carried out for the soil samples from different biotopes. The soil microflora exhibited distinct patterns for all biotopes. The rhizosphere and non-rhizosphere of the actinorhizal plant *Alnus nepalensis* provided an interesting observation which showed the prevalence of diazotrophs in the rhizosphere. This might be due to the phyto signals which attract the selective microbial community.

Another very important and significant findings from one of the metagenomic analyses was that chemical fertilizers are influencing the pathogenic population in the tea garden. Contrastingly, organic manure favors the growth of beneficial microflora in the tea garden. This observation emphasizes the benefit of organic manure thereby influencing microbial communities which may indirectly affect the health of the human population. The amplicon sequencing studies of other biotopes highlight the diverse population of the microbiome in the respective biotopes. These studies indicate that rubber plantation is displaying unique microbial population, whereas paddy field and vegetable field were exhibiting more or less similar micro biome .The tea garden and mangroves were showing analogous behavior in microbial distribution, this may be due to the extremophilic nature of the habitat.

It is apparent from the current work that actinobacteria has the potential to deliver innumerable metabolites which could solve the problem of emerging antibiotic resistance, help in novel drug production. The quality of tea can be improved by the use of organic manure and biofertilizers which will reduce the MRL. This will in turn boost the tea industry which aids in the development of the tea garden community.■

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Appendix-A

List of Publications

Sen G, I Sarkar, S Chetrri, P Kar, A Roy, A Sen, M Bhattacharya (2022). Rhizospheric soil metabarcoding analysis of *Alnus nepalensis* from Darjeeling hills reveals the abundance of nitrogen-fixing symbiotic microbes. *J Forest Res.* DOI: 10.1080/13416979.2022.2037813 **IF: 1.269.**

Sen G, S Sur, D Bose, U Mondal, T Furnholm, A Bothra, L Tisa and A Sen (2007) Analysis of codon usage patterns and predicted highly expressed genes for six phytopathogenic Xanthomonas genomes shows a high degree of conservation. *In silico Biol.* 7:0039 http:// www.bioinfo.de/isb/2007/07/0039/

Sarkar I, **G Sen** and **A Sen** (2020) Methods for whole-genome analysis of Actinobacteria through Bioinformatics approaches. In Methods in Actinobacteriology, Springer Protocols Handbooks (Springer Protocols), Springer Nature, New York.

Chhettri S, **G Sen** and A Sen (2022). Isolation and identification of Streptomyces sp. Producing agroactive enzymes with biocontrol potential. *Ind J Applied and Pure Bio*. 140 -146

Chhettri S, **G Sen** and A Sen (2022). Morphology, diversity and importance of Actinobacteria. *In* 'Biodiversity and Sustainable Resource Management (Basic to Research)' Ed.: D Das. Bharti Publications

Sarkar I, **G Sen**, M Bhattyacharya, S Bhattacharyya and **A Sen** (2021) *In silico* inquest reveals the efficacy of Cannabis in the treatment of Post-Covid-19 related neuro-degeneration. *J. Biomolecul. Struct. Dyna.*. https://doi.org/10.1080/07391102.2021. 1905556. **IF: 3.31**

Sur S, **G Sen**, S Thakur, AK Bothra and **A Sen** (2009) *In silico* analysis of evolution in swine flu viral genomes through re-assortment by promulgation and mutation. *Biotechnol*. 8:434-441.

Sarkar I, P Kar, **G Sen**, S. Chhetri, M Bhattacharya, S Bhattacharyya, **A Sen** (2021). Metagenomic outlooks of microbial dynamics influenced by organic manure in tea garden soils of North Bengal, India. *Archives Microbiolo.* DOI: 10.1007/s00203-021-02635-6. **IF: 2.552**

Ramachandran, P., et al. (2000). Evidence for association of a viroid with tapping panel dryness syndrome of rubber (*Hevea Brasiliense*). *Plant disease*, 84(10), pp.1155-1155.**IF: 4.438**

Appendix-B

Genome Projects

Whole Genomes

Sen,G., Sen,A., Chhettri,S., Sarkar,I. and Bhattacharya,M. Whole genome sequencing of *Streptomyces* sp. Tea02. https://www.ncbi.nlm.nih.gov/nuccore/JAIGNW000000000.1

Sen,G. and Sen,A. Whole genome sequencing of *Streptomyces actuosus* VRA1 https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAFFZS000000000.1

Sen,G., Sen,A., Chhettri,S., Sarkar,I. and Bhattacharya,M. Whole genome sequencing of *Streptomyces* sp. Tea10. https://www.ncbi.nlm.nih.gov/nuccore/jaignv000000000.1

Sen,G., Bhattacharya,M., Sarkar,I., Chettri,S. and Sen,A.Whole genome sequencing of *Streptomyces* species from India. https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAFVLN000000000.1

Partial Genomes

VRA-1	MW332556.1	Streptomyces actuosus
VRA-3	MW585686.1	Streptomyces sp. CT9210B3
VRA-9	MW585687.1	Streptomyces tanashiensis
VRA-10		Streptomyces
VRA-12	MW332566.1	Streptomyces
VRA-14	MW332567.1	Streptomyces
VRA-16	MW332568.1	Streptomyces sp.
VRA-17	MW332569.1	Streptomyces sp.
VRA-19	OL 851820.1	Streptomyces
VR-05		Streptomyces
MTA.1	MW332570.1	Streptomyces
MTA.13	MW332571.1	Streptomyces
Tea02	MK299993.1	Streptomyces
TEA10	MK290326.1	Streptomyces
ARA.1	MW585688.1	Streptomyces fimbriatus
ARA.2		Streptomyces microflavus
ARA.3	MW585689.1	Streptomyces sp. PDP
ARA.4	MW585690.1	Streptomyces viridifaciens
KL02		Streptomyces

Genome Projects
Meta-sequence Submission

Sen,G., Comparative 16s metabarcoding of Alnus rhizosphere and nonrhizosphere. https://www.ncbi.nlm.nih.gov/biosample/SAMN25729411

Sen,G., Comparative 16s metabarcoding of Alnus rhizosphere and nonrhizosphere. https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN25729412

Sen,G., Comparative 16s metabarcoding of Alnus rhizosphere and nonrhizosphere. https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN25729413

Sen,G., Comparative 16s metabarcoding of Alnus rhizosphere and nonrhizosphere. https://www.ncbi.nlm.nih.gov/biosample/SAMN25729414

Sen,G., Comparative 16s metabarcoding of Alnus rhizosphere and nonrhizosphere. https://www.ncbi.nlm.nih.gov/biosample/SAMN25729415

Appendix-C

Buffers & Chemicals

CTAB- buffer

100mM Trizma Base (Sigma, Cat# T1503) (pH-8.0)

20mM EDTA (Merck India, Cat# 60841801001730) (pH-8.0)

1.4 M NaCl (Merck India, Cat#60640405001730)

2% (w/v) CTAB (Hexadecyl cetyl trimethyl ammonium bromide) (Sigma, Cat# H6269)

12.11g of molecular grade Trizma base was dissolved in 400 ml double distilled water, pH was adjusted to 8.0 and was divided into two parts of equal volume. To one part 7.44g EDTA was added and to the other part 81.8g NaCl and 20g CTAB. Both the parts were than mixed and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

Note: Add 1% PVP (Polyvinylpyrrolidone) (Sigma, Cat #P5288) and 0.3% β-mercaptoethanol (Sigma, Cat# M3148) just before use.

5X TBE (Tris-borate-EDTA) buffer

Trizma base (Sigma, Cat# T1503) = 27 gm

Boric acid (Sigma, Cat# 15663)= 13.75 gm

0.5M EDTA (pH 8.0)=1.86 gm

All the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

1X TE:

Tris- Cl (pH 8.0) (i.e. 10Mm) =0.6055gm

EDTA (pH 8.0) (i.e. 1mM) =0.186 gm

Both the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

3M Sodium Acetate (Sigma, Cat# S9513):

The required amount of sodium acetate i.e.12.31 g was dissolved in 50ml double distilled water prior to autoclaving. The solution was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

6X gel loading buffer:

TYPE 3:

0.25% Bromophenol blue (Sigma, Cat# B0126)

0.25% Xylene cyanol FF (Sigma, Cat# X4126)

30% Glycerol (Merck India, Cat#61756005001730) in water

Store at 4ºC.

RNase A:

The RNase A enzyme (Sigma, Cat# R4875) was dissolved at a concentration of 10mg/ml in 0.01M sodium acetate (Sigma, Cat# S9513) (pH 5.2). The solution was heated at 100ºC for 15 minutes in a water bath and allowed to cool slowly to room temperature. The pH was adjusted by adding 1/10 volume of 1M Tris- Cl (pH 7.4) and stored at -20ºC for further use.

Note: Both 0.01M sodium acetate and 1M Tris-Cl were prepared and autoclaved at 121ºC and 15 psi for 20 mins prior to use.

The other chemical used for the molecular work are:

Ready Mix TM Taq PCR Reaction (Sigma, Cat# P4600)

Proteinase K (Sigma, USA. Cat #P2308

Chloroform (Merck India, Cat #822265):

Isoamyl alcohol (Merck India Cat# 8.18969.1000)

Phenol (Sigma, Cat #P4557)

Isopropanol (Merck India, Cat#17813)

Absolute ethyl alcohol (BDH, Cat#10107)

Agarose (Sigma, Cat#A9539)

Ethidium bromide (10mg/ml) (Hi Media, Cat# RM813)

Lambda DNA/ EcoRI/ HindIII double digest (Promega, Cat# PR-G1731)

100 bp ladder (Sigma, Cat#1473)

Culture Media

ISP 4 medium (HI-MEDIA) M359

Ingredients Gms / Litre

Starch, soluble 10.000

Dipotassium hydrogen phosphate 1.000

Magnesium sulphate heptahydrate 1.000

Sodium chloride 1.000

Ammonium sulphate 2.000

Calcium carbonate 2.000

Ferrous sulphate heptahydrate 0.001

Manganous chloride, heptahydrate 0.001

Zinc sulphate heptahydrate 0.001

Agar 20.000

Final pH (at 25°C) 7.2±0.2

Streptomyces Agar (HI-MEDIA) M1352

Ingredients Gms / Litre Malt extract 10.000 Yeast extract 4.000 Dextrose 4.000 Calcium carbonate 2.000 Agar 12.000

Actinomycetes isolation Agar(HI-MEDIA) M490

Sodium caseinate 2.000 L-Asparagine 0.100 Sodium propionate 4.000 Dipotassium phosphate 0.500 Magnesium sulphate 0.100 Ferrous sulphate 0.001 Agar 15.000 Final pH (at 25°C) 8.1±0.2

Nutrient Agar (HI-MEDIA) MM012

Peptone 10.000 Meat extract B # 10.000 Sodium chloride 5.000 Agar 12.000 pH after sterilization 7

Bennet's Agar (HI-MEDIA) M694

Yeast extract 1.000 HM peptone B # 1.000 Tryptone 2.000 Dextrose (GLucose) 10.000 Agar 15.000 Final pH (at 25°C) 7.3±0.2

ISP 7 medium (HI-MEDIA) M362

L-Asparagine 1.000 L-Tyrosine 0.500 Dipotassium hydrogen phosphate 0.500 Magnesium sulphate heptahydrate 0.500 Sodium chloride 0.500 *Trace salt solution (ml) 1.000 Agar 20.000:

*Trace salt solution contains - Ferrous sulphate heptahydrate 1.360mg Copper chloride, 2H2O 0.027mg Cobalt chloride, 6H2O 0.040mg Sodium molybdate, dihydrate 0.025mg Zinc chloride 0.020mg Boric acid 2.850mg Manganese chloride, tetrahydrate 1.800mg Sodium tartarate 1.770mg Final pH (at 25°C) 7.3±0.1

Appendix D
Software & Databases

Name	Executable	Description
CodonW	Windows	Program for codon and amino acid usage
XLSTAT	Windows	Statistical and data analysis software package
CMG-biotools	Linux	Stand alone OS for comparative microbial genomics
MEGA	Windows	Tool for sequence alignment and phylogeny
ClustalW	Windows	Multiple sequence alignment program
Bioedit	Windows	Biological sequence alignment editor
SPSS	Windows	Software package used for statistical analysis
FigTree	Windows	Graphical viewer of phylogenetic trees
BLAST	Windows/Linux	Algorithm for local similarity between sequences
R-Package	Windows/Linux	Functional language for statistical analysis
KyPlot	Windows	Software for plotting graphs

Name	Web Address	Description
JGI-IMG	www.img.jgi.doe.gov	Integrated Microbial Genomes system
NCBI	www.ncbi.nlm.nih.gov/	For molecular biology information
CAI Calculator2	http:// userpages.umbc.edu/ ~wug1/codon/cai/ cais.php	Calculation of codon adaptation index
RAST	rast.nmpdr.org	For the annotation of whole genome sequence
KEGG	www.genome.jp/ kegg	Kyoto Encyclopedia of genes and Genomes
DAMBE (v.5.0)	Windows	Software for sequence analysis
SwissDock	http:// www.swissdock.ch	To predict the molecular interactions that may occur between a target protein and a small molecule. ·
Open Babel	Windows/Linux	
MG RAST	http:// www.mg-rast.org	Phylogenetic and functional analysis of metagenomes

Appendix E

Abbreviations

%	Percent
/	Per
°C	Degree celsius
µmol l^{-1}	Micro mole per liter
3'→5'	3 prime to 5 prime
5'→3'	5 prime to 3 prime
v/v	Volume by volume
w/v	Weight by volume
α	Alpha
µg	Microgram
µl	Microlitre
µmol	Micromole
gm	Gram(s)
gm/l	Gram(s) per litre
hr	Hour(s)
ha	Hectare(s)
mg	Milligram
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar
mm	Millimeter
µM	Micromolar
cm	Centimeter
kb	Kilo base pair
M	Molar

sec	Second(s)
rpm	Revolution per minute
ddH_2O	Double distilled water
O.D.	Optical Density
CLSI	Clinical Laboratory Standards Institute
NCCLS	National Committee for Clinical Laboratory Standards
CTAB	Cetyl Trimethyl Ammonium Bromide
DAS	Days after sowing
IS	Intermediately Susceptible
PGPR	Plant Growth Promoting Rhizobia
IAA	Indole-3-acetic acid
DNA	Deoxyribose Nucleic Acid
RNA	Ribose Nucleic Acid
PCR	Polymerase chain reaction
16SrRNA	16S ribosomal ribose nucleic acid
RAxML	(Randomnized Axelerated Maximum Likelihood, version
EMBL	European Molecular Biology Laboratory
NCBI	National Centre for Biotechnology Information
IMG	Integrated Microbial Genome
KEGG	Kyoto Encyclopedia of Genes and Genomes
CAI	Codon Adaptation Index
RSCU	Relative Synonymous Codon Usage
BLAST	Basic Local Alignment Search Tool
RAST	Rapid Annotations using Subsystems Technology

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20th International Meeting on
Frankia and Actinorhizal Plants
29 – 31 May 2021
Online

Certificate of Participation

This is to certify that Dr. Gargi Sen participated in an online conference of the "20th International Meeting on *Frankia* and Actinorhizal Plants" organized by Ken-ichi Kucho (Kagoshima University), Takashi Yamanaka (Forestry and Forest Products Research Institute), Hiroyuki Tobita (Forestry and Forest Products Research Institute), Shunsuke Utsumi (Hokkaido University) and Toshiki Uchiumi (Kagoshima University) from 29 to 31 May 2021, and presented a paper on "Rhizospheric metagenomic analysis of *Alnus nepalensis* reveals the abundance of nitrogen-fixing symbiotic microbes" (oral presentation).

Ken-ichi Kucho
Graduate School of Science and Engineering,
Kagoshima University

Kenichi Kucho

Assam Botany Congress (ABC-02)
&
International Conference on Plant Science

(on blended mode)

3-5 December, 2021

Venue: Cachar College, Silchar, Assam

Organized by

Botanical Society of Assam, Guwahati

Department of Botany, Cachar College, Silchar, Assam

CERTIFICATE

Certified that **Ms. Gargi Sen** of **University of North Bengal** participated in the "Assam Botany Congress (ABC-02) and International Conference on Plant Science" held on 3-5 December, 2021 at Cachar College and delivered an oral presentation entitled **Microbial dynamics study depicts the importance of organic manure use in the tea garden soil.**

Dr. Tapan Dutta	**Dr. Mukul Kr. Baruah**	**Prof. Manabendra Dutta Choudhury**	**Prof. Partha Pratim Baruah**
Secretary (BSA)	Organising Secretary	Convenor (Technical Program)	Convenor (Technical Program)

JOURNAL OF FOREST RESEARCH
https://doi.org/10.1080/13416979.2022.2037813

ORIGINAL ARTICLE

Rhizospheric soil metabarcoding analysis of *Alnus nepalensis* from Darjeeling hills reveals the abundance of nitrogen-fixing symbiotic microbes

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ABSTRACT

Actinorhizal plants are employed as successional plants for ecological restoration mainly due to their nitrogen-fixing ability. *Alnus nepalensis* or Nepali alder of Darjeeling hills is one of the potential plants used for agroforestry and known for its symbiotic association with an actinobacterium, *Frankia*. In this study, we performed a comparative16S rRNA amplicon analysis among six soil samples of *Alnus* rhizosphere and Non-rhizosphere of different altitudes of Darjeeling hills. Bioinformatics analyses were performed through the MG-RAST web server. Results revealed a set of 32 core bacterial genera among both rhizosphere and non-rhizosphere. Interestingly, *Alnus* rhizospheric soil samples were more populated with nitrogen-fixing taxa than non-rhizospheric or bulk soil. Nitrogen-fixing bacteria like *Frankia* and *Cyanobacteria* may play an important synergistic role in the proper growth and developmental stages of these plants. They are crucial for increasing the nitrogen amount of the soil through nitrogen fixation and gradually help in increasing the soil fertility and thus help in the proper progression of *Alnus* through the seral stages of succession. Nonrhizospheric soil samples were having a distinct population of other soil bacteria like *Streptomyces, Rubrobacter, and Xanthomonas* with higher Alpha diversity (54.14) than *Alnus* rhizospheric soil (42.18). This result was also validated by the rare fraction curve, which indicated more biodiversity in Non-rhizospheric soil rather than *Alnus* rhizosphere.

ARTICLE HISTORY
Received 6 July 2021
Accepted 30 January 2022

KEYWORDS
Metabarcoding; rhizosphere; alpha diversity; nitrogen fixation; *Alnus nepalensis*; *Frankia*

Introduction

Soil is one of the prime requisites for life on earth, which is a natural medium for the growth of plants, microbes, and other organisms. Soil can be considered as an ecosystem itself, as they support a variety of life and all accompanying complex interactions among organisms (Curtis and Sloan, 2005). The density of the microbial population in the soil is highly influenced by the interaction between host plants and microbes in the rhizosphere. The rhizodeposits (e.g. exudates, border cells, mucilage, etc.) are the major factors regulating the diversity and function of microbes on plant roots. The interactions are mainly mediated by Phyto signals released by the host plant (Mendes et al., 2018). The plants may moderate the microbiome of the rhizosphere to their advantage by selectively stimulating microorganisms with qualities that are beneficial to plant growth and health (Cook et al., 1995). A definite understanding of the microbiota of an ecosystem helps in sustainable agriculture, ecological restoration, reclamation of land, pathogen resistance, nutrient acquisition, and stress tolerance of the host plant (Liu et al., 2019). However, the traditional approach of microbiology fails to tap the microbial resources in their entirety and was not competent enough to provide clear estimates of the diversity. Hence, the study of microbial diversity using culture-independent approaches becomes necessary. In this context, one good approach is 16S metabarcoding analysis. It aids in the direct analysis of genomes contained within an environmental sample by providing sequence data of microbial communities as they exist in nature. It is a promising approach in describing the functional potential of the soil microbial community, which might yield greater insight into the health of soil than taxonomy-based metrics (Mardanov et al. 2018).

The present study focuses on 16S metabarcoding of soil samples from the *Alnus* rhizosphere and non-rhizosphere of natural forest areas of different altitudes of Darjeeling hills, India. The selected study regions of Darjeeling hills are part of the Siwalik Range or Outer Himalayas. The nature of the soil varies from loam to clayey loam and sandy loam. The main vegetation comprises tall and large trees like Alder (*Alnus*), Himalayan Birch, Alpine Fir, Oak, Chestnut, Walnut, Maple, etc. *Alnus* is an actinorhizal plant, a pioneer species, and acts as the primary successional plant in degraded habitats. *Alnus* belongs to the family Betulaceae which is locally known as "Utis", provides wood for poles, fuel, etc. Alders are fast-growing, show vigorous growth even in acidic soil and damaged sites such as burned areas and mining sites, further adding to their importance as the species of choice in forest restoration programs (Rana et al., 2018). *Alnus* plantation with cardamom (Sharma et al., 2002; Mukherjee, 2012), tea (Mortimer et al., 2015), and mandarin oranges as shade trees (Duke, 1983) signify its importance in the agroforestry system. The common Alder variety present in Darjeeling hills is *Alnus nepalensis,* which is widely distributed throughout the hills. Another species *Alnus nitida* is highly restricted to moist sandy areas and is rare (Shaw et al., 2014). *Alnus* is mainly associated with *Frankia, an* actinobacterium that is involved in nitrogen

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Supplemental data for this article can be accessed here.

fixation. The symbiont *Frankia* uses carbohydrates from alder trees to convert atmospheric N_2 into reactive nitrogen, a nitrate form directly available to plants (Huss-Danell et al., 1997), thereby enriching the soil.

Our current study is an attempt to assess the distribution and variation of microbes in the *Alnus* rhizosphere to the non-rhizosphere of different altitudinal regions of Darjeeling hills. This will be probably the first report of *Alnus nepalensis* rhizospheric soil 16S amplicon sequencing from India.

Material and methods

Sample collection

The sampling site was Darjeeling hills, West Bengal, India, with an altitudinal range that varies from 130 to 3660 m and covers an area of 1,200 sq. miles surrounded by the majestic peaks of Himalaya, characterized by a subtropical highland climate. The temperature varies from a minimum of −4°C to 25°C and the average annual precipitation of 3620 mm. Composite soil samples containing at least 3 sub-samples from the depth of 5–25 cm (collected in close proximity of around 1–2 meters) were taken from three locations belonging to different altitudes of Darjeeling hills from *Alnus* rhizosphere (R) and bulk soil (minimum distance of 10 meters from *Alnus*) or non-rhizospheric soil (NR) (Figure 1). Detailed sampling data including parameters like altitude, latitude, longitude, etc., and other biochemical factors of the soil are summarized in the Table 1. Sampling was performed in August 2019, a period in which the average temperature was about 18–21°C and the precipitation was 65 mm. The collection of soil samples was on a rainy day and nodules were not found. The samples were collected in sterile polythene bags and stored at 4°C till further processing.

DNA extraction

The soil samples (1 g each) were subjected to DNA extraction by using the MoBioPowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA; cat # 12888–100) as per the manufacturer's protocol. The purified DNA in

Figure 1. Map of the study area.

Table 1. Organic matter and organic carbon were analysed after the procedures of Walkley and Black (1934); the amounts of nitrogen, phosphorus and potassium were determined after the methods of Jackson (2005).

Sample name	Place	Altitude(m)	Latitude	Longitude	Soil Temp.	pH	OC(%)	OM(%)	N(%)	P(ppm)	K(ppm)
R1	Ghoom	2282	27°0′11″ N	88°0′13″ E	18°C	3.62	3.28	5.64	0.28	16.1	214
NR1	Ghoom	2282	27°1′15″ N	88°0′9″ E	18°C	3.68	3.26	5.6	0.26	16	194
R2	Pashupatinagar	1877	26°0′55″ N	88°8′48″ E	20°C	3.71	2.35	4.04	0.21	15.5	156
NR2	Pashupatinagar	1877	26°1′25″ N	88°7′68″ E	20°C	3.73	2.26	3.88	0.18	15.3	145
R3	Mirik	1650	26°52′54″ N	88°11′19″ E	21°C	3.74	2.74	4.71	0.25	15.3	85.4
NR3	Mirik	1650	26°72′34″ N	88°15′29″ E	21°C	3.92	2.52	4.33	0.22	15.3	87.3

R: Rhizosphere, NR: Non-rhizosphere, OC: organic carbon, OM: Organic matter, N: Nitrogen, P: Phosphorous, K: Potassium

triplicate was pooled into a single sample to obtain enough DNA that collectively represented the microbial community composition in the soil samples. The quality and concentration of the pooled DNA from each sample were determined by using a Lambda II spectrophotometer (Perkin Elmer, Norwalk, Conn.) followed by agarose gel electrophoresis (1% w/v agarose in 1XTris-Acetate-EDTA (TAE) buffer, pH 7.8). Then, the purified DNA samples were dried and stored at 4°C until further use. Forty nanograms of extracted DNA was used for amplification along with 10 pM of each primer. The DNA was subjected to 16S amplicon sequencing targeting hypervariable V3-V4 region using 5' AGAGTTTGATGMTGGCTCAG 3' primer for forward sequence and 5′TTACCGCGGCMGCSGGCAC 3' primer for reversed sequence. Initial denaturation was done at 95°C followed by 25 cycles with denaturation at 95°C for 15 seconds, annealing at 60°C for 15 sec, and elongation at 72°C for 2 mins. Final extension at 72°C for 10 mins and hold at 4°C. 16S amplicon sequencing analysis has been performed using the Parallel-META pipeline (version 3.5; Jing et al., 2017).

16S rRNA sequencing and analysis pipeline

The 16S rRNA sequencing was done using the Illumina™ Nextseq platform (paired-end, 2 × 250 mode). The raw reads qualities were checked using FastQC. Sequence reads with >5 low-quality base pairs (<15 Phred score) were removed. Mean quality score for each base, per sequence quality score, per sequence GC contents and per base, N contents are calculated. Post QC, adapters are identified using the BBmerge function from the BBmap tool. Reads are trimmed with BBDuk tool, followed by quality check again. Post trimming, the quality check was done with the Fastqc tool for adapter content and reading the content of fastq files). Filtered reads were arranged into operational taxonomic units (OTUs) using Kraken v1.2.3 via The Galaxy Project (URL: usegalaxy.org) (Wood and Salzberg, 2014; Afgan et al., 2018). The phylogenetic architecture of all reference sequences is built by FastTree (Price et al., 2010). Since the 16S rRNA gene copy number varies greatly among different bacterial species, Parallel-META 3 (version 3.5; Jing et al., 2017) also calculates the precise relative abundance of each organism by 16S rRNA copy number calibration using IMG database (Markowitz et al., 2012). Besides, considering that the uneven sequencing depth (number of sequences) among multiple samples may introduce bias in detecting diversity patterns (Koren et al., 2013), an optional sequence rarefaction for sequencing depth normalization at the OTU level is provided after the taxonomic profiling. MG-RAST server (version 2.0) was used to analyze the high-quality reads from each sample for taxonomic profiling. The sequence data were uploaded to MG-RAST for further processing. For taxonomic annotation of sequence reads, the RefSeq database (Pruitt et al., 2005) was used and for functional gene annotation, and the SEED database (Overbeek et al., 2014) was used. Sequences were annotated using default settings of MG-RAST (maximum e-value cutoff of 10^{-5}, minimum % identity cut-off of 60%, and minimum alignment length cut-off of 15 bp). Subsystem Function level (the finest level) data was sub-sampled to 963693 bp reads per sample for diversity analysis. All the comparison was done following Spearman's Rank statistical analysis with $p < 0.01$.

Results

Sequence processing, quality filtering, and annotation

FastQC report revealed good quality reads indicating successful amplicon sequencing. Read adapters were trimmed and post trimming adapter content fell between 0% and 1% for all samples. Following quality check with FastQC tool, the paired-end reads from soil samples gave 54–56% average GC content and reads were 0.3–0.4 Million sequence reads with duplication values ranging from 47.30% to 82.90% (Table 2).

Taxonomic abundance analysis

Out of the six samples under study, the major phyla present in the rhizosphere (R1, R2, R3) and bulk soil or non-rhizosphere (NR1, NR2, NR3) are Proteobacteria, Bacteroidetes, Acidobacteria, Verrucomicrobia, Planctomycetes, Actinobacteria, Chloroflexi, Firmicutes, OD1, TM7, and others. Proteobacteria was the most abundant phylum (37.25%) with no significant difference in the distribution in the rhizosphere and non-rhizosphere region of different altitudes. This phylum is an important component of the soil microbiome, which is involved in biogeochemical cycles (Hunter et al., 2006). Bacteroidetes was the second abundant phylum with a distribution of 10–12% among all samples, but for the non-rhizosphere of Mirik (NR3), its population was exceptionally high (35%) ($p < 0.001$). It is a known fact that this phylum is adapted to different ecological niches and is a part of the gut microbiome (Moore and Holdeman, 1974; Leng et al., 2011). Interestingly, the increase in the Bacteroidetes population in NR3 area drastically reduced the presence of other phyla (Supplementary figure 1). It is also observed that the population of Acidobacteria, Verrucomicrobia, and Planctomycetes were reducing with a decrease in altitudes in the non-rhizosphere region, but it remained more or less the same in the rhizosphere region of different altitudes (Supplementary figure 1). The Actinobacteria phylum represented a uniform distribution in the rhizospheric region irrespective of the different altitudes (Supplementary figure 1).

At the genus level, we obtained a total of 2728 genera (apart from the unclassified taxa) from the six samples (R1, R2, R3, NR1, NR2, NR3). From Table 3, it is evident that the genera *Nocardia, Isosphaera, Gemmata, Gemmatimonas*, etc., including *Frankia* were the most abundant common genera with uniform distribution in the rhizosphere region of *Alnus* irrespective of their difference in altitudes. This

Table 2. Number of reads after adapter trimming and filtering for high-quality bases.

Sample Name	%age Duplicates	GC %	Mseqs
R1_Rd1	38.50%	55	0.1
R1_Rd2	89.30%	53	0.1
R2_Rd1	40.20%	55	0.1
R2_Rd2	89.50%	54	0.1
R3_Rd1	39.30%	54	0.1
R3_Rd2	89.50%	53	0.1
NR1_Rd1	39.50%	55	0.1
NR1_Rd2	88.80%	54	0.1
NR2_Rd1	39.70%	54	0.1
NR2_Rd2	90.00%	54	0.1
NR3_Rd1	41.10%	54	0.1
NR3_Rd2	90.50%	52	0.1

R: Rhizosphere, NR: Non-rhizosphere, Rd: Read

Table 3. Major microbial communities in the rhizosphere and non-rhizosphere.

R-1	Count	R-2	Count	R-3	Count	NR-1	Count	NR-2	Count	NR-3	Count
Nocardia	3413	***Nocardia***	3420	***Nocardia***	3416	*Xanthobacter*	2879	*Xanthomonas*	2222	*Xanthomonas*	2225
Isosphaera	2223	*Isosphaera*	2230	*Isosphaera*	2226	*Streptomyces*	1849	*Rubrobacter*	2122	*Rubrobacter*	2125
Gemmata	2133	*Gemmata*	2140	*Gemmata*	2136	*Rubrobacter*	1780	*Streptomyces*	1888	*Gemmata*	1848
Cyanobacteria	1780	***Cyanobacteria***	1787	***Cyanobacteria***	1783	*Polaromonas*	1580	*Ureaplasma*	1561	*Streptomyces*	1824
Gemmatimonas	1580	***Gemmatimonas***	1587	***Gemmatimonas***	1583	*Sideroxydans*	1561	*Thermodesulfo bacterium*	1511	*Borrelia*	1564
Clostridium	1561	*Clostridium*	1568	*Clostridium*	1564	*Thiobacillus*	1511	*Bilophila*	1457	***Cyanobacteria***	1514
Corynebacterium	1511	*Corynebacterium*	1518	*Corynebacterium*	1514	*Curvibacter*	1411	*Anaeroplasma*	1205	***Gemmatimonas***	1208
Burkholderia	1050	*Burkholderia*	1057	*Burkholderia*	1053	*Methylobacillus*	1050	*Kosmotoga*	1050	*Anaeroplasma*	1053
Rhodopirellula	798	*Rhodopirellula*	805	*Rhodopirellula*	801	*Candidatus* Accumulibacter	798	*Thermosipho*	879	*Clostridium*	882
Frankia	637	***Frankia***	644	***Frankia***	640	*Desulfatibacillum*	617	*Petrotoga*	798	*Ureaplasma*	801
Acidobacterium	631	*Acidobacterium*	638	*Acidobacterium*	634	*Bilophila*	614	*Rubritalea*	621	*Corynebacterium*	624
Staphylococcus	614	*Staphylococcus*	621	*Staphylococcus*	617	*Desulfotalea*	611	*Aspergillus*	526	*Thermodesulfobact.*	617
Saccharopolyspora	536	*Saccharopolyspora*	543	*Candidatus* Cloacamonas	539	*Desulfuromonas*	516	*Paracoccidioides*	460	*Burkholderia*	529
Pseudomonas	460	*Pseudomonas*	467	*Vaccinium*	463	*Pelobacter*	460	*Nectria*	426	*Kosmotoga*	463
Ktedonobacter	426	*Ktedonobacter*	433	*Bacteroides*	429	*Corallococcus*	426	*Trachelomonas*	282	*Rhodopirellula*	429

R: Rhizosphere, NR: Non-rhizosphere; (Diazotroph taxa are in bold)

indicates that a specific microbial community is being attracted towards the rhizospheric region and also found that the majority of the core microbial community in the soil shows nitrogen-fixing properties. On contrary, the non-rhizosphere region exhibited no specific choice in microbial population and had a high diversity index (Supplementary Figures 2–4). We have also measured the soil temperature at different collection sites. However, taxon distribution was not found to be influenced by the temperature of the soil.

Rhizospheric effects on microbiome diversity

Based on the OTU number, Chao1 bacterial species abundance index, and Shannon microbial diversity index, α-diversity analysis was conducted on various samples. Results indicate that indigenous microbial community diversity was significantly higher in non-rhizospheric soil than *Alnus* rhizosphere, which holds good for all three altitudes. This validates the previous report that shows rhizospheric diversity is lower than bulk soil (Costa et al., 2006; Hein et al., 2008). A significant rhizosphere effect was reflected by reduced microbiome diversity in the *Alnus* rhizosphere compared with that of the non-rhizosphere. The Simpson index of alpha diversity for the rhizosphere ranges was found to be 76.31–125, and in the non-rhizosphere, it varies from 25.38 to 69.45. The microbial diversity of the rhizosphere decreases from high to low altitudes, but intriguingly, diversity in non-rhizosphere regions decreases from low to high altitudes.

The assessment of the Beta diversity of microbial communities across different rhizosphere and non-rhizospheric soil indicates the effect of altitudes among the microbial population of the rhizosphere and non-rhizosphere. On the functional front, both the altitudes and soil conditions such as temperature and pH play an equal role in microbial diversity. PCA analysis was used to study the similarity among various rhizosphere and bulk soil samples in the structures of bacterial communities exploring the main influencing factors driving the differences in micro-community compositions. The axis in the PCA plot indicated the total sequence reads for each soil sample. The subsequent multivariate analysis shows the clustering of non-rhizosphere and rhizosphere on the same axis, indicating similar environmental conditions of the soil. Nonetheless, the formation of separate clusters in the same axis demonstrates the specific choice of the microbial community in these regions (Figure 2). The rarefaction curve for the rhizosphere and non-rhizosphere (Figure 3) shows that the microbial population of rhizosphere soil is getting saturated at a higher level (5000 sequence reads) compared to the non-rhizosphere (3500 sequence reads). This is indicative of the volatile microbiome of the *Alnus* rhizosphere due to the host plant's interaction with the microbial population. Fascinatingly, it is observed that the non-rhizosphere presents a stable scenario where it gets saturated at a lower level (Figures 2 and 3).

The functional profiling analysis showed that KEGG Ontology (KOs) were mainly involved in 23 KEGG level 2 pathways and 25 KEGG level 3 pathways for both rhizosphere and non-rhizosphere. This may be because microbes are evolved in the same physical environment, which has resulted in the lack of variation in metabolic pathways.

The microbial interaction network is constructed to explore co-occurrence and co-exclusion patterns of organisms or functional genes across microbial communities. In the interaction network, each node represents a single organism (or gene), and nodes are connected by links that represent their correlation coefficient of abundance variation among multiple samples (Faust and Raes, 2012). The network analysis shows that the major phyla were represented as 23 nodes belonging to Proteobacteria, Cyanobacteria, Nitrospirae, Firmicutes, Acidobacteria, Planctomycetes, Actinobacteria, Verrucomicrobia, Chloroflexi, Bacteroidetes, etc. Furthermore, when we analyzed the bacterial count of rhizosphere versus non-rhizosphere (bulk soil), we found that approximately 50% of the microbial population was associated with nitrogen metabolism

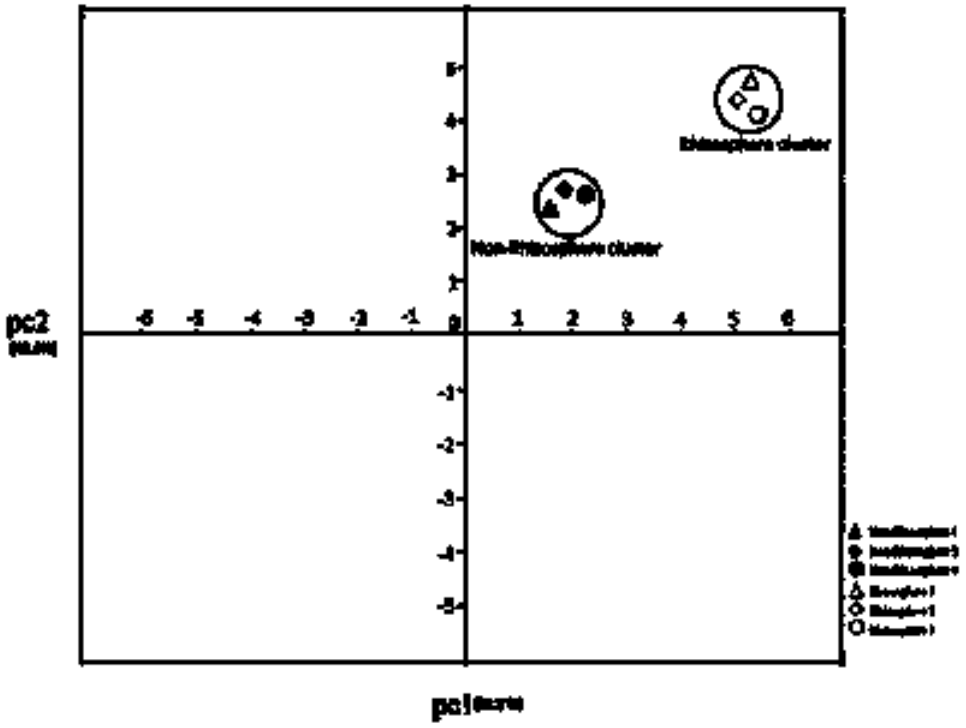

Figure 2. PCA plot for rhizospheric and non rhizospheric sample.

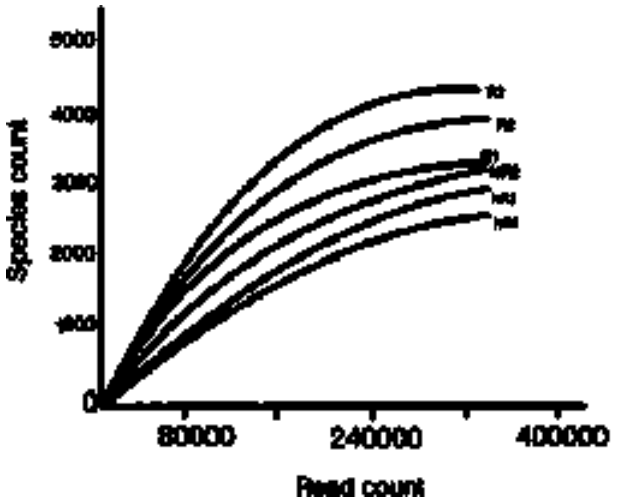

Figure 3. Rarefaction curve for rhizospheric and non rhizospheric sample.

in the rhizosphere (Table 3). This substantiates our findings that the *Alnus* rhizosphere is enriched with a specific microbial community that exhibits nitrogen fixation properties. Contrary to that, the non-rhizospheric soil exhibit rather neutral behavior in the case of microbial populations. This corroborates with the earlier findings where allelochemical inhibition of nitrification in nondiazotrophic plants was reported by Rice and Pancholy (1972) and Gökceoğlu (1988).

Discussion

In this present study, we compared the microbial diversity of *Alnus* rhizospheric and non-rhizospheric soil from different altitudes of Darjeeling hills, India, using amplicon sequencing approaches. Earlier reports show that though there is great diversity in the bacterial communities, very few taxa are predominant in any given soil sample (Xu et al., 2018). In consensus with this, we have also found a few bacterial taxa, such as Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia, etc., that are abundant in both the bulk soil and *Alnus rhizosphere*. This trend of selective abundance was also found by other workers (Mendes et al., 2014; Edwards et al., 2015). Bacteroidetes, Gemma timonadetes, and all proteobacterial classes *(α, β, δ, γ)* were identified as potential copiotrophs, which are involved in carbon metabolism (Elliott et al. 2014). Further analysis showed that the diversity index of the rhizosphere is more in higher altitudes than the lower altitudinal levels. However, the reverse scenario was observed in the non-rhizospheric region. One possible explanation could be that human intervention is reduced at higher altitudes, so the specific microbiome nurtured by the actinorhizal plant, *Alnus* forms a stable community. This is in agreement with the earlier studies that, root exudates from plant species select specific microbial populations in the rhizosphere region (Carvalhais et al., 2011). As the altitude decreases, there is more intrusion from man and animals, which disturbs this equilibrium. The reverse condition in the non-rhizosphere could be because of the lack of host-specific interactions and the change in the available organic matter due to the wide range of involvement of domestic animals, humans, etc. It has already been mentioned that the Bacteroidetes were found to be abundant in the non-rhizosphere soil of Mirik (NR3), which is involved in cellulose degradation and is a common gut microbe. Soil pH increased from high to low altitudes, which also correlates with the reducing population of *Acidobacteria*, an oligotroph in lower altitudes. Different bacterial taxa such as *Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria*, etc., coexist in the soil indicating their minimal competition for resources. The abundance of nitrogen-fixing and nitrogen metabolizing microbes in the *Alnus* rhizosphere underlines the fact that the host plant is instrumental in maintaining distinct rhizobiome. This corroborates with the previous reports that a plant's root exudates can influence the diversity of microorganisms (Igiehon and Babalola, 2017, 2018). Another interesting observation was that *Frankia* was found to be associated with several nitrogen-fixing microbes in the rhizosphere. This may be an indication of their evolutionary association, where the nitrogen-fixing microbes might have existed as symbiotic organisms in the past. As the evolution progressed, they could have established themselves as free-living but tend to remain in the *Alnus* rhizosphere. This association of *Frankia* and other microbes as well as the host-induced signals may act synergistically to create a specific microbiome for the *Alnus* rhizosphere.

Conclusion

In the present study, we intended to assess the microbial populations of the *Alnus* rhizosphere and that of non-rhizosphere (bulk soil) in different altitudes of Darjeeling Hills. We performed meta-barcoding of the soil samples and found that despite altitudinal variations, the *Alnus* rhizosphere was found to be enriched with the diazotrophs, whereas the bulk soil was relatively neutral in this regard. This study is probably the maiden comparative analysis of the *Alnus nepalensis* rhizosphere and non-rhizosphere of varying altitudes, which is purely based on the soil metabarcoding data. However, this is a very interesting topic and demands detailed study on the aspects of nodule formation, diversity of taxa in nodule and rhizosphere, etc., which was beyond the scope of the present study.

Acknowledgments

We acknowledge Mr. Moinak, Soil testing laboratory, Department of Tea Science, NBU and University of North Bengal (NBU) for supporting this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contribution

GS, conceived the idea. GS, SC, PK, AS, MB did the soil collection and preparation. GS, IS & AR performed the Bioinformatic analysis. All authors contributed to the manuscript preparation and approved the final draft.

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In Silico Biology 7 (2007) 547–558
IOS Press

Analysis of Codon Usage Patterns and Predicted Highly Expressed Genes for Six Phytopathogenic *Xanthomonas* Genomes Shows a High Degree of Conservation

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Edited by H. Michael; received 20 April 2007; revised 8 August 2007; accepted 9 August 2007; published 21 August 2007

ABSTRACT: Members of the genus *Xanthomonas* are significant phytopathogens, which cause diseases in several economically important crops including rice, canola, tomato, citrus, etc. We have analyzed the genomes of six recently sequenced *Xanthomonas* strains for their synonymous codon usage patterns for all of protein coding genes and specific genes associated with pathogenesis, and determined the predicted highly expressed (PHX) genes by the use of the codon adaptation index (CAI). Our results show considerable heterogeneity among the genes of these moderately G+C rich genomes. Most of the genes were moderate to highly biased in their codon usage. However, unlike ribosomal protein genes, which were governed by translational selection, those genes associated with pathogenesis (GAP) were affected by mutational pressure and were predicted to have moderate to low expression levels. Only two out of 339 GAP genes were in the PHX category. PHX genes present in clusters of orthologous groups of proteins (COGs) were identified. Genes in the plasmids present in two strains showed moderate to low expression level and only a couple of genes featured in the PHX list. Common genes present in the top-20 PHX gene-list were identified and their possible functions are discussed. Correspondence analysis showed that genes are highly confined to a core in the plot.

KEYWORDS: Codon usage, codon adaptation index, correspondence analysis, pathogenicity, *Xanthomonas*

INTRODUCTION

Members of the γ-proteobacteria genus *Xanthomonas* are plant-associated bacteria and most species are significant plant pathogens that cause disease in various economically important plants [1]. Among them, *X. oryzae* pv. *oryzae* is a pathogen of the staple crop plant rice (*Oryza sativa*), *X. axonopodis* pv. *citri* causes citrus bacterial canker in many citrus producing tropical and subtropical countries around the world [2], *X. campestris* pv. *vesicatoria* is responsible for bacterial spot or black spot of tomato or

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Electronic publication can be found in *In Silico Biol.* **7**, 0039 <http://www.bioinfo.de/isb/2007/07/0039/>, 21 August 2007.

capsicum, while bacterial black rot of canola is caused by *X. campestris* pv. *campestris*. The genomes for all the above plant pathogens have been sequenced [3–6] and shown to contain circular chromosomes that have coding densities very similar to other sequenced bacteria. The availability of these complete genome sequences opens the door to the potential use of bioinformatics approaches that focus on the codon usage profile and to investigate gene expression and regulation in the context of global cellular network.

Codon usage and codon preferences vary significantly within and between organisms [7–9]. Across the genome, the G+C composition resulting from mutational bias and/or translational selection has been hypothesized to determine the major trends in codon usage by high or low G+C organisms [10]. Within a given genome, codon bias is much higher in highly expressed genes than in lowly expressed ones [11–15]. The bias of highly expressed genes is more affected by translational selection than the lowly expressed genes, which are directed by mutational bias [16]. To dissect the patterns and causality of codon usage, many indices have been proposed to measure the degree and direction of codon bias [12]. Among these indices, the codon adaptation index (CAI) was proposed as a measure of codon usage within a gene relative to a reference set of genes (usually ribosomal protein genes) [12]. This index has been shown to correlate best with mRNA expression levels [13]. Besides CAI, the effective number of codons (Nc), which is defined as the number of equal codons that would generate the same codon usage bias as observed, and the frequency of optimal codons (Fop), which is defined as the fraction of synonymous codons that are optimal codons, are also used for the same purpose. The codon bias index (CBI), which measures the extent that a gene uses a subset of optimal codons, is used as an indicator of the cause of codon bias. A low CBI value indicates that the biasness may be to mutational selection whereas elevated CBI may point to the translational efficiency as the cause [17,18].

Xanthomonas species are plant pathogens. Lee *et al*. [3] recognized three major groups of genes related to pathogenesis: (1) effector or avirulence genes (*avr*), (2) hypersensitive response and pathogenicity (*hrp*) genes, (3) the *gum* gene cluster. With phytopathogenic bacteria, a type III protein secretion system (TTSS) encoded by *hrp* genes plays a central role in eliciting defense responses, including rapid cell death response. On non-host or resistant host plants, this response is called the hypersensitive reaction (HR), and leads to pathogenesis on susceptible hosts [19]. Some Hrp proteins form a pilus that has been proposed to function as conduit to directly translocate effector proteins such as avirulance factor into plants [20]. In addition to the TTSS, the type II secretion system may play a role in secretion of other virulence factor with many *Xanthomonas* species, the *gum* gene cluster involved in exopolysaccharide synthesis functions as a virulence determinant [21]. Among the six sequenced *Xanthomonas* genomes, the *hrp* genes occur in frequencies of 2 to 20 while the *gum* genes and *avr* genes occur in frequencies of 13 to 16 and 3 to 18 respectively [3,4,19–21].

The aim of the present study was to perform a comparative analysis of the codon usage patterns and predicted expression levels for the protein coding genes in these phytopathogenic bacteria with special reference to those genes associated with pathogenesis.

METHODS

Genome sequences for six *Xanthomonas* strains (*Xanthomonas axonopodis* pv. *citri* 306, *Xanthomonas campestris* pv. *campestris* 8004, *Xanthomonas campestris* pv. *campestris* ATCC 3391, *Xanthomonas campestris* pv. *vesicatoria* 85-10, *Xanthomonas oryzae* pv. *oryzae* KACC10331and *Xanthomonas oryzae* pv. *oryzae* MAFF 311018 (hence forth, these strains will be referred to as XAC, XCC1, XCC2, XCV, XOO1 and XOO2, respectively) were obtained from the IMG website (http://img.jgi.doe.gov). Table 1

Table 1
Salient features of *Xanthomonas* genomes analyzed in this study

Organism	XAC	XCC1	XCC2	XCV	XOO1	XOO2
DNA, total number of bases	5274174	5148708	5076188	5420152	4941439	4940217
G+C content (%)	64.71	64.96	65.07	64.56	63.69	63.70
Genes total number	4487	4333	4240	4786	4140	4431
Protein coding genes	4427	4273	4181	4726	4080	4372
RNA genes	60	60	59	60	60	59
rRNA genes	6	6	6	4	6	6
tRNA genes	54	54	53	56	54	53
Genes with function prediction	2751	2664	2690	3389	2872	2776
Pseudogenes	0	0	0	0	0	0
Genes assigned to enzymes	593	583	587	905	486	506
Genes in COGs	3210	3133	3133	3286	2921	3059
Number of plasmids	2	0	0	4	0	0
Total number of genes in plasmids	115	0	0	241	0	0

XAC, *Xanthomonas axonopodis* pv. *citri* 306; XCC1, *Xanthomonas campestris* pv. *campestris* 8004; XCC2, *Xanthomonas campestris* pv. *campestris* ATCC 3391; XCV, *Xanthomonas campestris* pv. *vesicatoria* 85-10; X001, *Xanthomonas oryzae* pv. *oryzae* KACC10331; X002, *Xanthomonas oryzae* pv. *oryzae* MAFF 311018.

shows some of the general features of these genomes. Only two of the strains contain plasmids. Strain XAC has two plasmids, pXAC64 (64.9 kb) and pXAC33 (33.7 kb), which have 73 and 42 genes, respectively. Strain XCV maintains four plasmids, pXCV183 (182.6 kb), pXCV38 (38.1 kb), pXCV19 (19.1 kb), and pXCV2 (1.85 kb), which have 173, 43, 23 and 2 genes, respectively.

All of the protein coding genes and those genes associated with the ribosomal proteins, plasmids, virulence/avirulence related traits, hypersensitive response and pathogenesis (*hrp*), and *gum* gene clusters were identified from the available literature [3,4,19–21] and were analyzed by the use of CodonW software (http://sourceforge.net/projects/codonw/) and CAI Calculator 2 (http://www.evolvingcode.net/codon/CalculateCAIs.php).

The software CodonW [18,22] was used to calculate GC3s, Nc [23], RSCU [12], CBI, and Fop values [21,22]. GC3s symbolize the frequency of guanine and cytosine at the synonymous third positions of codons. The effective number of codons (Nc) is a simple measure of overall codon bias [23]. Its value represents the number of equal codons that would generate the same codon usage bias that was observed. Nc values range from 20 (when only one codon is per amino acid) to 61 (when all codons are used in equal probability). The relative synonymous codon usage (RSCU) is defined as the ratio of the observed frequency of a codon to the expected frequency if all the synonymous codons for those amino acids are used equally [12]. The values of RSCU, which are greater than 1, reveal that the corresponding codons are used more often than the expected frequency and *vice versa* [15]. The codon bias index (CBI) [24] is a quantum of directional codon bias and measures the extent to which a gene uses a subset of optimal codons. In a gene with extreme codon bias the CBI value may be equal to 1. Fop is the fraction of synonymous codons that are optimal codons. Its value ranges from 0 (meaning a gene has no optimal codons) to 1.0 (when a gene is entirely composed of optimal codons).

Another very widely used measure of codon bias in prokaryotes and eukaryotes is the codon adaptation index (CAI). It is a measurement of relative adaptiveness of a gene's codon usage towards the codon usage of highly expressed genes. The relative adaptiveness of each codon is the ratio of the usage of each codon, to that of the most abundant codon within the same synonymous family. The CAI value vary from 0 to 1.0 with higher CAI values indicating that the gene of interest has a codon usage pattern more similar to that in the reference genes.

Correspondence analysis (COA) was also performed using Codon W 1.4.2. This method investigates the major trends in codon and amino acid variations among the genes.

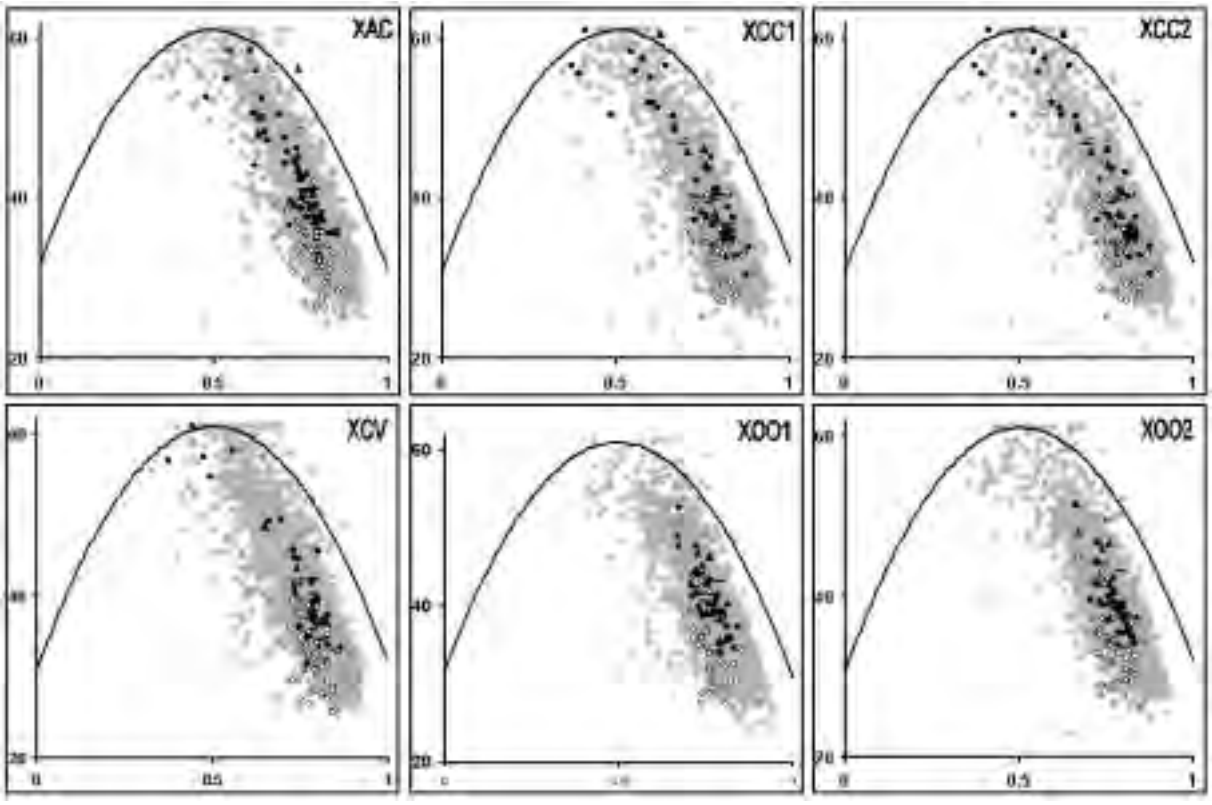

Fig. 1. Effective number of codons used (Nc) in each gene plotted against the G+C content at synonymous third position of codons (GC3) for all six *Xanthomonas* genomes. The continuous curve in each plot represents the null hypothesis that the GC bias at the synonymous site is solely due to mutation but not selection [23]. Gray circle = protein coding genes; hollow circle = ribosomal protein genes; '−' = *gum* genes; '▲ = *hrp* genes and '•' = *vir* genes. In all the plots x and y axis correspond to GC3 and Nc respectively.

RESULTS AND DISCUSSION

Heterogeneity in codon usage in Xanthomonas genomes

Our first aim in this study on the codon usage patterns among various *Xanthomonas* genomes was to determine the degree of heterogeneity in codon use. Most bacteria with a balanced AT/GC genome content have a considerable amount of codon heterogeneity [25]. Codon heterogeneity is usually associated with gene expression level. Thus, highly expressed genes contain a higher frequency of codons that are considered translationally optimal [13,14,25]. Since *Xanthomonas* have a moderately high G+C content, the GC3s and Nc values for all of the genes in these genomes were calculated to determine if codon heterogeneity exists among genes of various *Xanthomonas* species. The results from this analysis were plotted and shown in Fig. 1.

These Nc *vs* GC3s plots have been suggested to be an effective means to investigate the codon usage variations among genes in the same genome [22]. The Nc values of the *Xanthomonas* genes range from 22 ± 2 to 61 ± 0 for all genomes suggesting that these moderately GC-rich genomes exhibited considerable heterogeneity in codon usage. The genes encoding ribosomal proteins, which are expected to be expressed at high levels during rapid cell growth, were identified and are highlighted in the Nc

plots. Most of the ribosomal protein genes of all *Xanthomonas* genomes clustered at the low ends of the plot, which is similar to the results observed with genomes from *Escherichia coli* and two *Streptomyces* species [26], and indicate a significant strong codon bias in the ribosomal protein genes which was the result of selection for translational efficiency [27]. The location of the *gum*, *hrp*, and *vir* genes are also indicated in the Nc plots (Fig. 1). For the XAC, XCC1, XCC2 and XCV genomes, the *gum* and *hrp* genes were relatively clustered together with the ribosomal protein genes, except for one gene each in the XAC, XCCI and XCC2 genomes (in all three cases it was *HrpE* gene), while as expected considerable codon heterogeneity was found among the *vir* genes [26]. However, contrary to other four genomes, *vir* genes in XOO1 and XOO2 were clustered together. If GC composition is not the only factor influencing codon usage bias, analysis of the distribution of the genes in an Nc/GC3s plot would indicate the other factors. If synonymous codon bias was absolutely dictated by GC3s, Nc values should fall on the expected curve the GC3 and Nc plot [22]. However, we found that except for a very few genes, the values obtained for the majority of the genes were well below the expected values (Fig. 1). This result indicates that codon usage bias for the majority of *Xanthomonas* genes is affected independently of overall base composition.

Table 2 shows the mean values of different indices used to study codon usage patterns. Variation in the mean Nc values for the different gene groups was observed within the same species as well as other species. As expected, a correlation between Nc and GC3 was observed: Nc values decreased with a corresponding increase in GC3 values and *vice-versa*. These low Nc values indicated a high degree of codon bias. Ribosomal protein genes had a lower mean Nc value than the mean value obtained for all of the protein encoding genes for all of the genomes, the mean Nc values for the virulence and hrp genes were higher, except for the virulence genes of X001 and X002. Thus, ribosomal protein genes were more highly bias compared to those associated with pathogenesis.

An analysis of the Fop values for the different gene sequences among different species of *Xanthomonas* showed some variation (Table 2) The mean Fop values of the potentially highly expressed ribosomal protein genes were higher than the mean Fop of the total protein coding gene sequences for these genomes, while the mean Fop values of the different gene groups were lower. This result indicates that ribosomal protein genes have higher proportion of optimal codons. If mutational bias only influenced codon bias, these genes would have had a low Fop value. Since that was not the case for these *Xanthomonas* genomes, there may be other factors acting on codon bias. These data were analyzed by the use of the codon bias index (CBI) [24] which is a measure of directional codon bias and measures the extent to which a gene uses a subset of optimal codons. In our study, we found that the CBI values for the gene sequences were low in the range of 0.192 to 0.354 (Table 2). These low CBI values indicate lower level of biasness. Ribosomal protein genes had comparatively higher CBI values compared to other gene groups. CBI values correlated with Nc values similar to their correlation with GC3 values. Genes having lower Nc values had higher CBI values. Those gene sequences showing low CBI values suggest the possibility that they are influenced by mutational pressure [17].

Correspondence analysis

Correspondence analysis of the relative synonymous codon usage (RSCU) of all protein coding genes in *Xanthomonas* strains was performed. Correspondence analysis of genes encoding ribosomal proteins and three categories of pathogenicity-related protein genes are shown in Fig. 2.

The first axis strongly correlated with GC3s, whereas axis 2 correlated with G3s. Scatter plot using the first two axes showed genes were focused within a similar region and had very few outliners. Our results with these *Xanthomonas* genomes had a different shape of distribution compared to previous

Table 2
Mean values of Nc, GC, GC3, CAI, CBI and Fop for the genes in six *Xanthomonas* strains

Organism	Genes	Mean Nc	Mean GC%	Mean GC3%	Mean CAI	Mean CBI	Mean Fop
XAC	PCG	37.95	64.81	78.68	0.583	0.293	0.582
	RPG	33.64	61.94	79.12	0.714	0.348	0.621
	GUM	38.43	62.61	78.09	0.560	0.279	0.566
	HRP	43.24	62.27	74.07	0.518	0.211	0.541
	VIR	44.09	60.36	71.53	0.479	0.184	0.523
XCC1	PCG	36.80	65.31	79.95	0.611	0.309	0.592
	RPG	33.88	61.84	79.17	0.725	0.354	0.625
	GUM	39.61	62.91	77.98	0.558	0.274	0.564
	HRP	41.95	63.07	75.54	0.544	0.255	0.563
	VIR	45.34	59.00	68.88	0.474	0.197	0.532
XCC2	PCG	36.54	65.45	80.31	0.620	0.313	0.594
	RPG	33.88	61.84	79.17	0.725	0.354	0.625
	GUM	39.59	62.92	78.00	0.558	0.274	0.564
	HRP	41.96	63.10	75.63	0.544	0.256	0.563
	VIR	45.23	58.76	68.66	0.476	0.192	0.530
XCV	PCG	38.3	64.61	78.10	0.571	0.287	0.579
	RPG	33.40	61.81	79.16	0.716	0.352	0.624
	GUM	38.36	62.78	78.32	0.554	0.276	0.565
	HRP	39.98	63.24	76.31	0.541	0.249	0.563
	VIR	43.29	59.16	71.95	0.490	0.204	0.536
XOO1	PCG	39.12	64.00	77.07	0.583	0.281	0.575
	RPG	34.79	61.38	77.75	0.709	0.326	0.609
	GUM	40.97	60.88	75.23	0.545	0.242	0.544
	HRP	43.84	62.26	73.85	0.520	0.216	0.537
	VIR	38.82	65.30	78.01	0.570	0.264	0.561
XOO2	PCG	39.03	63.92	77.09	0.579	0.281	0.575
	RPG	34.44	61.27	77.61	0.717	0.343	0.618
	GUM	40.67	60.93	75.44	0.542	0.248	0.547
	HRP	43.58	62.93	73.58	0.525	0.224	0.546
	VIR	37.30	65.81	79.59	0.575	0.262	0.559

PCG = protein coding genes; RPG = ribosomal protein genes; GUM = *gum* gene cluster; HRP = *hrp* gene cluster; VIR = avirulence/virulence genes.

studies on genes from *Streptococcus pneumoniae*, *Escherichia coli* or *Nocardia farcinica*, which showed a core region with two ascending horns [28–30]. With all the *Xanthomonas* genomes, the genes were clustered tightly in a core and confined mostly in a narrow range of -0.5 to $+0.5$ of both the axes. No visible ascending masses or horns of genes were observed, but a few genes were found located away from the core. We calculated that on an average 99.43% of the protein coding genes were located in the core block. Genes located away from this core block included a number of hypothetical protein genes, ribosomal protein genes, YapH protein, histone H1 genes, and translation initiation factors (IF1, IF3, etc.). Only one gene associated with pathogenesis (*HrpE*) was found in this region for the XAC genome. These results imply that these six *Xanthomonas* genomes are relatively conserved with most genes lying within the core region. However, some recent reports based on G+C content and CAI values [31] and analysis of nucleotide templates of individual genes with clustering of horizontally transferred genes [32] predicted that a number of pathogenicity related genes acquired through lateral gene transfer. Further study is required to confirm this conclusion.

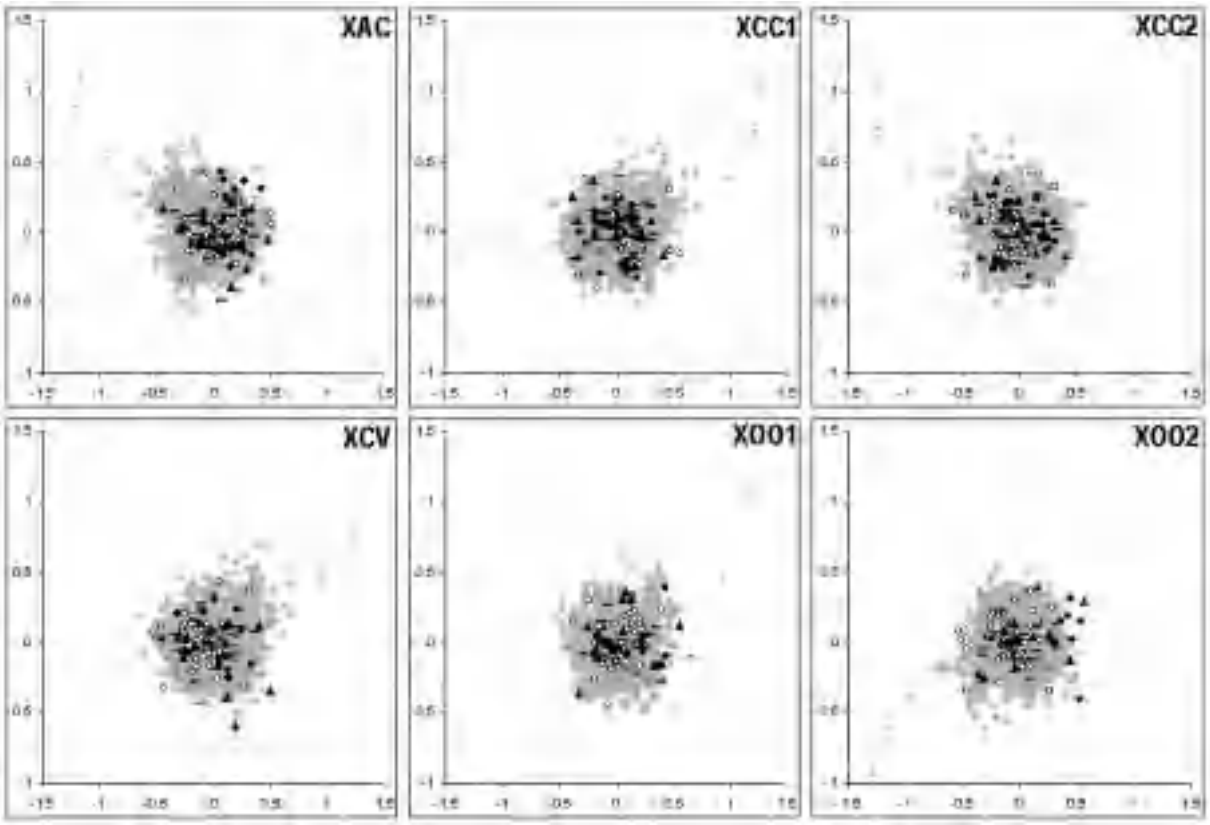

Fig. 2. Correspondence analysis of codon usage patterns of various *Xanthomonas* genomes. In all the plots x and y axis correspond to axis 1 and 2 of the analysis. Buttons are as per Fig. 1.

Identification of potentially highly expressed (*PHX*) *genes in Xanthomonas genomes*

Codon adaptation index (CAI) is a measure of directional synonymous codon usage bias. The index uses a reference set of highly expressed genes from a species to assess the relative merits of each codon, and a score for a gene is calculated from the frequency of use of all codons in that gene. The index assesses the extent to which selection has been effective in molding the pattern of codon usage. In that respect, this index is useful for predicting the level of expression of a gene [12]. Wu *et al.* [26] analyzed proteome results and validated the correlation between CAI values and expression levels showing experimentally that CAI predicted highly expressed genes highly expressed. Thus, we examined CAI values for these *Xanthomonas* genomes to identify the potentially highly expressed genes (PHX genes).

The CAI values for all genes in different *Xanthomonas* strains were calculated and their distributions are shown in Fig. 3.

The CAI values ranged from 0.103 to 0.870, 0.104 to 0.911, 0.104 to 0.911, 0.102 to 0.882, 0.252 to 0.851, and 0.190 to 0.861 in XAC, XCC1, XCC2, XCV, XOO1 and XOO2, respectively. The majority of the genes have CAI values between 0.4 and 0.8. The median CAI values for genes located in the chromosomes were 0.60, 0.63, 0.63 0.59, 0.59 and 0.58 for the aforesaid order of genomes. Plasmid-borne genes of strain XAC showed a CAI value ranging from 0.233 to 0.729 and 0.218 to 0.729 for plasmids pXAC64 and pXAC33 respectively, while strain XCV showed CAI ranges of 0.174 to 0.745,

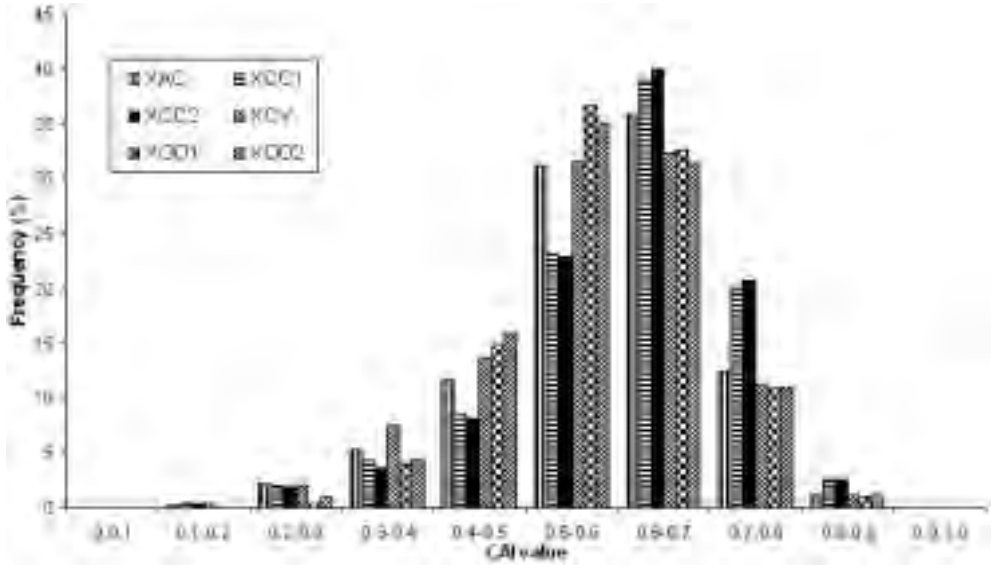

Fig. 3. Frequency distribution of CAI values for all coding genes in the *Xanthomonas* genomes.

0.288 to 0.707, 0.177 to 0.542 and 0.177 to 0.313 for plasmids pXCV183, pXCV38, pXCV19, and pXCV2 respectively. The median CAI values of these plasmid-borne genes were lower than the median value of the chromosomal genes. The median CAI of pXAC64 and pXAC33 were 0.46 and 0.45, respectively, while strain XAC chromosomal genes median CAI was 0.60. Strain XCV chromosomal genes had median CAI value of 0.59 and its plasmids pXCV183, pXCV38, pXCV19 and pXCV2 were 0.43, 0.51, 0.44 and 0.24, respectively.

The average CAI values for different gene groups associated with diverse functions varied (Table 2). Ribosomal protein genes showed high CAI values indicating high levels of gene expression. The predicted expression levels of the virulence genes for strains XOO1 and XOO2 were comparatively higher than those found for the other four species.

As defined by Wu *et al*. [26], the top 10% of the genes, in terms of CAI values, were defined as the predicted highly expressed (PHX) genes. This definition corresponded to CAI cutoffs of 0.716, 0.746, 0.748, 0.713, 0.708 and 0.711 in strains XAC, XCC1, XCC2, XCV, XOO1 and XOO2, respectively. Strain XAC had 445 PHX genes including 30 ribosomal protein genes (RPG) and each plasmid contained one PHX gene. Both plasmid-borne genes were partition protein genes. Strains XCC1, XCC2 and XCV had 25, 22 and 32 RPG in their pool of 433, 466 and 483 PHX genes, respectively. Strain XCV had three PHX genes, which were associated with pXCV183, including one single-stranded DNA-binding protein and two hypothetical protein genes. Strains XOO1 and XOO2 had 22 and 31 RPG among the identified 415 and 441 PHX genes, respectively. Genes associated with pathogenesis genes with two exceptions were found to be expressed at a moderate level and all the other genes were not in the category of PHX. The two exceptions were found in strains XOO1 and XOO2 and both the genes encode a virulence regulator protein. Frequency distribution of CAI values for all coding genes in the genomes of *Xanthomonas* (Fig. 3) showed that maximum numbers of genes were present in the CAI range of 0.6–0.7, but strains XCC1 and XCC2 had statistically significant numbers of genes present in 0.7–0.8 range. These results indicate that strains XCC1 and XCC2 have more highly expressed genes in their genomes than others four strains.

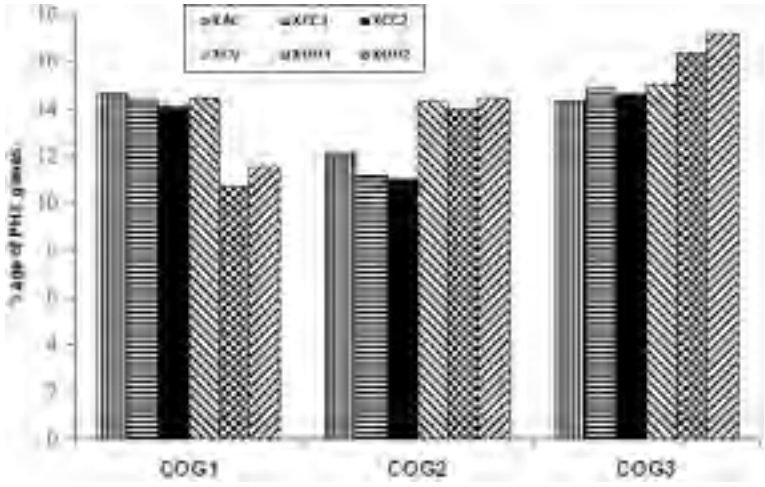

Fig. 4. Percentage of PHX genes in various COG functional groups of *Xanthomonas* genomes.

Clusters of Orthologous Groups of proteins (COGs) were delineated by comparing protein sequences encoded in complete genomes, representing major phylogenetic lineages. Each COG type consists of individual proteins or groups of paralogs from at least 3 lineages and thus, corresponds to an ancient conserved domain. For these *Xanthomonas* genomes, 22 to 23 COG categories were identified and the number of genes found in COG categories are shown in Table 1. To help the analysis, each of the COG categories were clustered in the following 4 COG functional groups: Information storage and processing consisting of COGs related to transcriptions, translation, RNA processing DNA replication and chromatin structure (group 1); cellular processes including, cell division, nuclear structure, defense mechanisms, signal transduction, cell envelop biogenesis, cell motility, cytoskeleton, extra cellular structures, intercellular trafficking and posttranslational modification (group 2); metabolism containing energy production and conversion, carbohydrate transport, amino acid transport, nucleotide transport, coenzyme metabolism, inorganic ion transport and secondary metabolites biosynthesis (group 3); genes with general function predictions and unknown functions (group 4). For this study, we analyzed genes from the first three groups to identify potentially highly expressed (PHX) genes. CAI values of all the genes present in different COG groups were calculated and the PHX genes were identified as per the cut off values of various *Xanthomonas* genomes mentioned above. Figure 4 shows the percentage of PHX genes in various COG functional groups.

It has been found that the percentage of PHX genes in all categories and genomes were above the expected values of 10%. This result implies that the genes in these COG categories are relatively better expressed than the rest of the genes in the genomes. Functional analysis showed that COG functional group 3 (metabolism) contained largest number of PHX genes in all the genomes except in strain XAC where group 1 (information storage and processing) was the largest group. Top 20 PHX genes of all the *Xanthomonas* genomes contain a large number of genes from COG functional categories. Among them, COG group 1 had a major cold shock protein gene and elongation factor P, which is involved in peptide bond synthesis and stimulates efficient translation on native or reconstituted 70S ribosomes *in vitro* [4]; COG group 2 had peptidyl-prolyl *cis-trans* isomerase, an enzyme that accelerates protein folding by catalyzing the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides [33]; GTP-binding elongation factor protein, promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome [34]; co-chaperonin GroES, binds to Cpn60 in the

presence of Mg-ATP and suppresses the ATPase activity of the latter; molecular chaperone DnaK takes part in bacterial nascent polypeptide chain-folded protein anabolism [35] and chaperonin GroEL, this double ring chaperonin binds unfolded protein, ATP and GroES to the same ring, generating the *cis* ternary complex in which folding occurs within the cavity capped by GroES (*cis* folding) [36] and COG group 3 contained ATP synthase subunit A and B, having hydrolase activity, act on acid anhydrides, catalyzing transmembrane movement of substances [4]; L-glutamine synthetase; spermidine synthase takes part in spermidine biosynthesis and dihydrolipoamide dehydrogenase takes part in alanine and aspartate metabolism, glycine, serine and threonine metabolism, pyruvate metabolism [3].

For the plasmid-borne genes of strain XAC, both pXAC64 and pXAC33 had the same single PHX gene, the partition protein gene, which produces proteins of part A protein family involved in chromosome partitioning [6]. With the plasmids of XCV, three genes on plasmid pXCV183 were PHX genes including two hypothetical protein genes and a short gene that codes for a single-stranded DNA binding protein. Genes from other plasmids were not in the PHX category.

CONCLUSION

The codon usage-based strategy has been successfully applied to the identification of highly expressed genes in various bacteria including G+C rich *Streptomyces* and pathogenic *Nocardia* [26,30,37,38]. In this study, the approach was used to estimate gene expressivity in six strains of phytopathogenic bacteria *Xanthomonas*. The results from this study indicate considerable heterogeneity exists among the genes of these moderately G+C rich genomes. Mostly genes predicted to be expressed highly were house keeping genes and only two out of 339 genes associated with pathogenesis were in the PHX category. Genes present in the plasmids of two strains did not contain many PHX genes. Another important finding was high degree of conservation for all of the genes. Correspondence analysis of these genomes showed that their genes clustered in a very narrow range in both the axes with a few genes outside the core block suggesting that lateral gene transfer was limited in these genomes. These results provided an estimation of the global gene expression patterns in *Xanthomonas* that will be useful in guiding experimental design for further investigation and will enhance our knowledge of the metabolism and pathogenicity of the *Xanthomonas* species.

ACKNOWLEDGEMENT

The work was partially carried out by the grant provided by DBT-India to establish Bioinformatics Facility at NBU. A. Sen acknowledges the receipt of DBT Overseas associateship.

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7 Morphology, Diversity and Importance of Actinobacteria

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Abstract

Actinobacteria is a gram-positive bacteria with high content of guanine and cytosine in their DNA. They share the characteristics of both fungi and bacteria. They are prokaryotic like bacteria and are filamentous like fungi. However, they produce more slender nonseptate mycelium. Classic actinobacteria are morphologically characterized by the formation of aerial and substrate mycelium. They are distributed widely in terrestrial and aquatic environments, even exist in some extreme conditions such as thermophilic, psychrophilic, acidophilic and alkaliphilic. Some are found associated with plants, animals and humans. They play important role in biogeochemical cycles and decompose organic matter from dead animals, plants and fungi. Actinobacteria is the source of diverse bioactive secondary metabolites that are important medically, agriculturally and industrially. Actinobacteria especially Streptomyces are exploited for potential applications. This chapter summarizes the morphological characteristics, diversity of actinobacteria in various habitats and their importance.

Keywords: *Actinobacteria, Morphology, Habitats, Importance*

INTRODUCTION

Actinobacteria are gram +ve group of bacteria with high GC content, found in various ecosystems on earth. They share characteristics of both fungi and bacteria. The type of habitat can greatly affect actinobacterial diversity. They are found in many diverse habitat including terrestrial, aquatic, aerial, and even in extreme environments such as *volcanic caves, marine sediments, desserts, hot springs, sediments of cold springs, lakes, etc.*[15] Some are also found in association with higher organisms *such as the genera Frankia present as the symbionts within the root nodules of many woody plants which are dicotyledonous.* [10] The actinobacterial diversity in various habitats can be studied by culture-dependent cultivation and high throughput sequencing techniques. Combining both the techniques is very helpful for the recovery of both the abundant as well as rare members of the microbial community even in extreme environments which were difficult to access [15].

They decompose organic materials such as dead animals and plants debris playing role in humus formation. They play a very important role in carbon cycling as they are known to decompose cellulose, the most abundant biopolymer in nature, followed by chitin.[1] Apart from carbon cycling they are also known for cycling phosphorous, nitrogen, potassium, and many other elements in soil and replenish the nutrient supply in the soil.[25] Geosmin and 2-Methylisoborneal are organic volatile compounds produced by actinobacteria that cause musty and earthy odours in water bodies. Geosmin is responsible for the slightly metallic scent of freshly turned soil. Actinobacteria such as *Streptomyces albidflavus, S. luridiscabei, Nocardia cummidelens* are gross producers of geosmin.[5]

They are known for producing various secondary metabolites such as antibiotics which find its great importance in medicine which includes antifungal, antibacterial and some antitumor drugs. [25] Actinobacteria mainly found in soil especially in the plant rhizosphere have very good plant growth-promoting activity thereby enhancing the growth of plants. They are also known to have antagonistic activity against various plant pathogens causing disease of agriculturally important crops. [13]

This chapter gives a summary of the morphological appearance of actinobacteria, their diversity in various habitats, and their importance.

MORPHOLOGICAL CHARACTERISTICS

Actinobacteria exhibit prokaryotic cell structure with the morphological differentiation highest among gram-positive bacteria. They are branching unicellular microorganisms with most of them being aerobic and characterized by the formation of the aerial and substrate mycelium. [11] Morphologically they appear compact, chalky white, often leathery and covered commonly with aerial mycelium. The substrate mycelium grows either on the culture medium surface or into the medium and is known as the primary mycelium or the vegetative mycelium. Nutrient absorption is the main function of substrate mycelium for actinobacterial growth. They show variation concerning shape, size, thickness and varied colorations which range from white to red, pink, brown, black green, orange or yellow. It is due to adequate differentiation, the thickness of aerial mycelium is frequently greater differentiation compared with substrate mycelium. The structure of aerial mycelium appears as powdery, velvety or cottony, forming concentric zones or rings may have varied colorations due to pigmentation. [1] Some form diffusible pigments. Some of the actinobacteria form spores, sporangia and sporangiophore. The position and the number of spore, ornamentation of spore surface, sporangia shape, sporangiophore with the presence or absence of flagella are some morphological characteristics important in the classification of actinobacteria. [11]

DIVERSITY AND IMPORTANCE OF ACTINOBACTERIA

Terrestrial Habitat

Actinobacteria can be found in both terrestrial and aquatic habitats. They are economically important to humans since the actinobacteria such as *Streptomyces* are the contributors of forest and agricultural soil systems as they decompose dead organic matter. They are also known for the biological buffering of soil. The growth of several pathogens that cause disease to plants is inhibited by actinobacteria in the rhizosphere thereby promoting the production of crops. [3] About 80% of the antibiotics in the world are from actinobacteria especially from the genera *Micromonospora* and *Streptomyces.* Antibiotics such as erythromycin, tetracycline and vancomycin are produced by actinobacteria. These soil dwellers are also the source of many anticancer drugs, insecticides and herbicides. Soil isolates such as *Nocardia bangladeshensis, Streptomyces rimosus, S. iakyrus*, *S. bingchengensis, S thermocarboxydovorans, S. luteospreus, S. alboniger* and *S. gilvosporeus* have antibacterial and antifungal properties. [4]

Aquatic Habitat

The biodiversity present on earth is contributed largely by the aquatic ecosystem. The basic freshwater ecosystem consists of lotic (rivers and streams), lentic (ponds, lakes and pools) and wetlands. Actinobacteria is among the abundant group of microbes found in freshwater habitats. However, maximum actinobacterial strains were found from the lake sediments compared to that from the river sediments. This may be because of the continuous running water in rivers whereas sediments in the lake are not much affected by the running water. Huge actinobacterial diversity was found in freshwater isolates from sediments of lakes and rivers which included common genera *Streptomyces* and eight rare genera included *Rhodococcus Amycolatopsis, Saccharopolyspora, Nocardiopsis, Micrococcus, Kocuria Prauserella, Promicromonospora*. [17] Acquatic actinobacteria have some ecological roles as they degrade some recalcitrant organic matter in the water bodies. They like soil microbes are capable of degrading organic matter from plant biomass. The ability of aquatic actinobacteria to uptake nutrients like phosphorous, nitrogen and carbon enhances their performance under nutrient-depleted or oligotrophic conditions. They also help in preventing eutrophication, as they can sequester inorganic phosphorous in water bodies. [8]

Symbiotic actinobacteria

Actinobacteria interact with both micro and macroorganisms. There is a symbiotic relation between actinobacteria and the hosts such as animals, plants, humans and insects where the actinobacteria protect the pathogens against the host organisms. [2] Actinobacteria such as *Frankia* form a symbiotic association with woody plants mostly from eight families and three orders namely Rosales, Fagales and Cucurbitales. The actinorhizal nodules comprise modified lateral roots with the cells which are infected in the expanded cortex. Nitrogen is present in the atmosphere as the most abundant element, however, most of the plants are not able to utilize the atmospheric nitrogen directly and are dependent on soil for nitrogen sources. The root nodules that are present on the plant root system contain symbiotic nitrogen fixing actinobacteria such as *Frankia* strains which promote the fixation of nitrogen. The oxygen sensitive enzyme nitrogenase is protected by *Frankia* and thus facilitating nitrogen nutrition in plants. [23]The symbiotic association between Frankia and actinorhizal plant belonging to *Casuarinaceae* family such as *C.equisetifolia* and *C. glauca*. Agroforestry widely uses

Casuarina trees for the reclamation of land and is also important for crop protection, windbreaks firewood, source of poles and charcoal. Actinorhizal plants are with ecological and economic benefits including stabilization of soil, reforestation, and reclamation of land. [14]

Endophytic Actinobacteria

Endophytic actinobacteria reside symbiotically within the plant tissues as endophytes. They play a role of importance in the development and growth of the host plant by producing large amount of bioactive natural products. Lately, they are of great interest because them being a good source of some novel compounds which may have applications in agriculture, medicine, and the environment. There is great diversity among the endophytic actinobacteria including those isolated from plants in specific ecological niches and extreme habitats. [22] In recent studies 169 endophytic actinobacteria were isolated from *Rhynchotoechum ellipticum* with 81 strains with antimicrobial potential. Some *Streptomyces* sp. among them including *Streptomyces olivaceus* and *Streptomyces thermocarboxydus* produced antibiotics such as miconazole, rifampicin, fluconazole, ketokonazole and erythromycin. Some of them also produced anticancer compounds such as paclitaxel. The antagonistic actinobacteria *Streptomyces olivaceus* and *Streptomyces thermocarboxydus* showed plant growth promoting traits such as phosphate solubilization. Endophytic actinobacteria belonging to genera such as *Streptomyces, Actinomyces, Micromonospora, Microbacterium, Leifsonia, Pseudonocardia, Kocuria, Amycolatopsis* and *Brevibacterium* are producers of plant growth hormone indole acetic acid. [16]

Actinobacteria Residing in the human body

Actinobacteria is found in diverse habitats including the human body. *Propionibacterium, Actinomyces, Rothia, Bifidobacteria* and *Corynebacterium* are the five important genera of actinobacteria residing in healthy individuals. Some commensals and opportunistic pathogens also belong to this phylum. [24] Actinobacteria is also one of the major phyla among the other phyla that constitute the gut microbes. Actinobacteria such as *Bifidobacteria* sp. are known for health-promoting effects and affect human nutrition and metabolism positively. They constitute the intestinal flora of mammals including animals and can be used as probiotics for the treatment and prevention of diseases.[9]

Extremophilic Actinobacteria

Apart from actinobacteria in temperate habitats, they are also distributed in some extreme habitats of the terrestrial and aquatic ecosystem. Extremophilic actinobacteria such as thermophiles can grow at a temperature range of 40ºC to 80ºC. *Streptomyces thermoautotrophicus*, *Acidithiomicrobium* sp., *Amycolatopsis methanolica* are some of the thermophilic actinobacteria. [21] Psychrophilic actinobacteria such as *Streptomyces, Lentzea, Amycolatopsis* did grow in the temperature range of 10 ºC to 25 ºC with the capability to produce enzymes such as cellulase, amylase, protease and antagonistic activities. Psychrotolerant Actinobacteria belonging to genera *Tsukamurella, Streptacidiphilus, Rhodococcus, Streptomyces, Arthrobacter, Actinoplanes, Nocardia, Kribbella, Pseudarthrobacter* and *Pilimelia* were identified from Antarctica. Some of them were producers of potential antitumor compounds.[20]

Actinobacteria are found to occur in a diverse range of pH with some acidophiles found to occur at lower pH. In the recent studies, it was found a large proportion of acidophilic actinobacteria compared to neutrophiles, exhibited good plant growth-promoting activities and played important role in phosphate solubilization, siderophore production and showed antifungal activities. Those acidophilic actinomycetes beneficial to plants were isolated from rhizospheric soil using the media pH of 5.5 and were identified as *Streptomyces misionensis* and non *Streptomyces* isolates such as *Verrucosispora, Sacchaopolyspora, Nocardia, Mycobacterium, Amycolatopsis, Allokutzneria and Nonomuraea*. [18]

Some Alkaliphilic actinobacteria are found in alkaline soil, alkaline lake, soda lake. Alkalitolerant actinobacteria grow in both alkaline and neutral environments. They grow in the pH range of 7 to 11. *Streptomyces caeruleus*, *S. canescens , S. cavourensi, S. hydrogenans and Nocardiopsis alborubida are some of the alkalophilic actinobacteria.* [21]

Actinobacteria are also adapted to grow in conditions of high salt concentrations. Their ability to adapt themselves to high osmotic pressures existing in the environment makes them salt lovers. They live in different halophilic environments such as Deep sea brines, soda lakes, marine sediments marsh soil with 10% NaCl and many other hypersaline regions *Nocardiopsis halotolerans, Saccharomonospora halophila* and *Streptomonospora alba, Nocardiopsis salina* are some halotolerant and halophilic actinobacteria growing at high salt concentrations. [12] Haloalkane and hypersaline conditions prevalent in the mangroves and lakes limit the ability of the microbes

to hydrolyze biomolecules like lignin, chitin and cellulose. Only haloalkalitolerant and haloalkaliphilic actinobacteria and bacteria can proliferate and decompose the recalcitrant biopolymers. [21]

The presence of genes for stress response, osmoregulation, heavy metal resistance and some genes for antibiotic production and antibiotics in the genome sequence of some *Streptomyces* isolates would perhaps make the survival of actinobacteria possible in extreme environments like halophilic. [6]However their mechanism of adaptation, biological activities and diversity is less studied. The extreme and special environments are the source of some novel species and rare actinobacteria. They are new sources for the exploitation in the field of agriculture, industry and medicine as they may be sources of some novel natural product. [19] *The major genera comprised of Streptomyces and Nocardiopsis among 200 actinobacterial strains isolated from an extreme environment such as Great Salt Plain with high salinity, high temperatures, and exposed to UV radiation.* [7]

CONCLUSION

Actinobacteria is the dominant group of microbes that are distributed in various habitats such as aquatic, terrestrial, and in extreme conditions such as thermophilic, psychrophilic, acidophilic and alkalophilic. Their survival in extreme and special habitats proves their adaptability. It can be inferred from the review that they have many practical applications as they are a source of many secondary metabolites which are medically, agriculturally and industrially important and is of commercial value for the welfare of humanity. However many rare genera of actinobacteria are yet to be explored from many diverse unexplored locations for their industrial and biotechnological potential and they can be of value in the discovery of new drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Indian J. Applied & Pure Bio. Special Volume 140-146 (2022).
Environment & Sustainable Development : Problems, Prospects & Mitigation Date : 25th-27th November, 2021
ISSN: 0970-2091
A web of Science Journal

Isolation and identification of *Streptomyces* sp. producing agroactive enzymes with biocontrol potential

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Abstract

The enzyme chitinase has potential application in the field of agriculture. The aim of the present study is the isolation, characterization and identification of the potential chitinase-producing bacteria from the Nagari farm tea garden of Darjeeling Hills. Around twelve actinobacterial strains were isolated and screened for their chitinase and cellulase activities followed by the antagonistic activity against fungal pathogen *Fusarium* sp. The potential isolate with antagonistic activity was then characterized and identified as *Streptomyces* sp. SDr 22. The isolate tolerated the NaCl concentration of 1% to 4% and could grow in the pH range of 5 to10 at an optimum temperature of 30ºC. The presence of chitinase-producing *Streptomyces* isolate in the tea garden of Darjeeling Hills with biocontrol activity has been explored in this study.

There is a great demand for agricultural land for the cultivation of crops to supply food to the ever-increasing world population. However, there are limitations of the land that can be cultivated which increases the immediate need to control crop diseases and increase the yield with the limited land resource. Both the abiotic and biotic factors are responsible for the productivity of crop plants. One of the major factors causing a decrease in the yield of crops is fungal plant pathogens.[12] The pathogens causing various diseases to plants are being controlled by various chemical insecticides and fungicides. However, these chemicals are also causing harm to the environment thereby affecting both animal and human health.[33] Global warming accompanied by climatic change has increased the incidence of various fungal diseases and causing damage to crop plants. This has posed a great loss in the agricultural sector and production of food.[2]

Chitin is present in the exoskeleton of arthropods such as crustaceans and insects and is the most abundant biopolymer in nature

after cellulose.[33] It is also one of the components of the cell wall of fungal pathogens. Chitinases play a role of importance in the degradation of chitinous waste.[35] Chitinases are glycosyl hydrolases that hydrolyze the chitin in the insoluble form to pharmaceutically valuable products such as chitooligosaccharides and glucosamines which are soluble forms. These chitooligosaccharides are known to have antifungal, antibacterial, antitumor and immune-enhancing effects. The bacteria producing chitinases with biocontrol potential against pathogenic fungi have been exploited.[17] Thus the role of chitinase producing microbes as biocontrol agents cannot be ignored in such a scenario.

Actinobacteria is one of the main sources of bioactive secondary metabolites and commercially important enzymes in both the medical and agricultural fields.[16] They play important role in the cycling of carbon sources such as chitin and cellulose.[14] Actinobacteria especially from the genus *Streptomyces* which are are filamentous, gram-positive, long rods have chitinolytic activity. Several *Streptomyces* sp such as *S. griseus, S. lividans, S. plicatus, S.aureofaciens, S. halstedii, S.glauciniger* are known for producing chitinolytic enzymes.[4,16,30] They have antagonistic activity towards many pathogenic fungal species. *Streptomyces* is known to control Fusarium wilt.[37] The considerable affinity of chitinase to chitin has lead several biotech companies to explore the potential for the development of disease-resistant seeds and transgenic plants.[12]

In the present study, we isolated the actinobacterial strains from Nagari farm tea garden soil. The strains recovered were screened for the production of the enzymes cellulase and chitinase. The potential enzyme producer selected and studied further for their antifungal activities against the phytopathogen *Fusarium* sp. This isolate with the good antagonistic activity was characterized morphologically, biochemically and physiologically and identified by 16s rDNA sequencing.

Isolation :

Tea rhizospheric soil samples were collected from Nagari farm tea garden located in Darjeeling hills, West Bengal, India. The soil samples collected were air-dried for five days at 30ºC. The soil sample dried and was serially diluted in saline water containing 0.85% NaCl.[31] The inoculation was done from each dilution by the method of spread plate. For isolation, the media used for growth were Starch nitrate agar[11] and Inorganic Salts, Starch Agar (ISP4).[31] The inoculated plates were incubated at 30°C for seven days. After purification, the isolates were sub-cultured using ISP4 slants and stored at 4°C. [24].

Screening for the production of chitinase and cellulase :
Chitinase assay :

The isolated strains inoculated into chitin agar media (Colloidal chitin 5g; KH_2PO_4 0.03g; K_2HPO_4 0.07g; $FeSO_4.7H_2O$ 0.001g; $ZnSO_4.4H_2O$ 0.0001g; $MgSO_4.7H2O$ 0.05g; $MnCl_2.4H2O$ 0.0001g; agar 2g; ddH_2O 100 ml, pH adjusted to 7.0). Colloidal chitin was prepared[7] and the plates after inoculation were incubated for 5 days and the plate flooded with gram's iodine solution and the halo around the colonies were recorded.[22]

Cellulase production :

The isolated strains were inoculated into CMC Agar (Carboxy-methylcellulose 0.5g, K_2HPO_4 0.1 g, $NaNO_3$ 0.1 g, yeast extract 0.05 g, $MgSO_4$ 0.05 g, Agar15 g, distilled water 1000 ml) plates and incubated at 30°C for 5 days. After completion of incubation, plates were flooded with Gram's iodine solution which resulted in the formation of a bluish-black complex with unhydrolyzed cellulose. The clearance zone around the colonies is the indicator of a positive result.[10]

Antagonistic activity against fungal pathogens:

The strains under study were tested in vitro for their antagonistic activity against fungal root pathogen, *Fusarium solani* (RHS/P388) with accession number NAIMCCF-02901 and other *Fusarium* species obtained from immunopathology Laboratory, Department of Botany, North Bengal University. The antagonism test was carried out in Potato Dextrose Agar (PDA) by the method of dual culture[36] with slight modification. Freshly grown *Fusarium* sp. inoculated on one edge of the plate and the isolated strain SDr 22 inoculated in the same plate at the extreme opposite edge of the plates by streaking, sealed tightly with parafilm. The control plate was only inoculated with *Fusarium* sp. After inoculation plates were incubated for 10 days at 30°C.

Morphological studies :

Morphological studies were done by growing the isolates at 30°C for 7 days in various International Streptomyces Project (ISP) Medium such as Tryptone-yeast extract broth[19], Yeast extract-malt extract agar,[20] Oatmeal agar,[13] Glycerol-asparagine agar,[21] Inorganic salts-starch agar[13] Peptone-yeast extract iron agar,[32] Tyrosine agar.[25] The formation of the substrate and aerial mycelium was studied.[26]

Biochemical and Physiological Studies :

The biochemical tests that were carried out were the formation of Melanin,[26] degradation of Casein Tyrosine, Xanthine,[5] hydrolysis of Esculin[9], production of Amylase,[8] Gelatinase,[27] Lipase,[3] reduction of Nitrate,[15] and Carbohydrate utilization test.[26] Growth of the isolate in various pH(5-10),NaCl concentration (1% to 7%) and various temperature (25°C,30°C,40°C) evaluated using Bennet Agar media.[28]

DNA isolation and 16srDNA sequencing :

The isolation and purification of DNA of the potential isolate were carried out following CTAB method.[34] The amplification of 16S rDNA sequence carried out by Polymerase Chain Reaction (PCR) using primers ACT235F (5'CGC GGC CTA TCA GCT TGT TG3') and ACT 878R (3'CCG TAC TCC CCA GGC GGG G5'.[29]

Isolation and Screening for the production of chitinase and cellulase :

Colonies of actinobacteria were found to be white and chalky in the isolation media used namely Inorganic Salts Agar medium and Starch Nitrate agar medium. Total twelve

colonies of actinobacteria were recovered and they were found to be a producer of chitinase and cellulase. The isolate SDr 22 with the most potential ability to produce cellulase (Fig 1a) and chitinase (Fig 1b) were also found to exhibit good antifungal activity against plant pathogenic fungus *Fusarium* sp (Fig 1c). The control plate was fully grown by the *Fusarium* sp. Whereas in the plate with both *Fusarium* sp and SDr 22 inoculated, the growth and spread of *Fusarium* sp. in the entire plate was suppressed.

Morphological, biochemical, physiological characterization and 16s rDNA sequencimg:

Morphological studies of the isolate showed the strain showed variation in the coloration of Aerial and Substrate mycelium in all the tested media with variation of spore mass coloration (Table-1).

In biochemical studies it was found that the isolate produced amylase, cellulase and lipase and utilized tyrosine and esculin however failed to produce gelatinase, reduce nitrogen and hydrolyze casein. In carbohydrate utilization studies it was found the isolate SDr 22 utilized all the tested sugars namely glucose, arabinose, fructose, sucrose, xylose, inositol, rhamnose and raffinose. The isolate tolerated NaCl concentration of 1% to 4% and showed growth in the pH range of 5 to 8.

The Molecular identification of the strain was done by Sanger sequencing of 16S rDNA gene fragment after amplification by PCR and the phylogenetic tree constructed by the neighbor-joining method with the sequence obtained from *Streptomyces* sp SDr 22 and other *Streptomyces* sp.(Fig 2).

The similarity of the sequence studied through the online NCBI BLAST program, where it was found the isolate SDr 22 showed 99.50 % similarity to *Streptomyces* sp. By the similarity-based searches in the ezBioCloud server the isolate showed a similarity of 97.25% to *Streptomyces flavovirens*. The 16S rDNA gene sequences were submitted to NCBI and the accession number provided was MK300088.1.

A good percentage of the soil microbial community comprises actinobacteria with *Streptomyces* being the major genus known for producing antibiotics, bioactive compounds and various extracellular enzymes of importance. This genus has huge potential to improve agricultural productivity in the future.[18] Our study supports the findings where the *Streptomyces and* non-*Streptomyces* species producer of hydrolytic enzymes such as cellulase, chitinase, protease, and pectinase could degrade fungal and bacterial cell walls thus causing antagonism against fungal pathogens.[6] The *Streptomyces* sp. with chitinolytic activity plays the role of importance in the biocontrol of plant diseases caused by *Fusarium* sp. and attention is also being given to identifying the bioactive metabolites that are accountable for biocontrol. *Streptomyces* is the major microbe responsible for the suppression of diseases in plants.[23] Plant growth-promoting rhizobacteria including *Streptomyces*, with antagonistic activity against *Fusarium* sp, induces the immune system of the plant against various biotic stresses and thus are the better alternative to chemical pesticides and fungicides in the agricultural system.[1]

Table-1. Morphological characterization of SDr 22 in various ISP media

Medias	Aerial	Substrate mycelium mycelium	Spore Mass	growth
Tryptone Yeast Extract Agar	Off White	Off White	No Spore Mass	++
Yeast Malt Agar	Off White	Off white to Brown	Whitish	+++
Oatmeal Agar	Off White	Brownish Grayish	Brownish	+++
Inorganic Salt Starch Agar	White	Greenish	Olive Green	+++
Glycerol Asparagine Agar	Off White	Off White	Off White	++
Peptone Yeast Extract Iron Agar	Off White	Off white to Light Brown	Off White	+++
Tyrosine Agar	Off White	Grayish Brownish	Grayish	+++

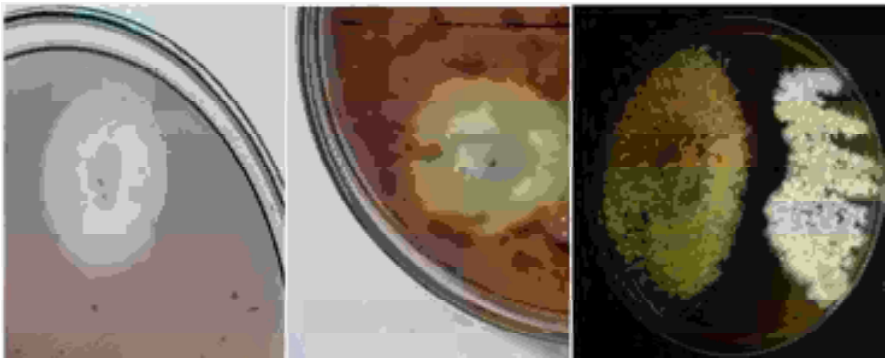

Fig1 (a). Cellulase production by strain SDr 22 in CMC agar after flooding with iodine solution (b). Chitinase production in Colloidal chitin agar by strain SDr 22, with a zone of clearance around colony after flooding with iodine solution. (c). Antagonistic activity of SDr 22 against *Fusarium* sp. after 10 days of incubation.

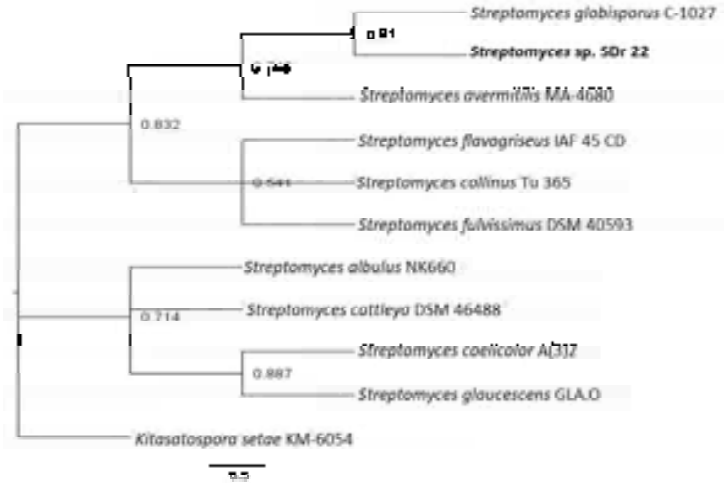

Fig 2: Phylogenetic tree based on the partial 16s rRNA sequences

In this study, the actinobacterial strains from tea garden soil were assessed for the production of cellulase and chitinase. The potential enzyme-producing isolate SDr 22, with good antifungal activity, was found to belong to genus *Streptomyces* based on morphological, biochemical and 16srDNA sequencing studies. Thus *Streptomyces* sp. SDr 22 represents the indigenous tea rhizosphere population of tea rhizospheric soil and can be explored for the preparation of biofertilizer in future. Chemical pesticides and fungicides are being used for many years however the use of eco-friendly alternatives will be a boon to the ecosystem thereby preventing the damage further.

Conflict of Interest :

The authors declare no conflict of interest.

Authors would like to acknowledge Bioinformatics Facility, the Department of Botany, University of North Bengal and Biswa Bangla Genome Centre, University of North Bengal for providing the facilities to carry out the research work.

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ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Bioteehnology 8 (4): 434-441, 2009
ISSN 1682-296X

In Silicn Analysis nf Evniutinn in Swine Fln Virai Genomes Through Re-assortment hy Promnlgation and Mntation

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Abstract: Availability of the sequences of latest strains of H1N1 virus and their comparison with other viral strains may provide signifieant clnes to the natnre of HIN1. The objective of the study was to look into the characteristics of genes and proteins of the swine fiu and related vimses to understand their lifestyle and evolntionary relationship. Seqnences of genome segments were analysed nsing ACUA, Codon W and DAMBE. Evolutionary relationships were determined via condensed matrix method. CAI values were qnite high in the studied vimses and pI values of proteins showed a bi-modal distribution. All HIN1 strains as well as Infiuenza C, H3N2 and H2N2 had pI in the range greater than 8.I with HINI CAL07/2009 having pI value of 8.87. Positive correlations of GC3 and GC content witb CAI values were noticed. Hydropatby and aromaticity levels increased with the decrease of GC3. Phylogram revealed a rooted tree, which shows two major clades, Clade A and Clade B with subclades. Majority of H1N1 lie together in the same clade with the exception of HINI CAL04/2009 that lies in a different elade altogether along with H1N1 Puerto-Rieo. Mutationai bias is the main factor driving codon nsage variation. High expression of pathogenicity related genes confirm its role as pathogen. Most of the HINI basic proteomes are infiueneed by mutational pressure. Genes associated with the hydropbilic proteins are favoured by transiationally optimal codons. Phylogenetic analysis portrays the role played by reassortment in controlling tbe evolution of the smdied strains.

Key words: H1N1, pathogenicity, isoelectric point, condensed matrix, phylogeny

INTRODUCTION

Infinenza or fiu is caused by a group of viruses called iufluenza viruses. These categories of A, B and C viruses are the common pathological agents (Snznki and Nei, 2002). Genomes of these viruses are segmented, single stranded and have (-) RNA. They are associated with epidemies and pandemies in mammals and birds. Wild waterfowI and other aquatic birds are tbe natural reservoirs of infinenza virnses (Holmes *et al*., 2005). Infiuenza epidemics are aeeonntable for causing I0,000-15,000 deaths in humans every year (Holmes *et al*., 2005).

Swine inflnenza also called swine fln is cansed by a strain of influeuza virus named HIN1 tbat usually infect pigs. Pandemic influenza has created havoe in 1918 (HINI), I957 (H2N2) and 1962 (H3N2) resulting in numerous deatbs worldwide (Cox and Subbarao, 2000; Webby and Webster, 2003), while H5N1 assumed epidemic proportions in Asia in the years 2003-2005 (Hoimes *et al*., 2005). Very recently, outbreaks of swine flu have sent shock waves in Mexico and United States, with the World Health Organization (WHO) issuing warning for possibility of worldwide pandemic. Altbough, the origin of this strain is stili unknown, some reports point ont that it has not been found in pigs. WHO reported (www.who.iut/mediaceutre/news/statemeuts/2009/H1NI-20090427) that the mntated form of the virus might have been transmitted between humans and causes symptoms of infiuenza, such as ruuny uose, fever, cougbing and headache etc. The HINi form of swine flu is reported to be a form of tbe causative agent that caused the pandemic in hnmans in 19I8-I9I9 (Taubenberger and Morens, 2006). The deseendants of the I918 HIN1 virus have persisted among humans as well as pigs throughout the 20th century, with some seasonal bonts of inflnenza (Tachenberger and Morens, 2006). New variants of influenza viruses arising out of reassortment of the segmented RNA genome pose severe threat to public health (Gog *et al*., 2007). The 2009 swine flu straiu of infinenza is reported (www.inspeetion.gc.ca/englisb/corpaffr/newcom/2009) to be a reassortment of four strains that ineludes influenza A virus subtype H1N1, one endemic in humans, one in birds and two in swine.

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The availability of the sequences of the segments of some of the latest strains of H1N1 virus has given an opportunity to look into the pattern of codon usage, gene expressiou levels, determine protein isoelectric poiuts, aromaticity and hydropathicity indicates the soluhility of the proteins: positive GRAVY (hydrophobic), negative GRAVY (hydrophllic) of the amino-acids In addition to studying their molecular phylogeny.

Syuouymons codons are unequally used between genomes. Compositional hlas, translational seleetlon and mutational pressure account for eodon usage variation amongst orgauisms (Sur *et al*., 2008). Highly expressed genes have tendency for eodons with high concentration of related tRNA molecules, whereas low expressed ones have consistent codon usage (Zhon *et al*., 2005). It has been reported that mutational pressure plays a significant role in inflnencing codon patterus in human viruses (Zhou *et al*., 2005). Estimation of the CAI (codon adaptation index) values (Sen *et al*., 2008) indicates the nature of gene expresslon levels of the respective genes. The physical properties of the proteins are fundamental in the normal functioning of an organism (Knight *et al*., 2004). Properties such as isoelectric point, hydropathicity and aromaticity play a role in protein functioning. Environment and GC content is known to play a crnclal part in influencing amino-acid usage in organisms (Tekaia and Yeramian, 2006). The correlation of GC3 and GC content of the organisms with isoelectric point and amino acid frequeneies of the proteins is expected to throw llght into the molecnlar natnre of swlne flu vlruses. The results obtained for the latest stralns will be compared with that of the older straius of H1N1 aud other flu vimses like H5N1, H2N2, H9N2, H3N2, influenza B and influenza C virus.

On the other hand phylogeny stndy of swine flu and other related viruses will shed some light on their evolntionary relatiouship. An importaut method of phylogeny developed by a group of laboratories including ours use nueleotide triplet based eondensed matrix method (Moudol *et al*., 2008). Phylogenetic studies using sequence alignment and structures are insufficient in pottraying the evolution of genes given that sequence comparison becomes unreliable at identity levels lower than 25% (Mondol *et al*., 2008). It also turns out to be tongh to distlnguish among properly aligned homologs and discrete seqnences. Structure based methodologies are also insufficient given that number of structures are scaree to represent any slgnlflcant conclusion. The condensed matrix method that relies on nucleotide triplet based phylogeny, is free from the aforesaid limitations as it takes into account, full length of the genes for creating phylograms (Mondol *et al*., 2008).

In a nutshell the aim of the present study is to look into the important charaeteristics of the genes and proteins of the swine flu and related vimses to infer upon their lifestyle and evolutiouary relatiouships.

MATERIALS AND METHODS

The research work was started in the spring of 2009. It was virtually doue iu two laboratories. The software was developed in Department of Chemlstry, Raiganj College. All bioinformatics analysis were performed at NBU Bioiuformatics Facility, NBU while iuterpretation of results and paper writing were done in both the laboratories.

Sequences of the genome segments of Influenza A vimses [A/Califorula/04/2009(H1N1); A/Califorula/05/2009 (H1N1); A/California/07/2009(H1N1); A/Texas/04/2009 (H1N1) and A/Texas/05/2009(H1N1)] were obtalned from the NCBI website (http://www.ncbi,nlm.nih,gov) and that of other Influenza A vlruses like [A/Goose/Gnangdong/1/96(H5N1); A/Korea/426/68 (H2N2); A/Hong Kong/1073/99(H9N2); A/New York/392/ 2004(H3N2); A/Puerto Rico/8/34(H1N1)] and Influenza B virus and Influenza C virus were obtained from the IMG database (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) (Markowitz *et al*., 2006).

The ACUA (Umashankar *et al*., 2007) was utilized to compnte GC content, GC3 composition (amount of G or C codons in the third position), Ne (Effective number of codons) and CAI (codon adaptatlon index) valnes. The Nc measures codon bias whose value ranges from 20 to 61 (Sur *et al*., 2009). The CAI computes the relative adaptation of codon usage of genes towards codon nsage of highly expressed genes (Wu *et al*., 2005). The CAI valnes vary from 0 to 1 with higher values signifying that, gene of concern has a codon usage pattern analogous to highly expressed genes. Certaiu viruses like hacteriophages have their own tRNA and it is inferred that though phages use most of the cells translational machinery and complement it with their own geuetic information to attain higher fitness (Bailly-Beehet *et al*., 2007). However, in swine flu viral genomes there is no gene coding for any tRNA assuming that the swine flu viruses entirely depend upon host cells translational maehinery. Therefore, we have nsed codon usage table of *Homo sapiens* as a reference for determining CAI valnes. Hydropathicity (GRAVY score) and aromaticity of the genes were determlned using Codon W (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/ portal.py?form = codouw) (Peden, 1999). Hydropathicity or GRAVY score is calculated as the arithmetic mean of the sum of hydropathie indices of each amino acid, whereas

aromaticity determines amino acid usage, provided ineqnality in amino acid composition lneIndes application for evaluating codon usage (Lobry and Gautier, 1994). Distribution of isoelectric points (pI) iu a proteome is one of the most important aspects of proteins (Kiraga *et al*., 2007). Protein isoeleetric points were ealculated using DAMBE (http://dambe.bio.nottawa.ea). Correspondence analysis was computed for codon usage on codon count usiug Codon W (http://mobyle.pasteur.fr/cgi-bln/MobylePortal /portal.py?form=codonw) (Peden, 1999). Major trends in codon usage variation among the genes within the genomes are estimated with this aualysis.

Determination of freqnency of triplets of nucleic acid bases: Our own program written in Turbo C++ was nsed to eount all the possible triplets of the nucleotide sequenees of the whoie genomes from the studied viruses. The matrices were formed using all the triplets. Introduction of a 4×4×4 cubic matrix having 64 possible entrles resolves the frequeney of lneidence of all the possible 64 triplets in a DNA sequence. Here, it is possible to obtain three gronps of 4×4 matrices {M_1, M_2, M_3, M_4], {M_5, M_6, M_7, M_8}, {M_9, M_{10}, M_{11}, M_{12}} each one having every entry of eubic matrix (Randic *et al*., 2001). Usnally, the gronp of 4×4 matrices {M_1, M_2, M_3, M_4] are taken as representative of the cubic matrix. Our teehnique gives equal weight to all positious siuoe it considers nucieotide tripiets, not only starting from ist codon position but all the three positions. Thus, addition or deletion of bases taking place during the course of evolution is taken eare of. The methodology depicts DNA by condeused matrix countiug the rate of preseuoe of adjoining base pairs (Randic, 2000).

Calculation of eigen value and eonstruction of phylogram: Leading eigenvalues were calculated using MATLAB (versiou 5.0.0.4069) software. Eigeuvalues are a special set of scalars associated with a linear system of equations, usually matrix equations that are often regarded as characteristic roots, characteristic values and proper values or latent roots (Mondol *et al*., 2008). Evaluation of DNA seqnences for similarity or dissimilarity is normally aided by the eonvenienee of leading eigeuvectors calculated by this method. Diversity betwecn eigenvalnes was nsed to study sequence similarity/dissimilarity keeping in mind the characterization of a seqnence by leading eigenvalue (Nandy *et al*., 2006). Matrices linked to each sequence are estimated and the leadiug eigeu values computed. Variatious in leading eigen values concnrrent to the string are estimated and the relationships between genes investigated, Distance matrixes of the studied sequeuoes were coustrncted hy summing up the square of the difference of eigen values. Phylograms were built by cluster analysis of the similarity matrix using PHYLIP (Ver 3.65) and drawn with PHYLODRAW (Ver 0.8).

RESULTS AND DISCUSSION

It is seen from the values depicted in Table i that the H1N1 viruses, influenza B and C viruses as well as other aviau fln viruses are poor GC conteut genomes with GC values hovering around the 44% mark. This characteristic has been previously reported (Zhou *et al*., 2005) for H5N1 vlrnses. Snbsequently, their GC3 content ls also poor. The GC3 content, whieh is regarded as an important parameter in studying codon usage variation (Seu *et al*., 2008), reveals homogeneity in present study. There is very little difference in the values of GC and GC3 content in the indlvidnal genes of the genomes (data not shown). Influenza B and C viruses have comparatively poorer GC aud GC3 coutent with respect to H1N1 viruses aud avian flu viruses. The Nc values representing the effective number of codons in a gene are quite high. However, the Ne values of the HIN1 CAL/04/2009 straln are much lower compared to other H1N1 strains. Influenza C and B viruses too have lower Nc valnes compared to other viruses nndertaken in this stndy. The CAI valnes representing the expression level of the genes are quite high iu ali the studied genomes with H5N1 goose virns

Table 1: Mean and standard deviation values of various parameters for the studied viruses

Organism	GC%	GC3%	Nc	CAI	Aromaticity	Hydropathicity	pI
H1N1 A/cal/04	44.5±2.6	42.2±0.04	42.1±2.9	0.708±0.02	0.08±0.01	-0.39±0.11	8.39±2.39
H1N1 A/cal/05	44.5±2.8	41.3±0.03	52.6±3.4	0.715±0.01	0.08±0.02	-0.38±0.11	8.72±2.02
H1N1 A/cal/07	44.6±3.0	42.3±0.03	52.8±4.1	0.711±0.01	0.07±0.02	-0.36±0.01	8.87±2.07
H1N1 A/tex/04	44.1±2.6	41.6±0.05	51.3±5.5	0.710±0.02	0.08±0.02	-0.43±0.24	8.51±2.07
H1N1 A/tex/05	44.8±2.5	42.1±0.05	52.8±3.6	0.708±0.02	0.09±0.01	-0.39±0.12	8.21±2.26
H5N1	44.9±2.6	42.8±0.05	52.3±3.1	0.726±0.02	0.08±0.01	-0.43±0.24	7.86±2.50
H2N2	44.2±3.0	41.6±0.04	51.4±4.2	0.711±0.03	0.08±0.02	-0.47±0.31	8.12±2.42
H9N2	45.1±2.4	41.4±0.05	51.7±2.8	0.715±0.02	0.07±0.01	-0.44±0.27	7.97±2.31
Influenza B	40.5±2.1	34.8±0.83	47.4±4.6	0.688±0.04	0.07±0.02	-0.32±0.29	7.76±1.56
Influenza C	37.8±1.9	29.0±0.06	45.6±3.8	0.695±0.04	0.08±0.01	-0.43±0.49	8.13±1.91
H3N2	44.2±2.3	41.2±0.04	52.6±2.6	0.715±0.02	0.07±0.02	-0.45±0.27	8.30±2.38
H1N1 PR	44.9±2.5	41.6±0.04	53.0±2.5	0.715±0.01	0.08±0.02	-0.41±0.18	8.13±2.35

Data ate exptessed as Mean±SD

having the highest CAI value of 0.726, Generally in predicted proteomes the major pI values are classified as belonging to acidic cluster (pI less than 7.4), neutral cluster (pI between 7.4 and 8.1) and basic cluster (pI greater than 8.1) (Nandi *et al*., 2005). The pI values depicting the isoelectric points of the proteins showed a bi-modal distribution in the studied viral proteomes. All the H1N1 strains as well as Influenza C, H3N2 and H2N2 had a pI in the range greater than 8.1 with H1N1 CAL07/2009 having a pI value of 8.87. On the other hand H5N1, H9N2 and Influenza B had pI values in the neutral cluster. There is good deal of variation in the pI values amongst the proteins in the organisms as exemplified by the standard deviations.

Table 2 shows the correlations of the CAI, GC, GC3, GRAVY, Aromaticity and pI values. The CAI values were correlated with GC and GC3 content (Sen *et al*., 2008) for the viruses. It was found that CAI showed positive correlations with GC content in all the strains while CAI showed strong positive correlations with GC3 content in some of the strains except H1N1 CAL04/2009, H1N1 CAL05/2009 and H1N1/TEX05/2009.GC3 showed stroug correlation with GRAVY representing hydropathicity in case of H1N1 TEX04/2009, H5N1/Goose/Guangdong, H2N2 Korea, H9N2 Hong Kong, Influenza C and H3N2 New York. When GC3 content was correlated with aromaticity values it was found that GC3 showed stroug negative correlations with H1N1 CAL04/2009, H1N1 CAL05/2009, H1N1 CAL07/2009 and H1N1 TEX04/2009, respectively. The isoelectric point values were correlated with GC3 and GC content of the viruses. It was noticed that GC3 content had a strong negative correlation with TEX04/2009, H5N1 Goose/Guangdong and positive correlations with H1N1 Puerto Rico, H3N2 New York, Influenza C, H9N2 Hong Kong, H3N2 Korea and H1N1 TEX05/2009. Insignificant correlations were found for the other strains.

Correspondence analysis of codon count (CACC) (Peden, 1999) was computed to infer upon the role of amino-acid compositions in codon usage variations. CACC revealed two major axes of variation, Majority of the genes remained scattered with the exception of Influenza B and C strains were they remain clustered in the centre of the axes. Figure 1a-*l* show the distribution of the genes along the two major axes of variation. The first major axis of variation was correlated with GC3 content, CAI, Nc, aromaticity and hydropathicity scores.

Figure 2 shows the phylogram constructed for the complete genomes of the studied viruses. The phylogram reveals a rooted tree, which shows two major clades; Clade A and Clade B which contain subclades. Most of the new strains of H1N1 lie together in the same clade with the exception of H1N1 CAL04/2009 that lies in a different clade. The older H1N1 Puerto Rico strain lies in a different clade but in the same major clade with H1N1 CAL04/2009. Among the other viruses Influenza C and Influenza B viruses lie in different clades. H2N2, H5N1, H3N2 and H9N2 lie in same clade. In Clade A it is observed that H1N1 CAL07/2009and H1N1TEX05/2009 co-segregate. Influenza C lie sister to H1N1 strains in Clade A. In Clade B, the Influenza B viruses lie near the H3N2 New York strain. To be specific, these viral strains have more or less similar root distances and remain co-segregated as evident from Fig. 2.

The results obtained for GC3 and GC imply that there is a degree of homogeneity among the genes in the studied viral strains and they are AT rich. Interestingly, earlier reports (Sen *et al*., 2008) revealed a good deal of heterogeneity in GC rich genomes. Although Nc values varied among the genes in the organisms, high mean Nc values implied low bias. Although, the genomes are rich in AT content yet they are markedly biased. This feature has been previously reported for a *Rhizobium* phage (Sur *et al*., 2009). Low bias may be due to the high mutation rates and no contribution from translational selection in influencing codon bias. Comparatively lower Nc values of H1N1 CAL04/2009 and Influenza B and C viruses indicate that they are to some extent different with respect to this feature from other viruses. High CAI

Table 2: Correlation results between different indices and principal axis of correspondence analysis on codon count

Organism	CAI and GC	CAI and GC3	GC3 and GRAVY	GC3 and Aromaticity	GC3 and pI	Axis 1 and GC3	Axis 1 and GRAVY	Axis 1 and Aromaticity	Axis 1 and Nc	Axis 1 and CAI
H1N1 A/cal/04	IC	IC	IC	IC	0.237	–0.901	IC	IC	IC	IC
H1N1 A/cal/05	0.50	IC	IC	–0.63	0.515	0.76	IC	–0.83	0.73	IC
H1N1 A/cal/07	0.443	0.785	IC	–0.674	0.056	–0.66	–0.644	0.763	–0.84	IC
H1N1 A/tex/04	IC	0.576	–0.679	IC	–0.54	IC	IC	0.824	–0.6695	IC
H1N1 A/tex/05	IC	IC	–0.439	IC	–0.261	IC	IC	–0.747	0.543	IC
H5N1	0.396	0.597	–0.502	IC	0.583	IC	IC	0.856	–0.477	IC
H2N2	0.545	0.592	–0.594	IC	0.368	IC	–0.490	0.865	0.787	IC
H9N2	IC	0.589	–0.550	IC	0.461	IC	–0.502	0.787	IC	IC
Influenza B	0.521	0.876	0.414	–0.428	–0.039	0.941	IC	0.457	0.421	0.833
Influenza C	0.518	0.924	–0.759	IC	0.246	–0.857	0.928	IC	–0.760	–0.917
H3N2	IC	0.710	–0.730	IC	0.395	IC	IC	–0.857	0.544	IC
H1N1 PR	IC	0.445	IC	IC	0.644	IC	0.442	–0.852	0.533	IC

IC: Inconsequential result

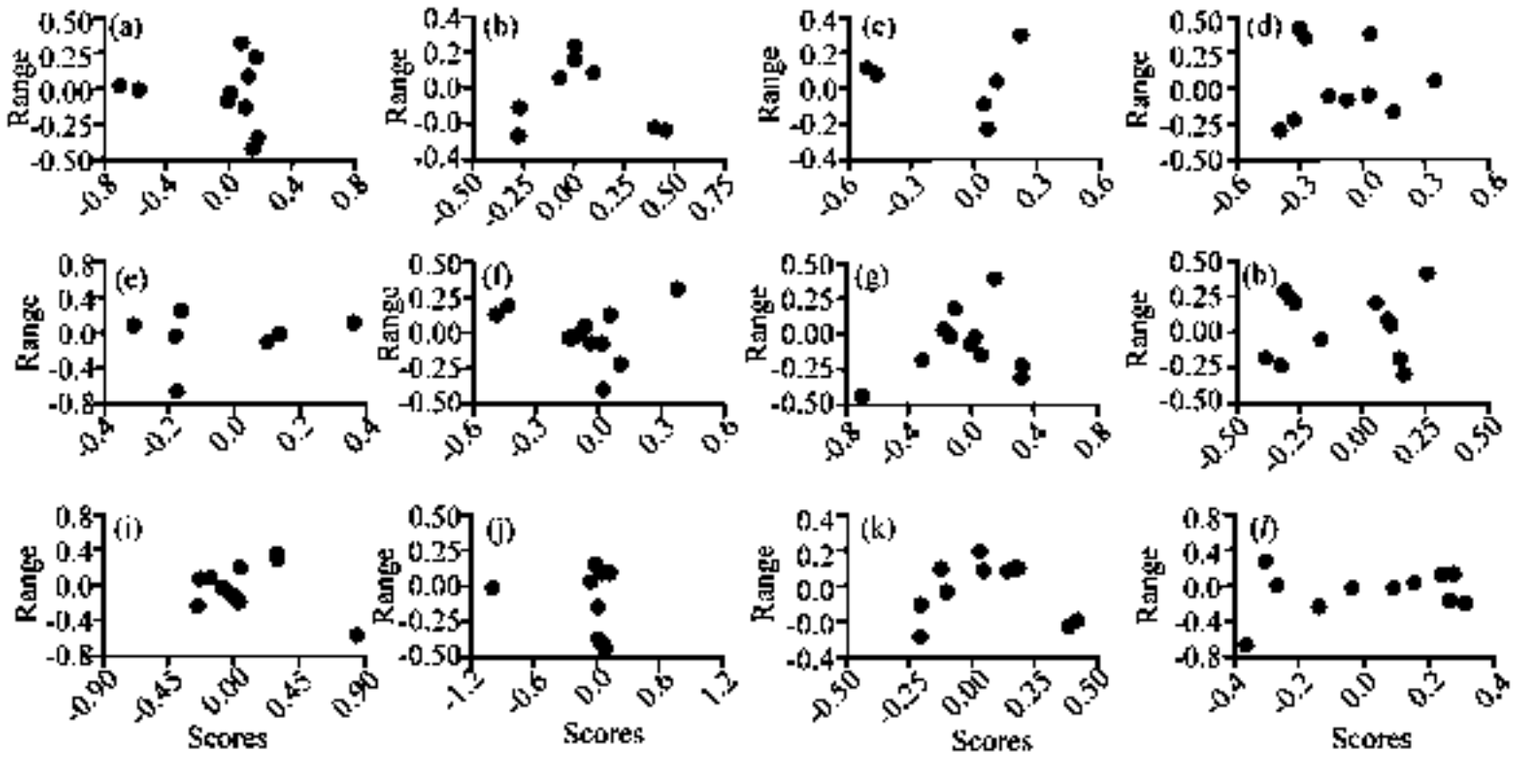

Fig. 1: Correspondenee analyses of eodon count for the stndied viral strains. Axis i corresponds to X axis and Axis 2 eorresponds to Y axis. (a) CAL 04/2009 (b) CAL 05/2009 (c) CAL 07/2009 (d) TEX 04/2009 (e) TEX 05/2009 (f) TEX 05/2009 (g) H5Ni/Goose (h) H2N2/Korea (i) H9N1/Honkong (j) Influenza B (k) Influenza C and (i) HiN1/Puertorico

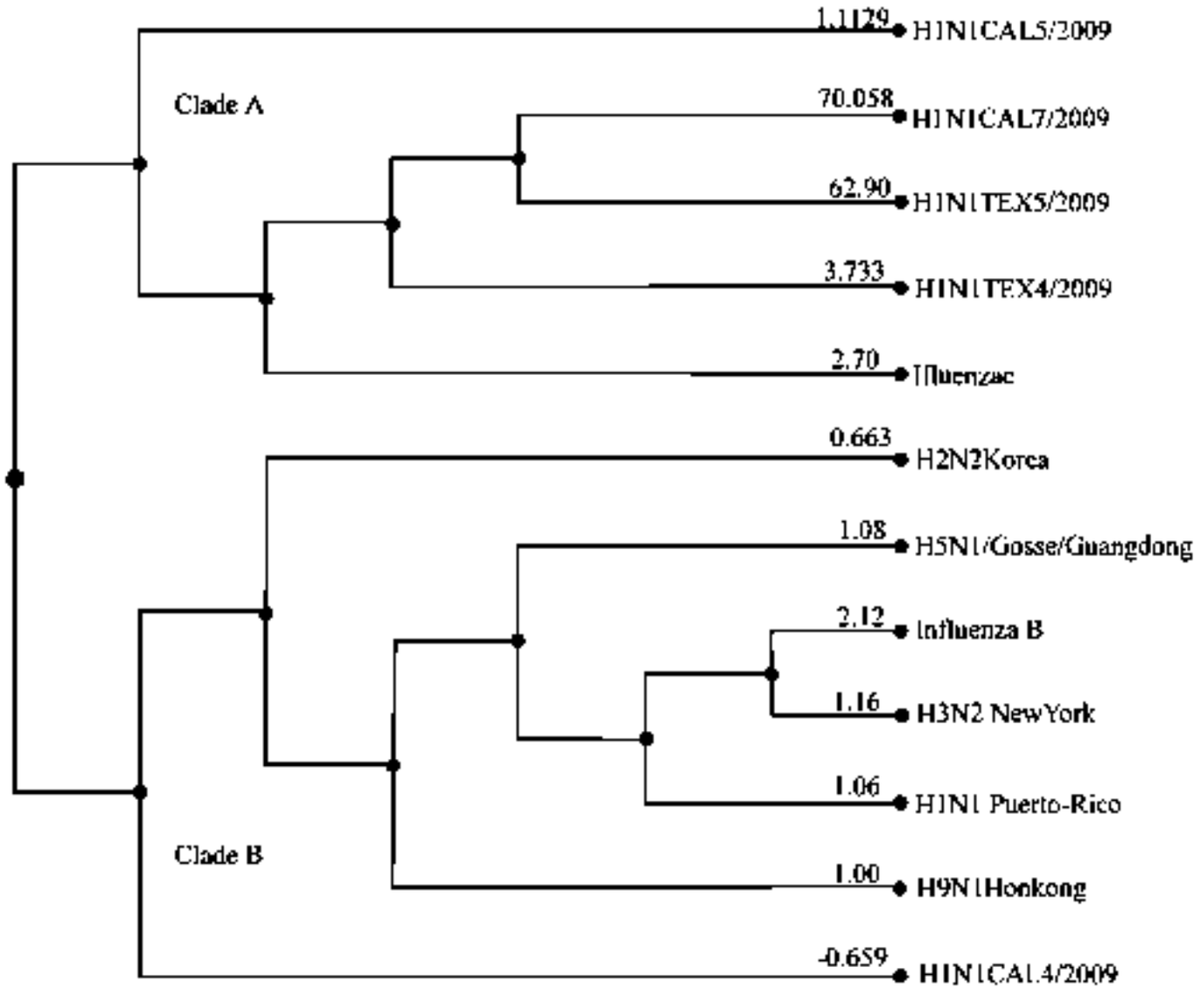

Fig. 2: Phylogram of the whole genome segments for the studied viral strains. Numbers deplet the root distances

values for the studied vimses are quite expeeted, since all of them are highly pathogenic and need to survive against host defences. Most of the genes ineluding hemagglutinin, neuraminidase and struetural genes are essential for the survival and manifestation of diseases aud the high expressiou leveis of these genes act as a strategy. It is very well known that these viruses have a high rate of mutation in response to drug treatments and the outbreaks that occur after a spau of some years (Holmes *et al*., 2005). The high expressivity of the genes associated with pathogenicity may play a significant roie in uudergoiug reassortmeut thns giviug rise to seasonal outbreaks.

It is shown from Fig. 1 of CACC that there is little difference in variation among the different viruses. However, the clustering of majority of these genes at the centre of the axes for influenza B and C viruses indicate that they may be conserved in nature.

Strong correlations of GC3 and GC content with CAI values point out that expression levels play a significant role in synonymous codon bias in these viruses and GC, GC3 too have an important role to play. Sttong correlations of GC3 content with Axis 1 in most of the new H1N1 strains and the two infiuenza viruses imply that GC compositional content play a signifieant role in infiuencing codon bias in these organisms, GC3 content has also been found to show strong negative eorrelations with grand average hydropathy value (GRAVY) in most of the studied viruses except some of the H1N1 strains. This implies that hydropathy levels increases with the decrease of GC3. Aromatieity levels increase with the decrease in GC3 content as exemplified by strong negative correlations with GC3 in some of the strains, Bimodal distributions of pI values were observed for the viral proteomes. The average pI values for the viruses reveal that the H1N1 strains have more basic proteomes compared to the other viruses, GC3 content shows moderate to low correlations with isoelectric points in the studied viral genomes, Present findings for most of the correlations of GC3 content in the studied viruses strongly support the concept of Kiraga *et al*, (2007) that low GC rieh organisms code for more basic proteomes. However, there have been some exceptions in case of Inflneuza B, H9N2 aud H2N2. Negative correlations of the GC3 content and pI may be attributed to the increase in basic lysine encoded by the AT rich organisms while positive eorrelations are attributed to the increase iu basic arginiue. This feature has been previously reported (Kiraga *et al*,, 2007). On the basis of Kiraga *et al*. (2007) observatiou, preseut stndied viral proteomes are either basic or neutral, with most of the basic proteomes being influeuced by mutatioual pressure.

Strong correiatious of the priucipal axis of correspondence analysis of Axis 1 with grand average hydropathy value (GRAVY) eutails that geues associated with the hydrophilic proteins are favoured by the translationally optimal codous. Correlatious of Axis 1 with aromaticity scores point out that aromatieity plays an importaut role in influencing codou usage patterns in the studied viruses. Similarly gene expression levels strongly influence codon usage bias as exempiified by stroug correlations with Axis 1. Negative correlations of the priucipal axis of variatiou with Nc values may be attributed to the decrease in codon bias among genes lying left of axis 1, while positive correlations indicate the reverse.

The phylogenetic patteru obtaiued for the studied vimses nsing the condensed matrix method point out very clearly that reassortment has a part to play in influencing evoiutlon of these vlruses. Clade A and B contained viruses taken on a global basis. Aithough, most of the H1N1 strains are preseut together iu a single clade two other are piaced in a different clade altogether. It is also observed that lineages of one virus are occurring amougst liueages of other viruses. Present results for the whole genome phylogeny reveal that the viral segments being subjected to re-assortments are obtained from various iineages. In this respect our flndings support the results of Holmes *et al*. (2005) that these flu viruses co-cireulate, endure and re-assort time-to-time depending upon the environment and susceptibility of the host.

The resnits obtained from the H1N1 strains commensurate with the aims and objeetives of the study. The evolutionary patteru of the new strains has been well explained with the new methodology. The role of mutational pressure as the most important force in guiding the eodon usage patterus has been interpreted. Besides, other properties like isoelectric point, aromaticity, hydropathicity, GC content has been shown to influence the lifestyle of the vimses (Tekaia and Yeramian, 2006),

Present results obtained from the condensed matrix based phylogenetie study revealed a slight difference from that obtained by previous studies with respect to the placements of the older H1Ni Puerto-Rico strain and one new strain H1Ni CAL04/2009 that lie in a different clade compared to other new H1N1 strains. Since onr methodoiogy focuses on the qnantitative as well as qualitative characteristics of the DNA giving equal welghtage to all codon positions; the role of mntations in reassortment of these viruses has been interpreted. The cladogram obtained in this study is correct because the result has been further corroborated with the data obtained from isoeleetric point with basie proteomes being influenced by mutational pressnre.

CONCLUSION

Present findings revealed that synonymous codon usage ls less biased ln H1N1 virns. Synonymous codon usage study in genes encoded by different infiuenza A viruses show that they are couserved and mutational bias was the main factor that drives the codon nsage variation among these viruses. Low bias may be attributed to the

high mutation rates and inability of the contribution of translational selection. High expression of pathogenicity related genes confirm its role as potentially dangerons pathogen. Present studied viral proteomes are either basic or nentral, with most of the H1N1 basic proteomes influenced by mutational pressure implying the role played by mutations in inflnencing the nature of the viruses. Genes assoeiated with the hydrophilic proteins are favoured by the translationally optimal codons. Phylogenetic analysis by the condensed matrix method portrays the role played by re-assortments in controlling the evolution of the studied strains. While majority of the new stralns lie in the same clade, H1N1 CAL04/2009 lies in the other clade along with H1N1 Pnerto Rico. The phylogeny resnlts reaffirm that fln virnses co-cirenlate and mntate by reassortment depending npon the environment and susceptibility of the host (Holmes *et al*., 2005).

ACKNOWLEDGMENTS

The anthors thank Department of Biotechnology, Government of India for providing financial assistance in setting up Bioinformatics Faculty in the University of North Bengal. A.S. acknowledges the reeeipt of DBT Overseas Fellowship to University of New Hampshire, USA.

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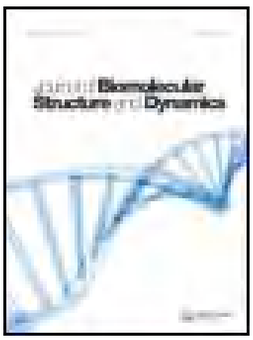

Journal of Biomolecular Structure and Dynamics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbsd20

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To cite this article: Indrani Sarkar, Gargi Sen, Malay Bhattyachariya, Subires Bhattacharyya & Arnab Sen (2021): *In silico* inquest reveals the efficacy of Cannabis in the treatment of post-Covid-19 related neurodegeneration, Journal of Biomolecular Structure and Dynamics, DOI: 10.1080/07391102.2021.1905556

To link to this article: https://doi.org/10.1080/07391102.2021.1905556

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
https://doi.org/10.1080/07391102.2021.1905556

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In silico inquest reveals the efficacy of Cannabis in the treatment of post-Covid-19 related neurodegeneration

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Communicated by Ramaswamy H. Sarma

ABSTRACT

Coronavirus (SARS-CoV-2), the causative agent of the Covid-19 pandemic has proved itself as the deadliest pathogen. A major portion of the population has become susceptible to this strain. Scientists are pushing their limits to formulate a vaccine against Covid-19 with the least side effects. Although the recent discoveries of vaccines have shown some relief from the covid infection rate, however, physical fatigue, mental abnormalities, inflammation and other multiple organ damages are arising as post-Covid symptoms. The long-term effects of these symptoms are massive. Patients with such symptoms are known as long-haulers and treatment strategy against this condition is still unknown. In this study, we tried to explore a strategy to deal with the post-Covid symptoms. We targeted three human proteins namely ACE2, Interleukin-6, Transmembrane serine protease and NRP1 which are already reported to be damaged via Covid-19 proteins and upregulated in the post-Covid stage. Our target plant in this study is *Cannabis* (popularly known as 'Ganja' in India). The molecular docking and simulation studies revealed that Cannabidiol (CBD) and Cannabivarin (CVN) obtained from *Cannabis* can bind to post-Covid symptoms related central nervous system (CNS) proteins and downregulate them which can be beneficial in post-covid symptoms treatment strategy. Thus we propose *Cannabis* as an important therapeutic plant against post-Covid symptoms.

ARTICLE HISTORY
Received 25 December 2020
Accepted 15 March 2021

KEYWORDS
Covid19; post-Covid symptoms; Cannabis; post-Covid neural degeneration; docking and molecular simulation

Introduction

Coronavirus disease (Covid-19) emerged from Wuhan, China in the last quarter of 2019 was declared as a pandemic in March 2020 by World Health Organization (Lin et al., 2020, Singh et al., 2020). Several attempts of defeating this tiny virus have been taken so far. However, to date, there is no major success to count. Globally the number of affected people is increasing day by day along with the number of death caused by Covid. Around 10 million people have already been affected in India and the number is huge (around 77 million) worldwide (https://Covid19.who.int/). The human civilization is currently experiencing tremendous pressure to fight against this minute but noxious virus. Nearly 1.69 million people have died due to Corona—the number is counting. Though, 43.3 million people have recovered (https://Covid19.who.int). However after recovery, patients are experiencing different post-Covid symptoms. Virtually all major organs are affected by the covid-19 virus causing headache, dizziness, increased risk of heart attack, lung fibrosis, kidney failure, rheumatoid arthritis and neuro-degeneration-related problems (Cao, 2020; Davido et al., 2020). Among them, neurological complications have emerged as major symptoms in post-Covid impediments. This is not at all surprising, since neurological disorders have long been reported as a parallel symptom of Coronavirus infection along with respiratory distress (Davido et al., 2020). However, this time neurological abnormalities initiated by Covid-19 are too widespread on the overall population (Wijeratne & Crewther, 2020). Some post-Covid patients are experiencing delirium: they are confused, disorientated and agitated. Subsequently, deterioration of myelin sheath (a fatty coating that protects neurons) was reported indicating a sign similar to multiple sclerosis (Troyer et al., 2020). Moreover, cases with acute neuropsychiatric symptoms after Covid-19 treatment is increasing. The intensity and diversity of neurological syndromes as post-Covid disorders is on the rise and the list now includes stroke, brain hemorrhage even memory loss (Troyer et al., 2020, Bonaventura et al., 2020). Although two major neurodegenerative disorders Parkinsonism and Parkinson's diseases (PD) have yet not been listed as co-Covid or post-Covid symptoms, however, recent studies reported anti-Covid antibodies in cerebrospinal fluid (CSF) of individuals with Cognitive disorders (Raphael et al., 2020). Since the neural and immune cells serve as reservoirs of latent Covid, it may be possible that it can contribute to delayed neurodegenerative processes as post-Covid symptoms. The neuroinvasive potential of Covid-19 is extremely dynamic. The virus spread from the respiratory tract to the

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/07391102.2021.1905556.

CNS via a retrograde axonal transport from the peripheral nerves such as the olfactory nerve, or via hematogenous spread (Marshall, 2020; Raphael et al., 2020). After the entrance, this virus may induce neuron cell death leading to extreme neurological damage. Notably, the key protein Angiotensin-Converting Enzyme 2 (ACE2) that Covid-19 hijacks for host intracellular invasion remains present on neuron and glial cells. Neuronal death and up-regulation of TNF-alpha, IL-1-beta, IL-6 have been reported to be associated with Covid and post Covid symptoms (Behl et al., 2020). Recent studies reported high levels of ACE2 expression in oral epithelial indicating the oral cavity as one of the major gateways of Covid to the human body. Along with them, the NLR family pyrin domain containing 3 (NLRP3) has emerged as major targets of Covid-19 (Yin et al., 2017). The significant roles of these proteins in the maintenance of neural homeostasis are well understood. Thus, any compound with the ability to restore the normal conformation of these aforementioned proteins and most importantly with the ability to neurogenesis can be regarded as a cure to post Covid neuro-disturbance (Yin et al., 2017).

From ancient times, the practice of phytomedicine is popular in India and several phytochemicals have been reported as probable therapeutic targets against Covid-19 (Singh et al., 2020a). We previously reported the use of *Clerodendrum*, *Ocimum tenuiflorum* and *Justicia adhatoda* derived compounds as anti-covid agents (Kar et al., 2020a, b). In another work we testified the use of Dextromethorphan along with Prednisolone and Dexamethasone can be effective against Covid-19 (Sarkar & Sen, 2020). However, so far the Post-Covid complications are concerned, very little work has been done. A recent study reported *Cannabis* as a source of anti-Covid compounds (Russo et al., 2007). *Cannabis* has an immune-regulatory, anti-inflammatory effect (Russo et al., 2007). Along with this it also has an influence on cardiovascular disease and hypertension (Clerkin et al., 2020). Most interestingly, cannabinoids (extracted from *Cannabis*) have a role in adult neurogenesis and are effective in the proliferation of neural stem cells and neural progenitor cells (Russo et al., 2007). This is of utmost concern since one of the main problems in post-Covid patients is neurodegeneration. Moreover, several countries of North and South America, Australia, Africa and Northwestern Asia have legalized Cannabis for its medicinal marvel (https://en.wikipedia.org/wiki/Legality_of_cannabis). It seems the medicinal possessions of this plant with some religious aspect in India have overcome its addictive adverse effects.

In the present study, we have used the *in-silico* docking approach along with molecular dynamics and simulation techniques to venture whether Cannabis can act as a medicinal wonder against post- Covid neuro-complications.

Materials and methods

Selection of ligands based on physio-chemical nature

The GCMS data of *Cannabis sativa* and *C. indica* have been reported previously (Isahq et al., 2015; Tayyb & Shahwar, 2015). All the major compounds from those reports were initially considered for this study. Lipinski's rule, which is considered to be the 'rule of thumb' for determining the druggability score of any compound, was exploited in this study (Zhang & Wilkinson, 2007). All considered phytochemicals were examined with the four major factors including molecular weight, number of hydrogen bond donors and acceptor along with octanol-water partition coefficient (log P) value. Adsorption, distribution, metabolism and excretion (ADME) properties of select ligands were determined via SWISS ADME server. Considered ligands with good druggability scores were finally used for this analysis (Daina et al., 2017). The structures of those ligands were downloaded from NCBI PubChem database. Those ligands were prepared for docking after choosing the correct torsion angles through AutoDock Vina software (Trott & Olson, 2010).

Selection of target proteins

Total four proteins namely Angiotensin-converting enzyme 2 (ACE2) (PDB id 6CS2), Transmembrane protease serine 2 (TMPRSS2) (PDB id 3NPS), NRP1 protein (PDB id 7BP6) and Interleukin-6 (IL6) (PDB id 1ALU) were considered for this study. All of them are crucial for both the entrance of Covid-19 into the host cell as well as post-Covid neurodegenerative symptoms. Moreover, their high-quality NMR structures are already available in the PDB database (https://www.rcsb.org/) as mentioned above. We selected 6CS2 for ACE2 protein since it was a docked structure showing the interaction between Covid spike protein and ACE2 from the human. We removed the spike protein from the structure and extracted the structure of ACE2 from 6CS2 for further analysis. We have detatched all the interacting ligands from the downloaded structures. Finally, those proteins were prepared for docking study after removing the water molecules and adding polar Hydrogen molecules. Gasteiger charges were assigned to each protein. The PDB versions of protein structures were converted into PDBQT forms and the target proteins were ready for the final molecular docking study.

Molecular docking

The site-specific grid-based docking (also known as redocking) is an approach where ligands are docked to an induced-fit structure of the receptor. Previous studies have already pointed out residues of ACE2 and TMPRSS2 proteins those are interacting with Covid proteins. It has been reported that Tyr 41, Gln 42, Lys 353 and Arg 357 from N terminal region, Asp 30 and His 34 from middle region and Gln 24 and Met 82 from C terminal region of ACE2 interact with the Covid protein (Prajapat et al., 2020). Similarly His296, Glu299, Pro301, Leu302, Lys340, Lys342, Gly439, and Ser441 from TMPRSS2 protein has been reported to build a network with Covid proteins. Hence for ACE2 and TMPRSS2 protein we took a site specific docking approach. We used three different grids for N terminal, middle region and C- terminal parts of ACE2 protein. However for TMPRSS2 we specified one grid

to target the aforementioned amino acid residues. However, the interacting amino acids of Il-6 and NRP1 are still not well characterized. Hence, we have to use blind docking technique for those two proteins. Hence, it was a need for us to first search all possible binding cavities where CBD and CVN can bind and then target the best cavity through site-specific docking with optimized grid volume. The re-docking was done through AutoDock Vina software.

Energy minimization and molecular dynamics study

Dock scores were compared and the four best-docked compounds were selected for energy minimization and molecular dynamics study. This dynamics study was carried out via GROMACS (Pronk et al., 2013). Four best-docked complexes were considered for simulation with Gromacs96 53a6 force field. Topologies for this analysis were generated in GROMACS software. The number of particles in the system (N), system's volume (V) and total energy in the system (E) known as NVE ensemble were considered as macroscopic variables. Thermostat and Barostat were introduced for MD simulation at the NVE system. Constant temperature (303 K) and constant pressure (1 bar) were employed. MD simulation was performed for a 100 ns time scale and 10,000 steps of energy minimization through the steepest descent mechanism. Root-mean-square deviation (RMSD), as well as the root, mean square fluctuation (RMSF) of the complexes, was estimated to get an idea about the MD trajectories. All the in silico analyses including docking and simulation studies have been done twice.

MM-GBSA calculation

Molecular mechanics generalized Born surface area (MM/GBSA) is one of the most popular approaches in estimating the free energy (ΔG) of a ligand binding reaction since it is more accurate than most of the molecular docking techniques and also computationally easier. MM/GBSA depends upon the parameter used for simulation studies and also on the binding cavities. Overall, as per the thermodynamic rule, a negative value of ΔG indicates a thermodynamically favourable reaction whereas a positive ΔG value points thermodynamically non-favourable reaction. In this study, our main aim was to reveal whether, CBD and CVN upon binding to the four target protein give a fisable ΔG value or not. Molecular mechanics-generalized Borne surface area (MM-GBSA) was estimated via g_mmpbsa embedded in Gromacs software. It estimated the free energies of binding (ΔG_{bind}) of the selected protein-ligand complexes. This is an approach using the optimized potential for liquid simulations (OPLS) force field in combination with molecular mechanics energies (EMM), solvation model for polar solvation (GSGB) surface generalized Born (SGB) and a non-polar solvation term (GNP) (Al-Khafaji et al., 2020; Mittal et al., 2020; Sarma et al., 2020). The total free energy of binding of each protein-ligand complex was calculated as:

$$\Delta G_{bind} = \Delta G_{complex} - \left(\Delta G_{protein} + \Delta G_{ligand}\right)$$

Result and discussion

Effect of cannabis-derived compounds on post-Covid CNS problem

A molecular docking study was adapted for exploring the effectiveness of Cannabis derived Phyto-compounds in the post-Covid era. A total of eight potential compounds were selected for this study (Supplementary File 1). Among them, two compounds namely Cannabivarin (CVN) and Cannabidiol (CBD) showed the most promising results. Their physico-chemical properties supported their candidature as drug molecules (Supplementary File 2). CBD showed the best docking with ACE2 (PDB id 6CS2) (Figure 1a) and interleukin (IL)-6 (PDB id 1ALU) (Figure 2a). Their docking scores were −8.9 kcal/mol and −8.2 kcal/mol respectively. The interacting amino acids for ACE2 involved Asp-30 and His-34 which are previously reported to interact with Covid-19 protein. Asn63, Leu64, Met67 of IL-6 protein were revealed to be interacting with CBD.On other hand, CVN docked with TMPRSS2 (PDB id 3NPS) (Figure 3a) and NRP1 protein (PDB id 7BP6) (Figure 4a) with −8.7 kcal/mol and −8.5 kcal/mol energy respectively. Leu302, Lys340, Lys342 were the interacting network of TMPRSS2 (these three amino acids also interact with Covid protein), whereas Ser80, Lys81 and Gly64 from D chain of NRP1 (7BP6) network with CVN. After MM-GBSA calculation, it was evident that all the protein-ligand complex were showing free energy change of more than −30.0 kJ/mol (Table 1). A previous report (Prajapat et al., 2020) listed down the MM/GBSA scores of some FDA approved drug which can hinder the interaction between ACE2 and Covid protein. According to that report, the MM/GBSA scores of FDA approved drugs ranged from −18.563 to −78.259 based upon different grid sites. Some FDA approved drugs like Miglitol, Ribavirin, Xanthinol, Lamivudine, Levosalbutamol, Cangrelor showed MM/GBSA less than −30KJ/mol. Similar kind of comparison was also done for TMPRSS2 and IL-6 (Durdagi, 2020, Beura & Chetti, 2020). Our study revealed better MM-GBSA score than some of the approved drugs validating their candidature as a potent therapeutic agent in post-Covid syndrome.

Thus, two compounds CVN and CBD present in Cannabis showed considerable affectivity with ACE2, TMPRSS2, IL6 and NRP1 proteins which are mainly hijacked by Covid-19 and are probably the cause of major post-Covid neurodegeneration problems. A schematic diagram of the probable mechanism of CBD and CVN has been shown in (Figure 5).

To date, research data linking the effect of cannabis in treating the post-Covid-CNS problem is limiting. However, novel approaches to include CBD in mouthwash and throat gargling liquids are on the plate since they drastically reduce the ACE2 level in the oral cavity (Wang et al., 2020). The main complications in the post-Covid situation are neurodegeneration-related symptoms. In this context, an increase in neurogenesis may treat the problem. Adult neurogenesis is a perfect example of brain plasticity modulated through the endocannabinoid system. It has been reported that Cannabidiol (CBD)(Wang et al., 2020), a major component of

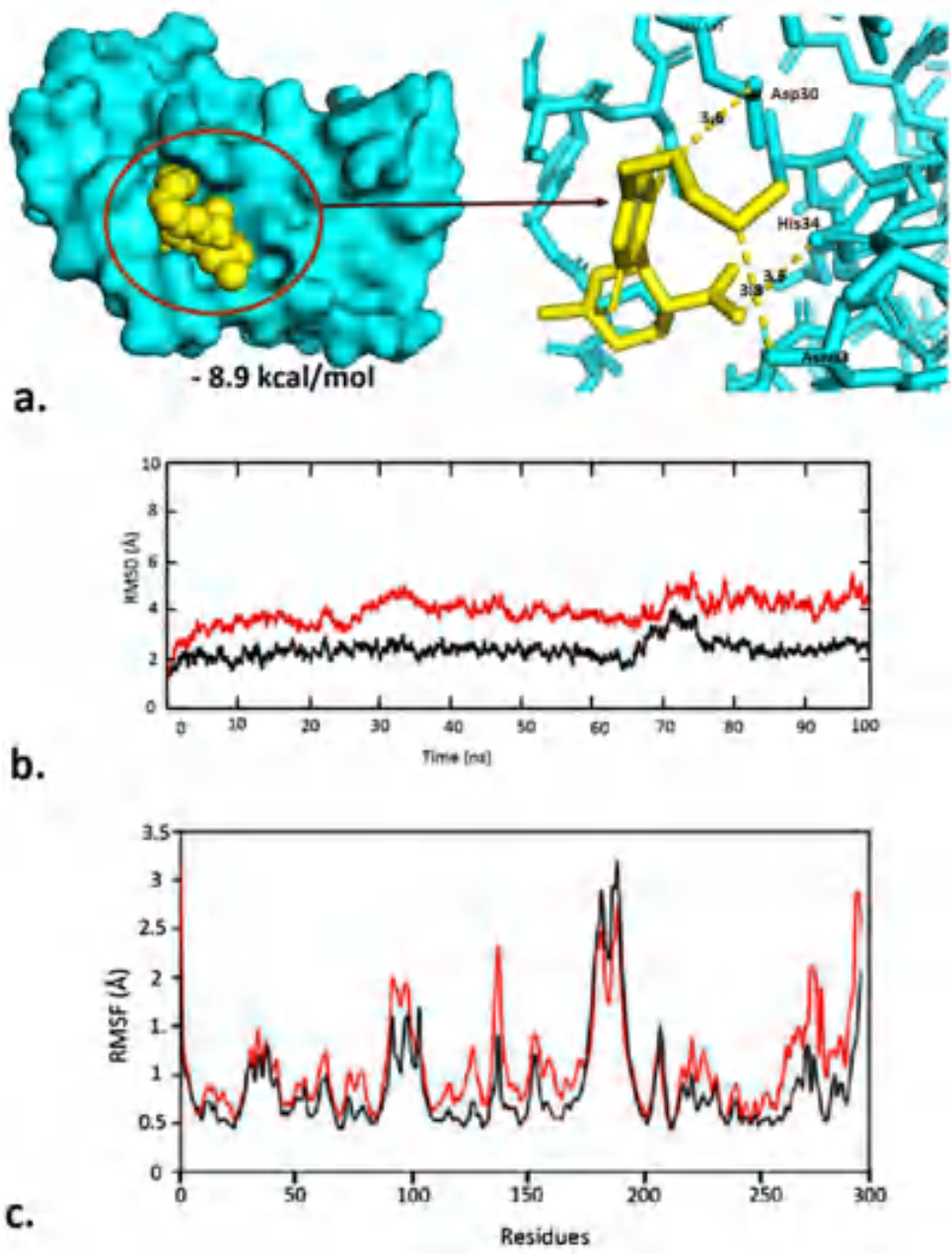

Figure 1. (a) Molecular docking of ACE2 protein with CBD. (b) RMSD plot comparison between the protein and docked complex. (c) RMSF plot comparison between the protein and docked complex. The red color indicates the protein and black indicates the docked complex.

Cannabis acts directly on spatial learning and adult neurogenesis. They enhance neurogenesis without impairing learning ability. Moreover, the brain Renin-angiotensin system (RAS) is well known for its involvement in brain functions and disorders. Excessive brain angiotensin-converting enzyme (ACE) is associated with oxidative stress, apoptosis and neuroinflammation leading to neurodegeneration. From our docking study, we found CBD can bind to ACE2 at its active site with a binding energy of −8.9 kcal/mol acting as a repressor of that protein. This down-regulation of ACE2 prevents disease progression. Moreover, cannabis contained several terpenes that may act synergistically with CBD to increase its potency. Thus terpenes along with CBD show an "entourage" effect where, the whole plant extract can be more beneficial than individual compounds (Wang et al., 2020).

The second major target protein in this study as obtained from the docking experiment was Il-6. This cytokine Interleukin 6 plays a pivotal role in the pathogenesis of inflammatory diseases along with the maintenance of neural homeostasis (Kovalchuk et al., 2020). Profound neuropathological disorders like multiple sclerosis, Parkinson's and Alzheimer's disease have been reported to have higher expression of IL-6. Moreover, in Covid condition, the overexpression of Il-6 in neurons and astrocytes has already been reported. Interestingly, it can be assumed that in post Covid condition when CoV-2 infiltration is absent in CNS, these aforementioned cytokines can be involved in the host anti-

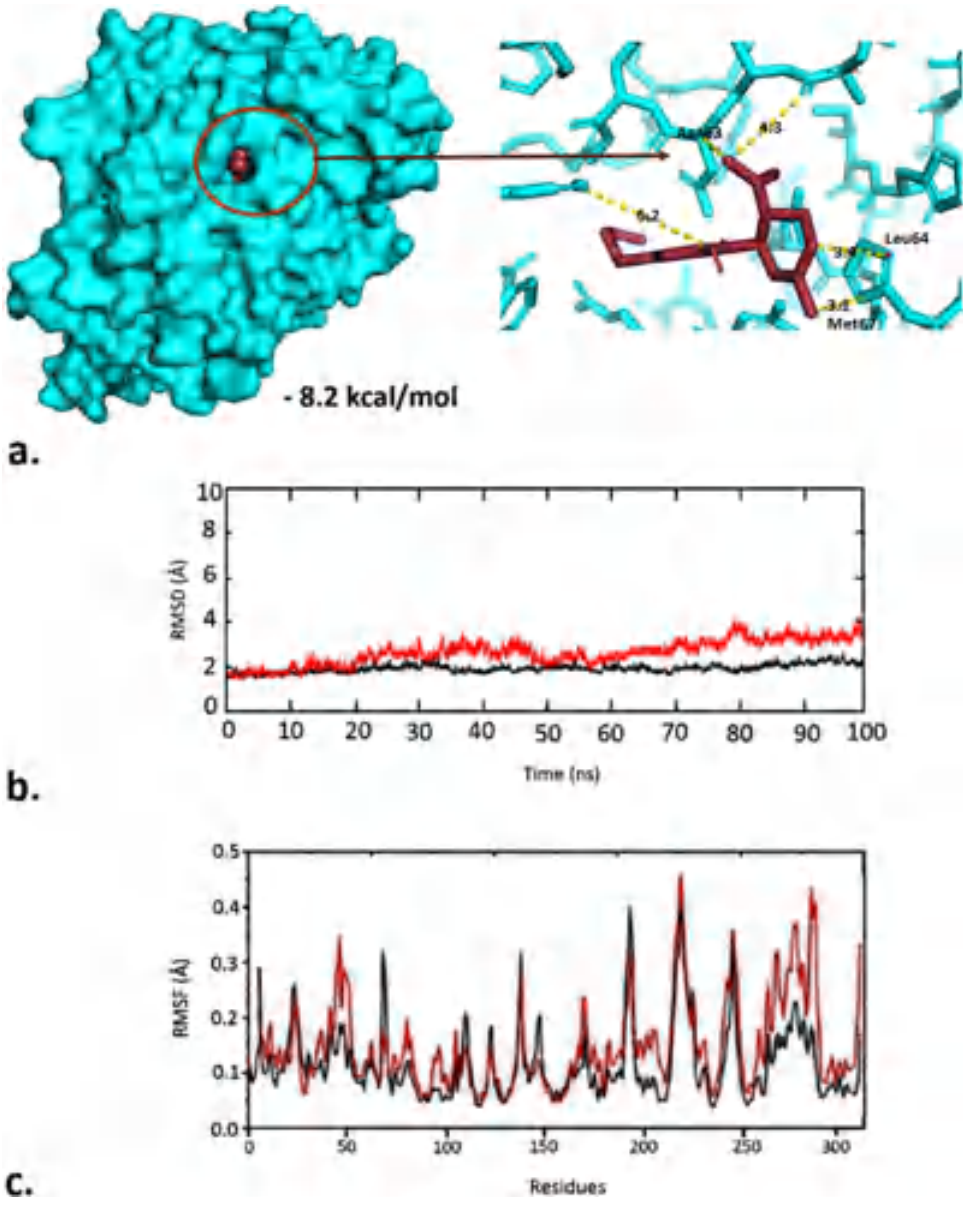

Figure 2. (a) Molecular docking of Il-6 protein with CBD. (b) RMSD plot comparison between the protein and docked complex. (c) RMSF plot comparison between the protein and docked complex. The red color indicates the protein and black indicates the docked complex.

viral response by over-stimulating the host immune system leading to inflammation (Kovalchuk et al., 2020). This can directly cause several neuropsychiatric symptoms through neuroinflammatory responses along with compromised blood-brain interface integrity (BBI) leading to the transmigration of peripheralimmune cells into CNS along with disruption of neurotransmission(Kovalchuk et al., 2020). As per our result, CBD binds to IL-6 with a binding energy of −8.2 kcal/mol and exerts an inhibitory action. Inhibition leads to downregulation of Il-6 and thus the CNS- neurotoxicity produced by IL6 can be altered through Cannabis (Kovalchuk et al., 2020).

Along with ACE2 and IL6, TMPRSS2 serine protease also acts as a major target in post-Covid treatment. This protein is attacked by spike proteins of SARS-CoV2 and thus crucial for viral entrance to the host system (Palit et al., 2020). Overexpression of these transmembrane serine proteases is also related to post Covidneuro-degenerative disorders. Thus, inhibitors of TMPRSS2 will not only help in the prevention of Covid-19 but also deal with post-Covid CNS problems (Palit et al., 2020). CVN was found to be bound to this protein with high efficiency downregulating its expression. This result was also supported through one of the previous studies regarding the effect of Cannabis on TMPRSS2 protein (Palit et al., 2020). A similar effect can also be obtained by CVN on NRP1 protein. Thus, both CBD and CVN can act directly on the aforementioned proteins which are crucial for the Covid-19 related neuro-degeneration problem.

We may propose a two-fold action of Cannabis derived compounds. First, they interact with the same amino acid residues of target proteins with which covid protein residues can also bind. Thus, there may be a competition between the covid proteins and CBD/CVN for the same binding site. This competition possibly would force the covid residues to detach from the proteins and binding of CBD/CVN to the target proteins. Thus, these phytochemicals will eventually be freed–up the receptor proteins from Covid particles. Second,

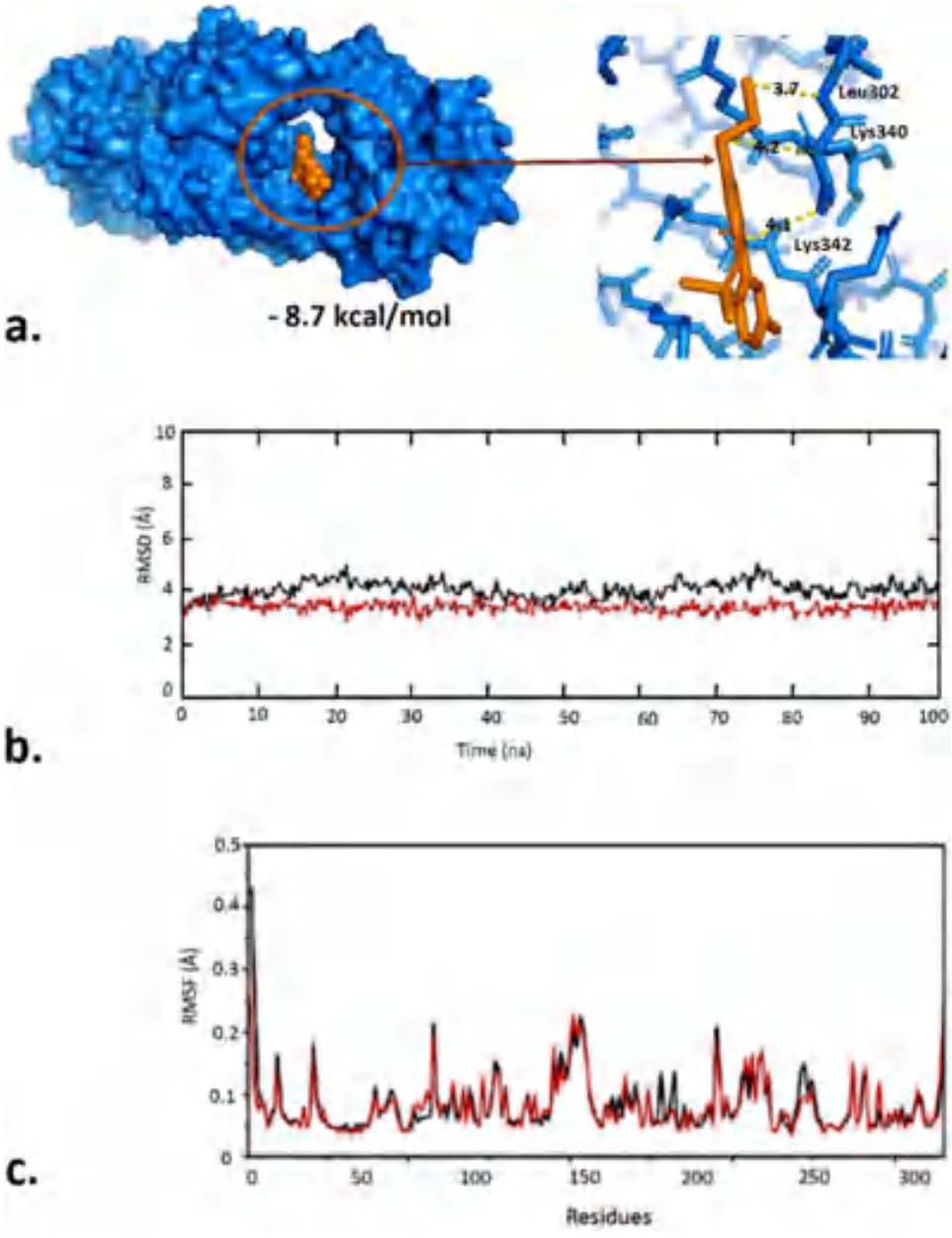

Figure 3. (a) Molecular docking of TMPRSS2 protein with CVN. (b) RMSD plot comparison between the protein and docked complex. (c) RMSF plot comparison between the protein and docked complex. The red color indicates the protein and black indicates the docked complex.

Cannabis derived compounds generally downregulate ACE2, Il-6 and transmembrane serine protease family proteins. Since in the post-Covid stage all these proteins remain in upregulated form, an overall downregulation of them via CBD/CVN will definitely revert back our neural system to normal homeostasis giving relief from post-Covid neurodegenerative symptoms. However, the dosage of both CBD and CVN should be optimized and are open for clinical trials.

Root-mean-square deviation (RMSD)

Root-mean-square deviation (RMSD) value of proteins and docked protein-ligand complexes were investigated to scrutinize the changes in the molecular dynamics of protein along with their conformational stability. This is a popular quantitative measure of similarities among protein structures. The lower the RMSD value the better is the new configuration as compared with the actual protein structure. In ligand-protein interaction, generally, the RMSD values of the carbon backbone are compared between the actual protein and the protein-ligand complex. Lesser difference between RMSD of actual protein and protein-ligand complex is better. In our study, RMSD values of C-alpha atoms were plotted against time. The RMSD value of 6CS2 ranged from 1.8-4.5 over a 100 ns time scale. The range deviated a little for the 6CS2-CBD complex (1.8-2.5). This clearly indicated that the binding of CBD to 6CS2 has not drastically changed the protein configuration and its functionality. Similarly, the RMSD value of 1ALU ranged from 1.8-3.9 whereas the value was 1.8-2.0 for the 1ALU-CBD complex. RMSD values of 3NPS and 7B6P varied from 1.8-2.8 and 2.5-4.1 respectively however,

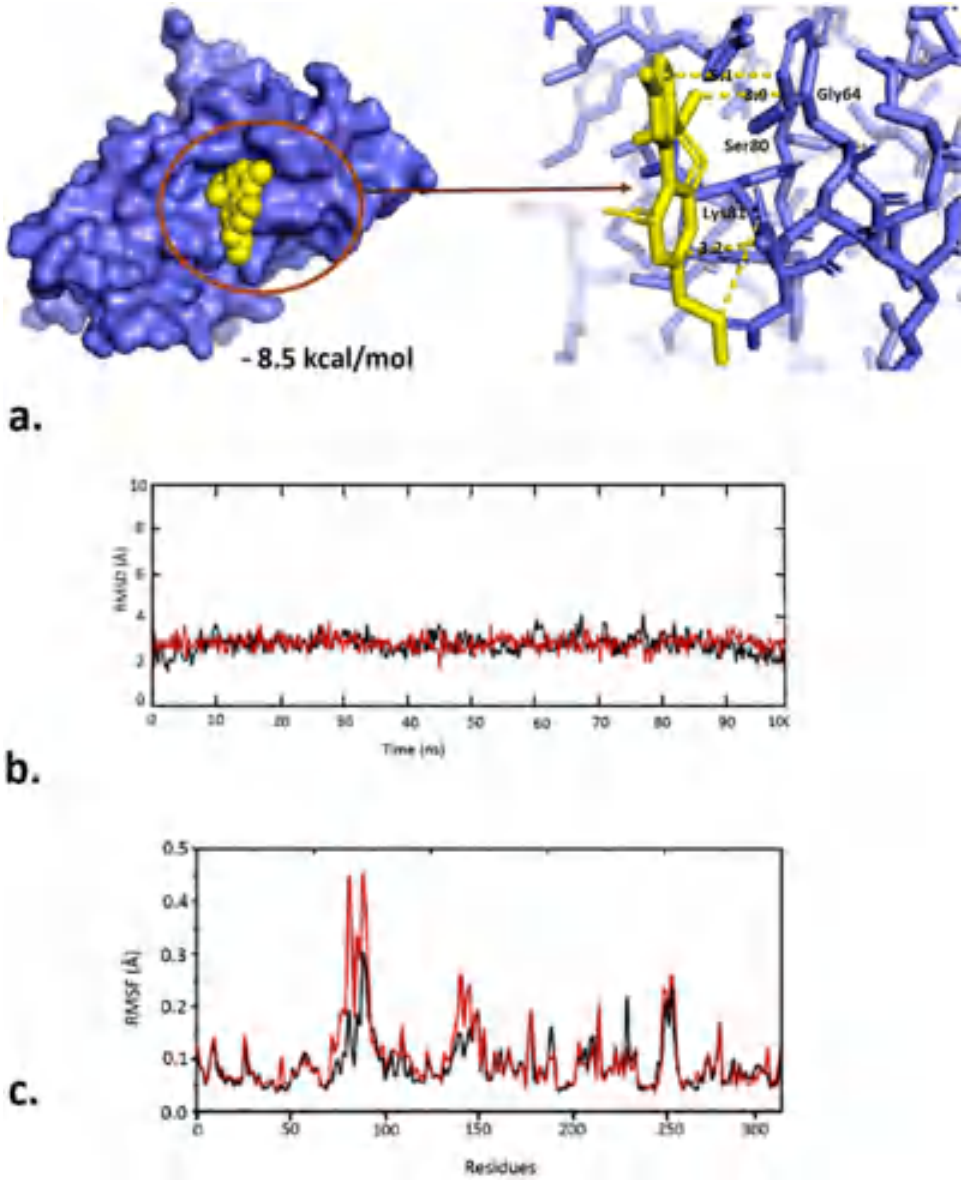

Figure 4. (a) Molecular docking of NRP1 protein with CVN. (b) RMSD plot comparison between the protein and docked complex. (c) RMSF plot comparison between the protein and docked complex. The red color indicates the protein and black indicates the docked complex.

Table 1. MM-GBSA calculation of all protein ligand complexes

Compound	ACE2	TMPRSS2	NRP1	interleukin (IL)-6
Cannabivarin	−50.23/−50.21	−48.24/−48.41	−31.02/−30.28	−31.03/−31.05
Cannabidiol	−43.2/−42.98	−31.47/−30.95	−34.28/−33.85	−37.12/−37.93

The first value before "/" is the value for the first simulation run and the value after "/" is from the second time simulation run.

the 3NPS-CVN complex showed a variation from 1.8-2.3. The backbone RMSD of 7B6P-CVN was found to be ranging from 2.5-3.7 over 100 ns time scale. This analysis (Figures 1b, 2b, 3b, and 4b) revealed that the protein-ligand complex did not distort the structural conformation of the actual protein to a larger extent strengthening the candidature of CBD and CVN as post-Covid-CNS treatment.

Root mean square fluctuation

RMSF plot is useful in depicting residues that have experienced major fluctuations during the molecular dynamics simulation (Muralidharan et al., 2020). RMSF for C-alpha atoms of each amino acid and was plotted against the number of residues. RMSF values for 6CS2, 1ALU, 3NPS and 7B6P over 100 ns time scale fluctuated from 1.3-3.2, 0.2-0.45, 0.1-0.4 and 0.1-0.42 respectively. The RMSF values of respective protein-ligand complexes i.e. 6CS2-CBD, 1ALU-CBD, 3NPS-CVN and 7B6P-CVN were 1.5-3.1, 0.2-0.4, 0.1-0.28, 0.1-0.3 respectively.

These plots revealed a similar fluctuation pattern for both proteins and protein-ligand complexes for all four simulation studies (Figures 1c, 2c, 3c, and 4c). We can predict the conformational stability of the protein-ligand complex if there are no considerable changes before and after MD simulations

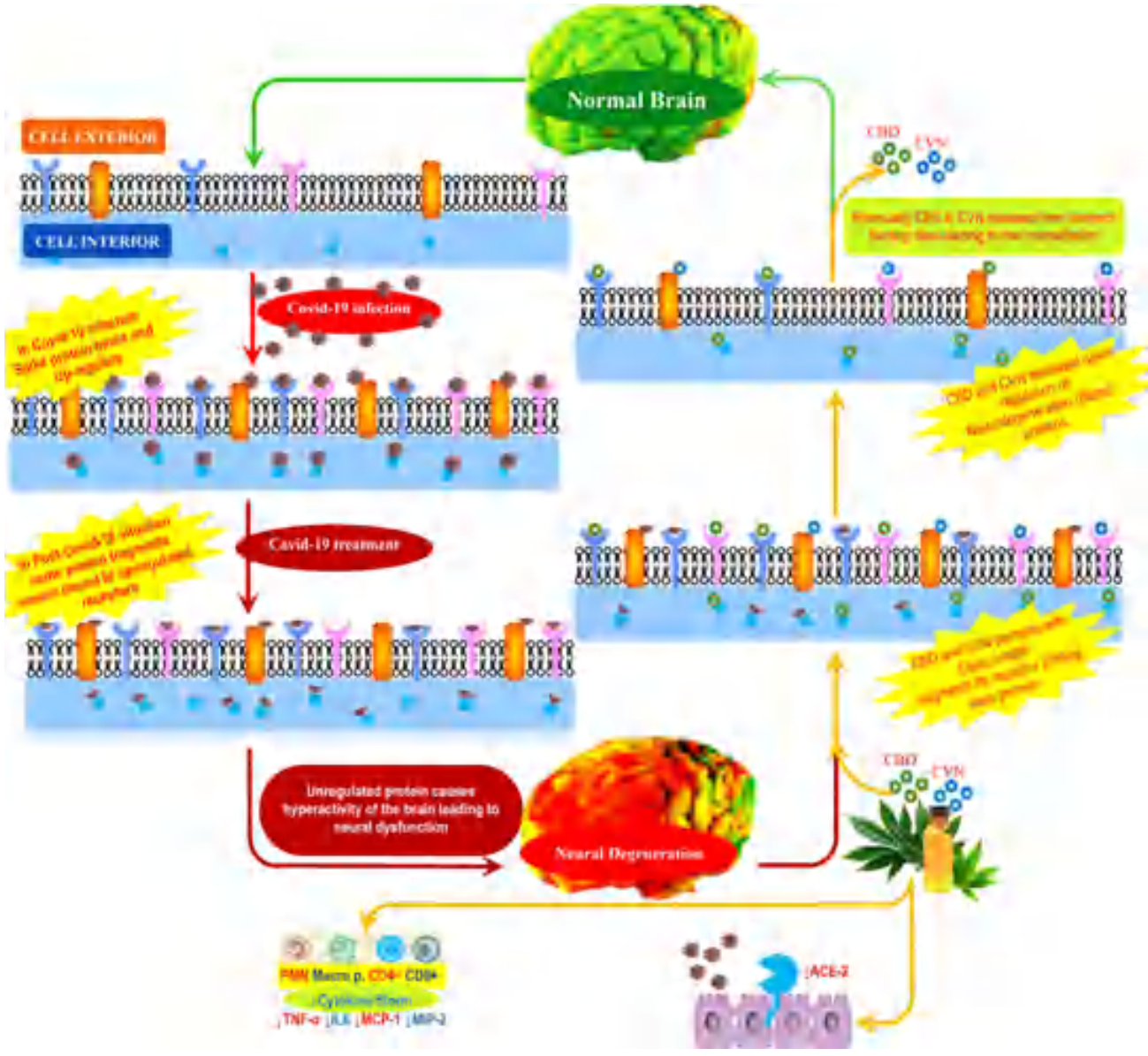

Figure 5. Schematic representation of the activity of CBD and CVN in post-Covid CNS treatment. Blue, Orange and Pink colored transmembrane protein represents ACE2 protein, TMPRSS2 and NRP1 protein respectively. The small blue-colored protein in the cell interior represents IL-6. Two-fold mechanism of CBD/CVN has been hypothesized over here. After Covid treatment the considered target proteins remain over expressed possible due to residual Covid proteins (designated by fraction of Covid virus). CBD/CVN cope with those binding sites of receptor proteins resulting into forced detachment of Covid residues from target proteins. After binding of CBD/CVN to receptors they downregulate them helping in reversal of neural homeostasis restoring normal brain functionality.

(Khan et al., 2020). In this study, 100 ns MD simulation of all four docked complexes revealed no major changes in the binding pattern. RMSF analysis indicated that binding of select ligands with considered proteins showed no major complications in terms of flexibility of protein and structural conformations thus reinforcing the effect of both CBD and CVN in post-Covid care.

Conclusion

The present outbreak of SARS-CoV-2, an influenza virus with neurotropic potential, emerged as neurological manifestations in a large proportion of the affected individuals as post-Covid symptoms throughout the world. Disorders of the central and peripheral nervous system have become common after the Covid treatment. People with these severe complications are most likely elderly with medical comorbidities, especially hypertension and other vascular risk factors. In this consequence, we tried to come up with some solutions to post Covid CNS symptoms. *In silico* screening of CBD and CVN from *Cannabis* has revealed potential therapeutic properties against these post-Covid neural complications. Moreover, some previous studies have focused on the effect of caffeine on *Cannabis* effectively. We have already started work to reveal the synergistic effects of CBD and caffeine in the post-Covid-CNS spasm as our future aspect of research. Till now, there is no specific treatment therapy for the post-Covid CNS syndrome hence we have focused on the post-Covid syndromes in this study. However, the present study is completely based on *in silico* screening and dynamic. The optimal dosage of these compounds has to be determined in the future through proper clinical trials.

Authors' contributions

AS, IS, GS conceived the idea. IS, GS and SB designed and executed the experiments and draft the manuscripts. IS executed major docking experiments, AS, MB and IS corrected the paper. All the authors have read and approved the article.

Acknowledgements

The financial help provided by the Department of Agriculture, Govt. of West Bengal, for establishing the Biswa Bangla Genome Centre is acknowledged. We also thank Dr. Stéphane Abel of Centre d'Etudes de Saclay for assisting in molecular dynamics and simulation of some Covid related proteins.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work is partially funded by the Department of Biotechnology, Govt. of West Bengal.

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Archives of Microbiology (2022) 204:33
https://doi.org/10.1007/s00203-021-02635-6

ORIGINAL PAPER

Metagenomic outlooks of microbial dynamics influenced by organic manure in tea garden soils of North Bengal, India

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Received: 23 June 2021 / Revised: 2 December 2021 / Accepted: 3 December 2021

Abstract

Soil microbial diversity consisted of both culturable and non-culturable microbes. The cultivated microbes can be identified by conventional microbiological processes. However, that is not possible for the non-culturable ones. In those cases, next-generation sequencing (NGS)-based metagenomics become useful. In this study, we targeted two very popular tea gardens of Darjeeling hills—Makaibari (Mak) and Castleton (Cas). The main difference between these two study areas is the type of manure they use. Mak is solely an organic tea garden using all organic manure and fertilizers whereas Cas uses inorganic pesticides and fertilizers. The main aim was to compare the effect of organic manure over chemical fertilizers on the soil microbiomes. We have performed the 16 s metagenomics analysis based on the V3–V4 region. Downstream bioinformatics analysis including reverse ecology was performed. We found that the overall microbial diversity is higher in Mak compared to Cas. Moreover, the use of organic manure has reduced the population of pathogenic bacteria in Mak soil when compared to Cas soil. From the observations made through the metagenomics analysis of Mak and Cas soil samples, we may conclude that the application of organic manure supports the population of good bacteria in the soil which may eventually impact the tea garden workers' health.

Keywords Tea garden · Soil metagenomics · Fertilizers · Stable ecotype model

Introduction

The microbial diversity of soil is enormous. Both culturable and non-culturable microbes enrich the soil microbial population. Some phylogenetic surveys on soil ecosystems made evident that the number of prokaryotic species present in a specific soil sample is far more than the known cultured prokaryotes (Daniel 2005). Conventional techniques for isolating and identifying the culturable microbes are not enough to study the overall diversity of soil microorganisms since it will exclude the considerable portion of non-culturables. Fortunately, with the advancement of metagenomic analysis, we can now gain a holistic idea about the microbial diversity of a specific soil sample.

Metagenomic analyses endow extensive information about the structure, composition, and predicted gene functions of varied environmental assemblages. Hence, 16 s metagenomic analysis based on the V3–V4 region has become a popular practice to unveil the effect of biotic and physicochemical factors on the overall microbial population of soil (Kakirde et al. 2010; Nesme et al. 2016).

There are some aspects of Metagenomics which are crucial to get the most accurate and relevant data from a sample. Extraction and purification of high-quality DNA is the major pre-requisite of any successful metagenomic study. The average insert size or the length of sequence reads for a high-throughput sequencing approach is also crucial. An appropriate metagenomics screening strategy should be adapted

I. Sarkar and P. Kar have contributed equally in this paper.

Communicated by Erko Stackebrandt.

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Published online: 19 December 2021

to address the specific question(s) of interest (Kakirde et al. 2010).

Despite all these obstacles, next-generation sequencing (NGS) based metagenomics provides us direct access to the uncultivated microbes. The high-throughput sequencing technology has equipped the field of microbiology with new phyla, class, genera, species as well as functional microbial genes (Nesme et al. 2016). The ability of metagenomics can answer numerous what, how who and why questions. For example, which type of soil hosts which kind of microbes? How the microbes interact with each other and also with the surrounding environment? Do they act synergistically or antagonistically? How the microbes act on changing environments and so on.

India is one of the major biodiversity hubs on this planet comprising of several different types of soils. This versatility ranges from snow soil to desert sand, from beach sands to most fertile riparian soil. The geographical location of this country has blessed it with a large area of agriculture friendly fertile soil with high yielding capacity. Paddy, wheat, green vegetables are common to grow in most of the Indian soils. However, one specific beverage crop that originally came to this country from outside and got very well adapted to the North-Eastern part of India is tea. Darjeeling tea has become world-famous for its brilliant aroma and color. Modern science has recognized the impact of the microbial population on the yielding capacity of the soil. Thus, it is now well known that soil along with its microbial communities can modulate not only the environment beneath the earth's crust but also above it including the crops that are grown in the soil along with other higher-order organisms and humans dependent on that soil in particular means.

In this study, we targeted two very popular tea gardens of Darjeeling hills—Makaibari (Mak) and Casselton (Cas). The main difference between them is the type of manure they use. Mak is solely an organic tea garden using all organic manure and fertilizers whereas Cas uses inorganic pesticides and fertilizers. Our main aim is to identify different sets of microbes that are present in these two tea garden soils using NGS-based metagenomic sequencing and to explore how the microbial population of both these tea gardens might be affected by the types of fertilizers being used. In this study, we deciphered the overall microbial population of both Makaibari (Mak) and Casselton (Cas) soil with special reference to their interaction among each other in terms of both complementation (synergy) or competition (antagonistic property). Along with that, we also tried to investigate whether the microbial population of two selected tea gardens may somehow affect the overall health quality of the tea garden workers or not.

Materials and methods

Field of study, sample collection, and soil testing

We have chosen two popular tea gardens from the Darjeeling hill region—Makaibari (26.8716° N, 88.2678° E) and Casselton (26.8659° N, 88.2777° E). The distance between these two tea gardens is only 12 min (4.0 km) via NH110 and they were on the same valley of the hill. Makaibari is solely an organic tea using organic fertilizers and manure whereas Casselton uses chemical pesticides and chemical fertilizers. Soil samples were randomly collected from the rhizosphere region of tea plants. Debris from the samples like roots, pebbles, etc. was removed by hand. Soil texture was assessed by the field method. The moisture percentage of soil samples was determined from the difference in weight of freshly collected and oven-dried soil samples. The clean air-dried samples were passed through a sieve and crushed with mortar and pestle. Soil pH, Electrical conductivity, and Loss of ignition were estimated following the protocol of (Baruah and Barthakur 1997). Other important parameters like organic carbon (Walkey and Black 1974), total soil Nitrogen (Jackson 1973) phosphorus as phosphate (Baruah and Barthakur 1997; Jackson 1973; Bray and Kurtz 1945) and potassium (Chapman and Pratt 1962), sulphur was determined during soil analysis. The level of micronutrients was qualitatively assessed by micronutrient kit. Information regarding the health of the tea garden workers were collected from a survey-based approach. The persons directly associated with the tea workers health of both organic and inorganic manure-based tea gardens were interviewed for collecting information regarding the present health scenario of those gardens.

DNA isolation

Soil DNA was isolated using rhizosphere soil of Makaibari and Casselton tea garden. Before initiating the isolation, 2 g of respective soil (three replicates per sample) was mixed with 4 ml of 1X Tris–EDTA buffer followed by proper vortexing (4–5 min) in 50 ml Oakridge tube. Before cell lysis, 150 µl of lysozyme (50 mg/ml), 100 µl of Proteinase K (20 mg/ml) and 600 µl of freshly prepared 10% SDS were mixed with the soil samples and the sample was incubated at 37 ºC for 90 min with gentle shaking every 15 min interval. After incubation, 1 ml 5 M sodium chloride, 1.6 ml CTAB/NaCl was mixed with respective solution and was further incubated at 65ºC for 30 min with occasional mixing in between to release the DNA from microbial cells. The supernatant containing microbial DNA was extracted with chloroform–isoamyl alcohol (24:1, v/v) and collected in a new tube after centrifugation at 6000 rpm for 15 min at

room temperature. The aqueous phase containing DNA was precipitated with 0.6 volumes of cold isopropanol and 0.1 volumes of 3 M sodium acetate followed by 2 h incubation at − 20 ºC. The DNA pellets were obtained by centrifugation at 10,000 rpm for 30 min at 4 ºC, washed with cold 70% ethanol, and dissolved in 100 µl of 1X Tris–EDTA buffer. To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/280 nm (DNA / protein) were determined and the DNA was sent for 16 s Metagenomics amplicon sequencing (V3–V4) to Genotypic Technology Pvt. Ltd.3.

PCR amplification of V3–V4 region of 16 s gene

About 40 ng of extracted DNA was used for amplification along with 10 pM of each primer (5' AGAGTTTGATG-MTGGCTCAG3' primer for forward sequence and 5' TTA CCGCGGCMGCSGGCAC3' primer for reversed sequence). The initial denaturation temperature was set as 95 °C. The denaturation was done for 15 s. Annealing was done at 60 °C for 15 s followed by elongation at 72 °C for 2 min. Final extension was done at 72 °C for 10 min. The final PCR product was stored at 4 °C. The amplified 16 s PCR Product was purified and subjected to GEL Check and Nanodrop QC. The Nano Drop readings of 260/280 at an approximate value of 1.8 to 2 were used to determine the DNA's quality.

Overall sequencing procedure

The amplicons from both samples were purified with Ampure beads to remove unused primers and an additional 8 cycles of PCR were performed using Illumina barcoded adapters to prepare the sequencing libraries. Libraries were purified using Ampure beads and quantitated using Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2 × 300PE v3 sequencing kit.

Processing of metagenomics data

Raw data QC was done using FASTQC and MULTIQC, followed by trimming of adapters and low-quality reads by TRIMGALORE. The trimmed reads were further taken for processing which includes merging of paired-end reads chimeria removal and OUT abundance calculation and estimation correction—this was achieved by Parallel-META pipeline. This workflow enabled highly accurate investigations at genus level. The databases used were SILVA (https://www.arb-silva.de/) / GREENGENES (https://greengenes.secondgenome.com/) and NCBI (https://www.ncbi.nlm.nih.gov/). Each read was classified based on % coverage and identity. A schematic diagram of the overall 16 s metagenomics process has been diagrammatically represented in Supplementary Fig. 1.

Reverse ecology analysis

Reverse ecology analysis is a simple yet effective way to study the interaction among microbes present in specific sample. This analysis considers both competition and complementation to assess the overall interaction among microbes.

The present metagenomic study identified microbes up-to genus level. We used a cut-off value of 200 sequence count i.e. if the count is less than 200 for a specific genus, we simply did not consider it. This was done purely to have a manageable amount of out-put data and to remove the possibilities of false-positives. We have used the same cut-off value for all further analysis in this study.

The whole genome sequences of the type strains from the identified genus (with count > 200) of both Mak and Cas were considered for reverse ecology analysis. Their KEGG Ontology (KO) information were retrieved from KAAS database. The KO information was fed into RevEcoR, an R-based package (Cao et al. 2016) to compute the competition and complementation indices among the studied strains.

Results

Physicochemical properties of Mak and Cas soil

Both the tea garden considered for this study had loam soil. Mak soil was light, friable loam with porous subsoil. This soil type is preferred for tea due to free percolation of water. The Cas soil was clay type. The low pH of both the soils indicated towards the acidic nature of the soil which is good for tea. Results of soil physicochemical analysis are shown in the table (Supplementary Table 1). The results were compared with soil physicochemical standards recommended by the Tea Board of India. The clayey soils of the tea plantations have low pH, sulphur but high organic carbon, organic matter, total nitrogen and P2O5. K2O was optimum in soil collected from Mak but high in soil from Cas. Micronutrients like boron, manganese, zinc, and copper were low while Molybdenum was moderate in both the plantations. Iron was optimum in Mak but low in Cas. The soil types of the tea gardens were not largely different. However, the difference arose in the fertilizers used by these two tea gardens. Mak is practicing with the organic manure filled with vermicompost, bio-fertilizers, and organic manure while Cas is totally dependent upon the inorganic manure and pesticides for maintaining sodium (Na):pottasium (K) ratio, weed control along with pest and disease management.

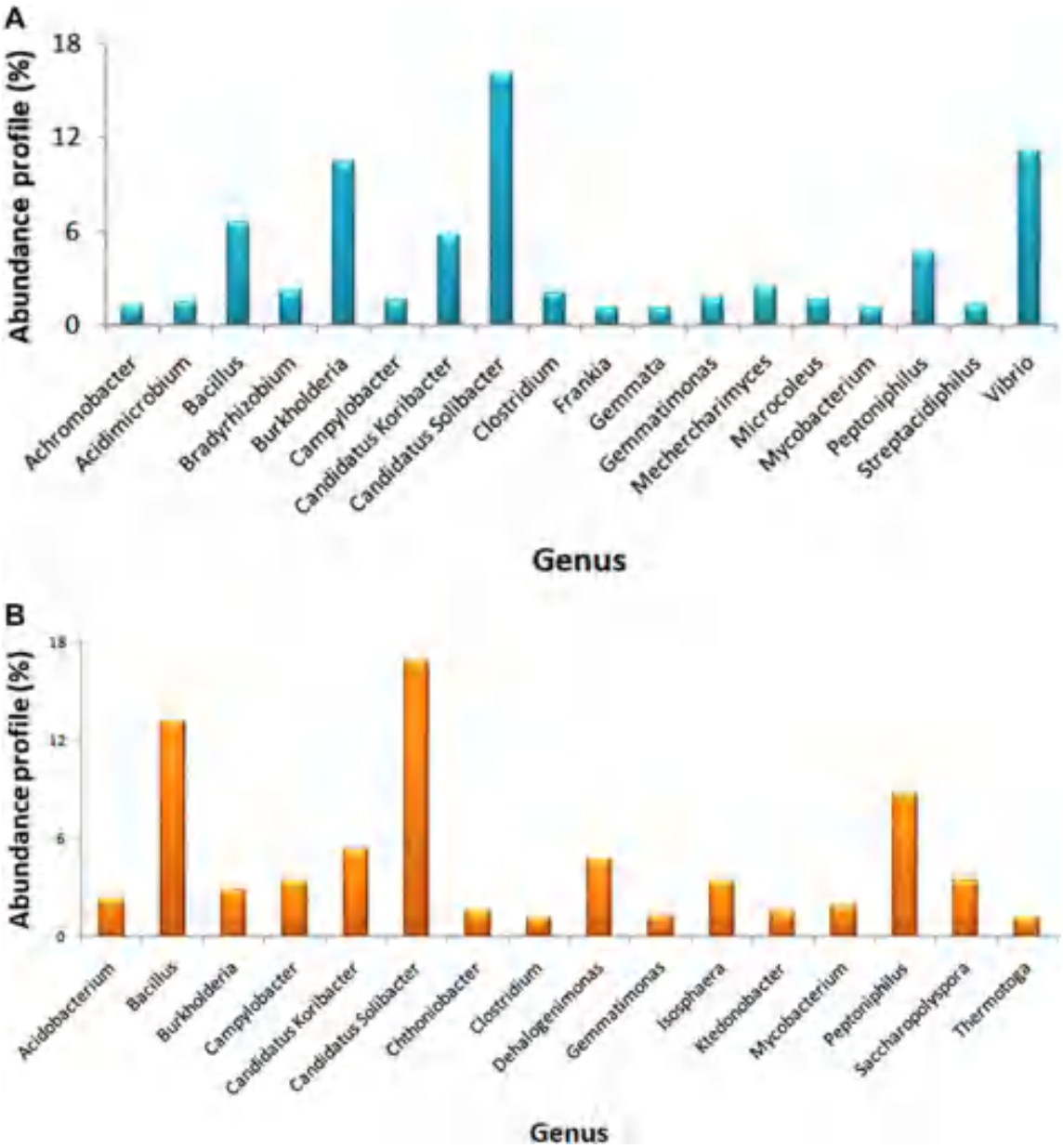

Fig. 1 (**a**) Microbial abundance profile of Mak. (**b**) Microbial abundance profile of Cas (color figure online)

Primary data summary

The paired-end reads from Mak and Cas soils gave 56% and 55% average GC, respectively. There were 0.2 M sequences for each read of Cas with 67.95% duplication value and 0.3 M sequences for each read of Mak with 72% duplication value. The read lengths were 256 bp and 205 bp for forward and reverse sequences of Cas samples. Mak forward and reverse sequence lengths were 258 bp and 189 bp, respectively. FastQC report revealed good quality reads indicating successful metagenomic sequencing. The 16 s metagenomics data for Mak and Cas has been submitted in NCBI SRA under the BioSample accession: SAMN21875714 with BioProject ID: PRJNA766783.

MAK is more populated than CAS with good bacteria leading to a stable ecotype model

The taxonomic abundance profiling identified the microbial abundance from phylum to genus level. It was found that both soils shared a large set of bacteria however, their relative abundance was not the same. For instance, *Cyanobacteria* and *Gemmatimonadetes* were present in Mak constituting 1.32% and 1.24% of the total microbial population,

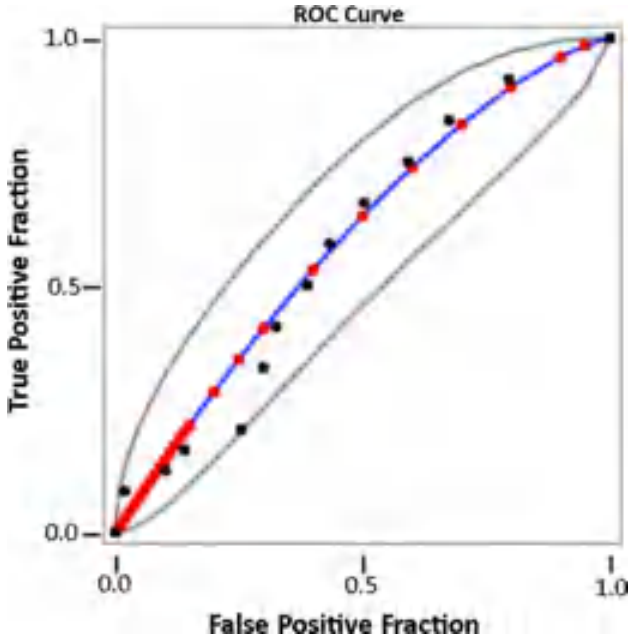

Fig. 2 The receiver operating characteristic (ROC) plot analysis among Mak and Cas (color figure online)

respectively, whereas they constituted less than 0.5% in Cas soil. The major microbial phyla identified in both soil samples were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Choloflexi*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes* (Fig. 1a, 1b). We did the one-way ANOVA test, however, found no significant difference among the overall microbial population of Mak and Cas. The f ratio value was found to be 0.00589 and p value was 0.939465 and it was not significant even at $p < 0.01$. The receiver operating characteristic (ROC) curve (http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html) was plotted based on the overall microbial diversity among Mak and Cas (Fig. 2). The analysis gave fitted AUC (area under ROC) as 0.59 and empiric AUC was 0.585 suggesting less discrimination between the overall microbial population of Mak and Cas.

However, a distinct pattern was observed between the two soil samples when only pathogenic microbes were considered. For instance, the relative abundance of the pathogenic microbial population was found to be more in Cas than Mak (Fig. 3). The abundance profile of pathogenic microbes like *Burkholderia, Campylobacter*, and *Bacillus* were much higher in Cas than in Mak. Moreover, the abundance of *Mycobacterium* was also more in Cas than Mak. Along with those mentioned genera, the presence of *Candidatus Solibacter, Candidatus Koribacter, Peptoniphilus, Peptoniphilus, Clostridium, Gemmatimonas* were found in both Cas and Mak. Their abundance was not very high in any of the soil samples. *Bradyrhizobium, Microcoleus, Acidimicrobium, Streptacidiphilus, Achromobacter, Gemmata and Frankia* were solely present in Mak but not in Cas however, *Dehalogenimonas, Saccharopolyspora, Isosphaera, Acidobacterium, Chthoniobacter, Ktedonobacter, Thermotoga* were solely present in Cas but not in Mak. This indicated the differential bacterial population among Cas and Mak. When the one-way ANOVA test was performed the f ratio came to be 7.75 with p value 0.010285 and the result was significant at $p < 0.05$ (at $p < 0.01$ the difference was non-significant). A PCA plot based on the pathogenic microbial population also supported the ANOVA results where Mak and Cas were placed in two different quadrants of the PCA plot (Fig. 4).

To find the species diversity between the two soil samples α diversity of both samples were exploited. Alpha (α) diversity is a direct measure of mean species diversity of habitat and a higher α diversity value indicates more diversity. The α-diversity value of Cas was 48.69 and for Mak it was 56.62 pointing to more species richness in Mak. Rare-fraction curve that allows us to calculate the species richness from a given number of individual samples was further implemented to support our aforementioned hypothesis. A common pattern of this curve is, it grows rapidly at first due to the most common species present in the samples and gradually becomes a plateau as the rarest species remain to be sampled. In Cas, the curve started to get a plateau state at species count 1400 (Fig. 5a) wherein Mak the stage came at species count 3000 (Fig. 5b). Hence, it is evident from taxonomical abundance profiling, α diversity and rare-fraction curve analysis that, Mak is more ecologically diverse with a higher microbial population rather than Cas.

Reverse ecology analysis was implemented to get a birds-eye view on the complex microbial interaction and signaling network going on within Mak and Cas soil samples. The reverse ecology analysis revealed that, the complementation index for Mak microbial population varied from 0.79–0.97 and that of Cas was 0.68–0.85. The competition index for both the samples were considerably low (0.21–0.39 for Mak and 0.32–0.41 for Cas). The differences between complementation and competition in both Mak and Cas were statistically significant (t test at $p < 0.001$). Moreover, the complementation among Mak population was more than Cas population ($p < 0.001$). This also supports that, the Mak microbial population has formed a more stable ecotype model than Cas which indicates a better natural selection forming and maintaining specific genetic clusters.

Discussion

Microbial population of Mak formed a stable ecotype model

We have found a clear picture about the microbial diversity of both studied tea gardens. Here are the major findings we

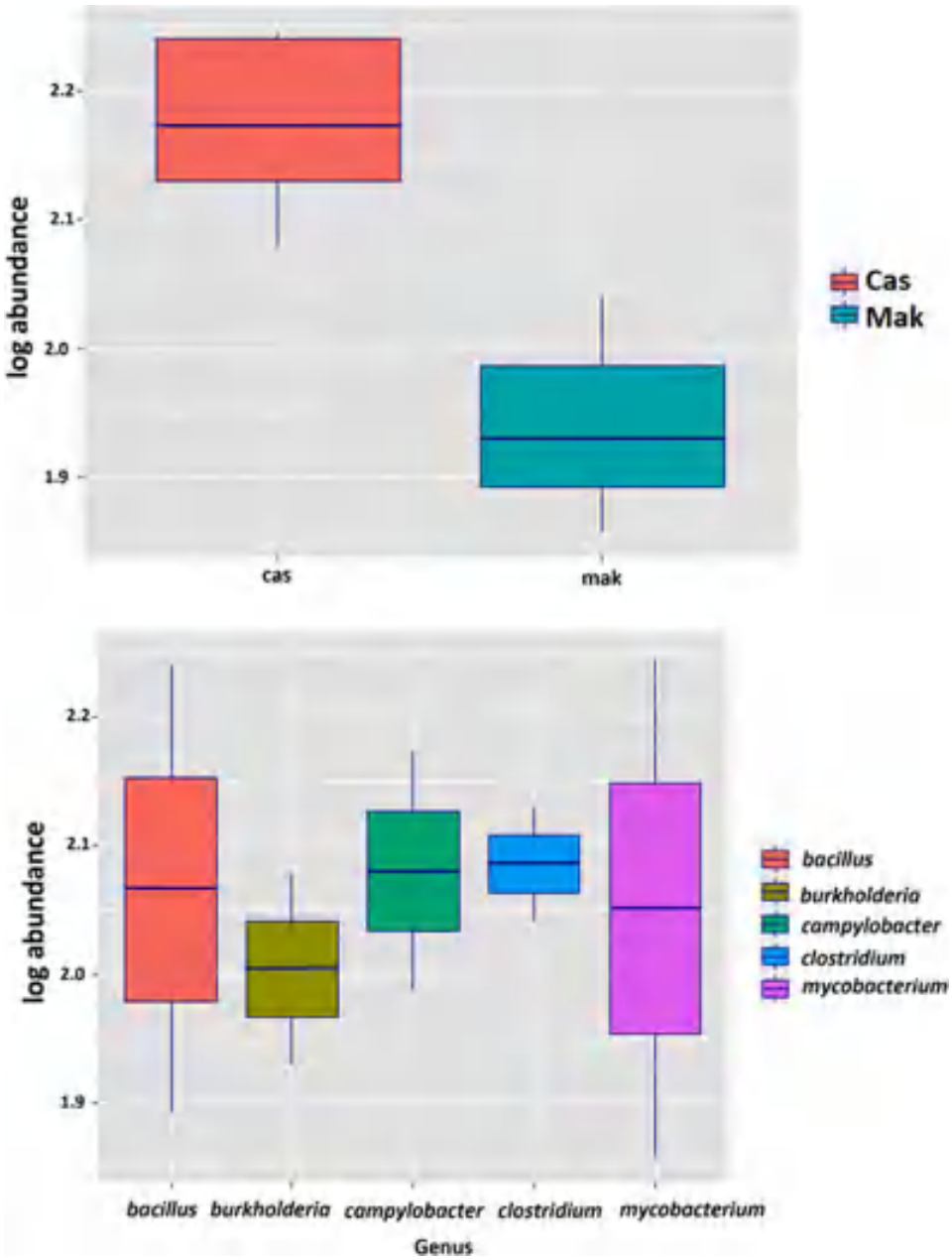

Fig. 3 Comparative pathogenic microbial abundance profile of Mak vs Cas (color figure online)

got (a) the overall soil physicochemical properties were alike as they belong to the same eco-geographical region and altitudinal level. (b) The overall bacterial diversity was more in Mak than Cas. (c) Moreover, Cas population contained more pathogenic genus than Mak. This clearly indicated a positive effect of organic manure in comparison to inorganic/chemical fertilisers. (d) The complementation values (obtained from reverse ecology analysis) among the Mak population was higher than Cas population. This may indicate a stable ecotype model (SEM) (Shapiro and Polz 2015) persisting in Mak where the main carbon source of soil is organic manure. It is a well-known fact that fertilizers have a direct impact on soil microbial population playing a pivotal role in both biogeochemical cycling and ecological processes (Li et al. 2017). Certain microbial taxa display ecological coherence in response to environmental variables. Based on substrate preference and life strategies, those microbes can be grouped into r-selected or k-selected categories. However, it is difficult to gain such knowledge at a lower taxonomic level (genus or species level).

Use of organic manure increased the microbial diversity of Mak

It has been documented previously that, continuous exposure of fertilization (both organic and inorganic) leads to the

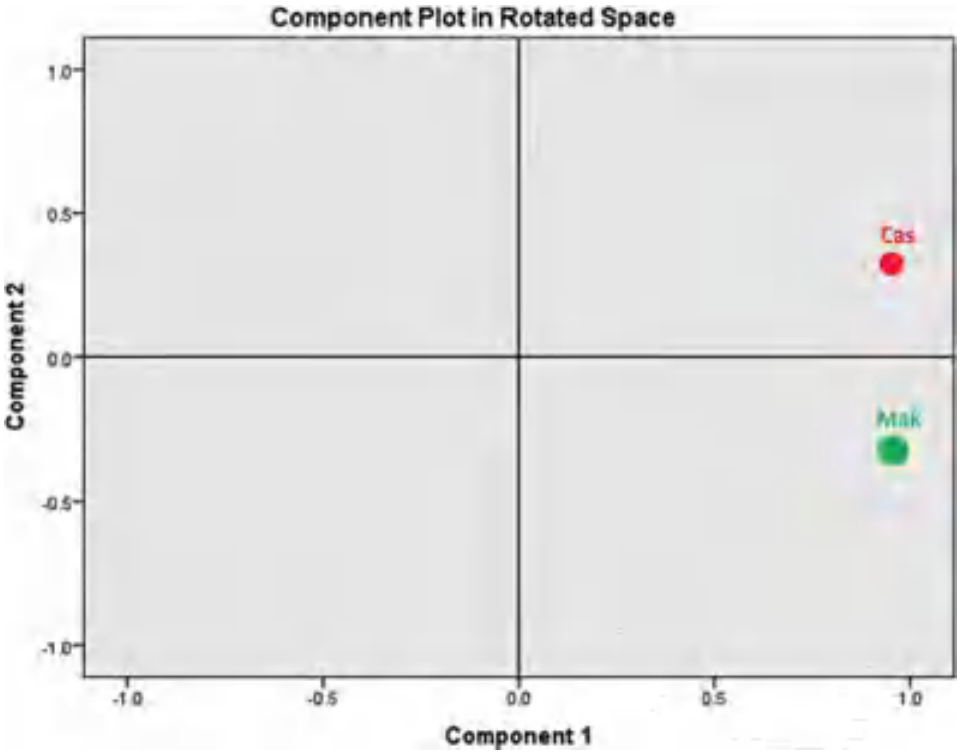

Fig. 4 PCA plot analysis of pathogenic microbial abundance profile of Mak vs Cas (color figure online)

addition of a specific category of carbon (C) and nitrogen (N) source to the soil. Over a period of time, a set of bacteria, capable to handle those specific C and N sources will proliferate in that agricultural field. This practice in the long run is good for providing agroecosystem stability. Organic manures are composed of different decomposing materials hence contain diverse C and N sources. On the contrary, chemical fertilizers are always well defined with their source of C and N (Li et al. 2017). As a result, it may well be predicted that a field exposed to long-term organic manure will house a more versatile microbial population utilizing various kinds of nutrient sources than a field exposed to defined inorganic manure. Makaibari (Mak) tea garden is popular for using organic manure since its inception whereas, Castleton (Cas) uses inorganic fertilizers. These differential practices are thus, playing a major role in the microbial population between these two tea gardens.

Microbial populations of tea garden soil bear a relation with tea garden worker health

In the tea plantation sector, safety and security issues of workers are overlooked widely (Roy Chowdhury et al. 2014). Most of the workers are ignorant about the consequences of the exposure to chemicals, environmental factors, etc. Extensive use of chemical fertilizers and pesticides results in the degradation of soil and water bodies. Agricultural chemical inputs gain access into human body systems through three major means: (1) oral ingestion, (ii) infiltration through the skin, and (iii) breathing (Roy Chowdhury et al. 2014; Rodríguez-Eugenio et al. 2018; Rajput et al. 2021; Bottone 2010; Ahmmed and Hossain 2016; http://www.tezu.ernet.in › project reports). Previous studies on tea garden workers showed the prevalence of neurological, gastrointestinal, renal and hepatic toxicity (Inglis and Sagripanti 2006; Frost 2001; Picard et al. 2005; Bae et al. 2002; Chenoll et al. 2015) among them. Most of the tea garden workers are prone to respiratory ailments such as tuberculosis and skin disorders (Gayathri and Arjunan 2019). Since the vast majority of workers in the tea plantation are women, concerns have centered on the potential reproductive hazards of chemical exposure and their impact on pregnant women, nursing mothers within their lactation period, and their children (Rajbangshi and Nambiar 2020). The difference in the soil micro-flora of Mak in comparison with Cas from our study indicated the role of fertilizers and chemicals in the development of soil microrganisms. The abundance of beneficial flora in Mak may provide a positive effect on the health aspect of tea garden workers further directed towards the advantages of organic manure over chemical fertilizers.

Conclusion

Human–microbe affiliation establishes even when the person is there in the mother's womb and the connection between human and soil microbes launches before it starts walking on the ground. It is believed that soil microbes contribute

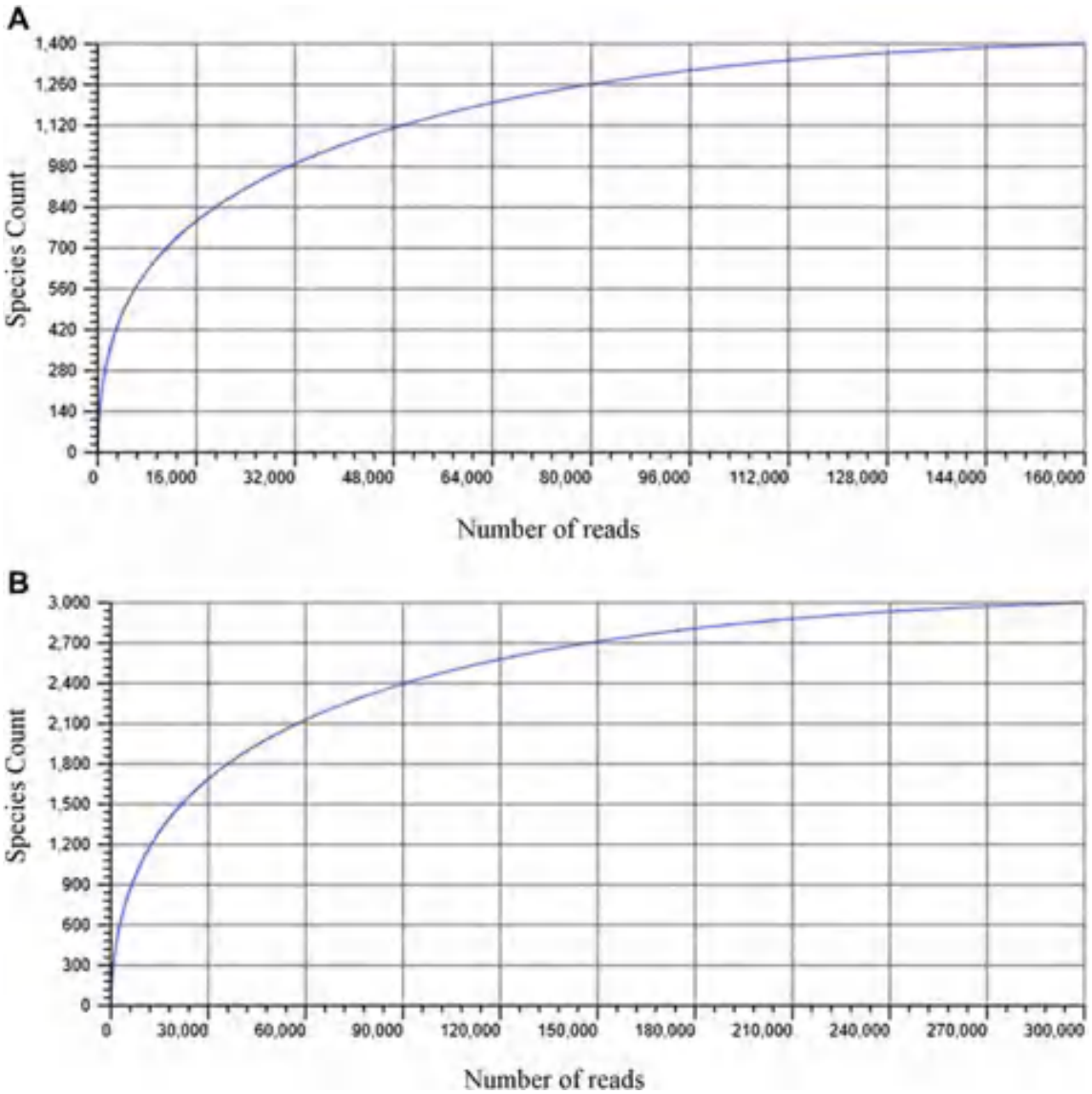

Fig. 5 Rare-fraction curve of (**a**) Mak and (**b**) Cas soil samples (color figure online)

considerably in developing the gut micro-flora and shape the overall human health. Soil dwellers, mainly soil micro-flora, play a paramount role in maintaining the biodiversity of a micro-habitat like tea gardens, paddy fields etc. The cultivable soil microbes are relatively easy to study while a large portion of culture-independent microbiomes remain largely illusory. In this consequence, soil metagenomics has become an important tool in studying the non-cultivable microorganisms present in a specific niche. In this present study, we did 16 s metagenomics of Makaibari (Mak) and Castleton (Cas) tea gardens from the Darjeeling region of India. The main difference between these two gardens is, Mak is an organic manure-based tea garden whereas Cas uses chemical fertilizers. Metagenomics revealed higher bacterial diversity in Mak than Cas. The pathogenic bacterial population was more in Cas than Mak indicating the positive feedback effect of organic manure on the overall bacterial population of soil. We investigated interactions among the identified genus from both Mak and Cas. A stable ecotype model was evident in Mak where microbes were showing synergistic effect (complementation) whereas in Cas soil, competition was more among the bacterial population revealing volatility of the ecosystem. Finally, the number of human pathogens was more in Cas than Mak which supported the better tea garden worker health report in Mak over Cas. Literature survey, as well as our own survey also supports this fact. Thus, this study indicates that organic fertilizers have a positive effect on the soil microbial population in particular and human health in general in that region.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02635-6.

Acknowledgements We acknowledge Makaibari and Castleton tea gardens for providing us samples and other supports. Especial acknowledgement to Dr. P.K. Dutta, North Bengal Medica Nursing Home, Siliguri for sharing his knowledge about the health aspect of tea garden workers.

Author contributions AS conceived the idea and did the experimental design. PK, GS, SC, MB collected and prepared samples for metagenomics. MB, PK and SB did the soil analysis related work. AS, GS and IS did the bioinformatics analysis. Figures and art works are mostly done by IS. All the authors contributed in manuscript writing and approved.

Declarations

Conflict of interest The authors declare that the research paper was written in the absence of any commercial or financial relationships that could be construed as real or potential conflict of interest.

Research involving human and animal participants No animal or human were treated as sample in this study.

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Methods for whole-genome analysis of Actinobacteria through Bioinformatics approaches

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Abstract

Actinobacteria is one of the most diverse groups of bacteria inhabiting virtually all different niches. Actinobacteria can be found in plants, animals, soil, water and extremophile situations like desert soil, arctic soil and thermophilic condition. Next-generation Illumina sequencing provided us the whole genome sequences of microorganisms including Actinobacteria. Genomics study on Actinobacteria has availed us with vast knowledge regarding the interaction of this group with its respective niche. In this chapter, we will discuss several important and interesting Bioinformatics techniques vastly used for microbial research.

Key Words: Actinobacteria, Codon Usage, Bioinformatics, Sequencing, Phylogeny

Introduction

Background of Actinobacteria

Actinobacteria which was previously known as ‘actinomycetes’ or ray fungi form an important constituent of the microbial biome. It comes next to proteobacteria in terms of number and distribution. Actinobacteria are mostly aerobic, gram-positive to gram variable with high G+C content and occupya diverse microbial niche. They share some characteristics with fungi, such as colony morphology, mycelial growth and musty smell, on the other hand, peptidoglycan cell wall structure is common with bacteria. Phylogenetic studies based on 16S rRNA classifies actinobacteria into six classes i.e. Acidimicrobia, Corniobacteria, Nitriliruptoria, Rubrobacteria, Thermoleophilia and Actinobacteria (1).

Actinobacteria are widely distributed in various biotopes such as soil, water, permafrost, mammals, arthropods, plants etc. (1). Furthermore, various lifestyles are encountered among *Actinobacteria*, and the phylum includes pathogens (e. g. *Mycobacterium* sp. *Nocardia*sp. *Tropherym*asp. *Corynebacterium*sp. and *Propionibacterium* sp.), soil inhabitants (*Streptomyces* sp.), plant commensals (*Leifsonia*sp.), nitrogen-fixing symbionts (*Frankia*), and gastrointestinal tract (GIT) inhabitants (*Bifidobacterium*) (2).

Actinomycetes identification in conventional systems is difficult, but it can be done by performing biochemical tests (3). It can also be done by advanced methods such as utilizing software 'Actinobase' for genus-level identification, using image files. Phylogenetic relationship determination is made easy by 16S rRNA studies, up to species level recognition using Blast search.

Biological research has undergone tremendous progress after the advancement of genome sequencing. Genomic database mining provides us with an opportunity in studying the genome profiles of different organisms and also helps in studying comparative genomics. The ongoing proliferation of whole-genome sequences is a stepping stone for systems biology (4), which aims to study the integrated network constituted by the complete repertoire of genes (genome), the population of transcripts (transcriptome),the population of proteins (proteome), the population of metabolites (metabolome),and fluxes of an organism or cell, concerning intrinsic and environmental stimuli (5). Bioinformatic tools become relevant in this aspect. The language of DNA which is the four-letter alphabet that is expressed as triplet codon for an amino acid is the key for fundamental gene expression. The degeneracy of codons for 18 amino acids except methionine and tryptophan showed that different sequences of DNA produce identical protein sequences. The degeneracy mainly occurred in the third position of the codon. Data from whole-genome sequences help to study the preference of codons among organisms.It has been noticed that variation of choice of codons to represent amino acids is not only observed among species from the different taxonomic groups but also showed significant variation among individuals of the same species, across different genes in the same genome and even across regions in the same gene. But, the codon bias is most prominent in species from different taxonomic groups even in proteins with identical functions. This phenomenon of species-specific codon choice is known as "codon dialect," which signifies the codon-usage bias observed across different organisms (6).

Bioinformatics in Genomic Research

Through the development of algorithms and statistical testing, research can be carried out faster and more accurately. Bioinformatics is used in different fields of research; however, it is especially important in genomics, such as in genome analysis, gene identification, genome-wide association studies and evolutionary studies. Traditional and next-generation processes aim to sequence the genome allowing the

analysis of DNA sequences. However, these methods produce many fragments of DNA, like fragments of a jigsaw puzzle, which need to be aligned and compiled to create a final complete sequence. The use of bioinformatics can align these fragments quickly and cheaply, aiding genomic sequencing.The human genome was initially sequenced between 1990 and 2003 and has since been uploaded online and extensively annotated. Annotation is the process whereby genes and their protein products are labeled directly onto the genome. The volume and complexity of the produced data would have taken many years to compile manually. However, with the advent of bioinformatics, scientists can carry out the compilation and annotation processes quickly and with better precision.

Bioinformatics and identification of mutations

Bioinformatics is vital in the research of*de novo*mutations. One example of a method that is used to identify these mutations is whole-exome sequencing. Whole exome sequencing is used to sequence only the protein-coding regions of DNA (the exomes), which makes up only 1% of the genome, thereby making it much faster than genome sequencing. However, large quantities of data are produced whereby bioinformatics application becomes vital for data curation, sequence alignment, and analysis. Using whole-exome sequencing and bioinformatics, 50% of rare disease genes have so far been identified, with the rest is expected to be sequenced by 2020. Another use of bioinformatics is in the identification of cancerous mutations. Through the development of automated systems, large volumes of sequential data can be produced and used to identify previously unknown point mutations. Bioinformatics also works to create new algorithms that can compare different sequences, thereby aiding in the identification of mutations.

Bioinformatics and genome-wide association studies

Genome-wide association studies (GWAS) carry out genomic scans inan attempt to identify specific markers that can indicate an individual's susceptibility to a genetic disease. Genetic association between a specific marker and the disease can improve detection and treatment. If used on a large scale, this can also aid in the development of prophylactic treatments. To carry out GWAS, the genomes of individuals with a disease and those without a disease are compared. The development of highly automated systems has led to the high-throughput identification of single nucleotide polymorphisms (SNPs).By comparing SNPs, those which are more common in individuals with the disease can be identified and used as disease markers. This information is then stored online and made available to scientists across the globe. The first published GWAS was age-related macular degeneration (AMD). Out of 116,204 SNPs that were genotyped, one study observed a link between the complement factor "H" (CFH) gene and AMD. Therefore, individuals susceptible to AMD can be screened for the presence of the CFH gene.Several

other disease genes have been characterized after that to help doctors and other health care professionals in identifying the possible risk of a genetic disease and allowing for appropriate disease management.

Bioinformatics and evolutionary studies

By studying the changes in DNA within organisms and comparing them to other species, the genetic changes associated with evolution can be classified. Evolution is the process that involves small, cumulative changes in DNA that eventually leads to the formation of novel species.Bioinformatics has aided research in the evolutionary process by allowing comparison of DNA sequences, sharing of data, prediction of future evolution and classification of complex evolutionary processes.When put together, the data can be used to create a phylogenetic tree that can trace several species to their original ancestry. These are only a few of the myriad applications of bioinformatics within genetics. Overall, bioinformatics has thrown open enormous opportunities in the field of genomics and targeted gene therapy.

Materials

1. Fasta Nucleic acid and Fasta amino acid sequences can be downloaded from IMG database or NCBI.

2. Sequences for 16s, 23s, Multi –Locus sequences (AtpA, DnaB, GyrA, FtsZ, SecA) sequences in fasta format is required for phulogeny analysis

3. Software mentioned here: CodonW, DAMBE, ClustalW, MEGA, Perl.

4. Statistical anysis can be done through SPSS.

Methods

Codon Usage

The codon usage pattern is a unique feature of a particular organism. It helps us to understand gene expression, horizontal gene transfer and also enablesus to determine phylogenetic relationships between organisms. The study of codon usage patterns of several genes and genomes is a popular technique to characterize and analyze genomic trends from a bioinformatics-based perspective. Codon usage patterns and preferences vary significantly within and between organisms (1,4).

CODON W software developed by Pedan,1999 became very popular and widely used for studying codon usage and multivariate analysis because of its error-free analysis. The parameters such as GC content

(amount of guanine-cytosine in the nucleotide sequences),GC3 content(frequency of either G or C nucleotides in the third position of synonymous codon), the effective number of codons used in a gene(Nc), frequency of optimal codons(Fop),CBI (codon bias index),GRAVY(hydrophobicity of amino acids) are included in this analysis.The most obvious factor that determines codon usage is a mutational bias that shapes genome GC composition. Mutational bias is responsible not only for the intergenetic difference in codon usage but also for codon usage bias within the same genome.Most of the organisms with a balanced AT/GC genome have codon heterogeneity(Sen et al.,2007).Highly expressed genes contain a higher percentage of translationally optimal codons (7). Codon heterogeneity in the genome can be studied by GC content, GC 3 content, effective number of codons (Nc). Nc measures the overall codon bias of synonymous codons (8). It ranges from 20(in the case of one codon for one amino acid) to 61(where all codons are used).

Whole-genome sequences of actinobacteria can be obtained from the IMG database (www.img.jgi.doe.gov) using their respective genome accession numbers. Nucleotide sequences are downloaded for the codon analysis.For codon analysis, the CODONW version1.4.2 and the selected genome is put together in a folder. Before running the program, file extension 'fna' has to be changed into 'dat' format. Several parameters such as GC content, GC3 content, Nc, CBI, Fop, GRAVY, RSCU, and aromaticity are obtained as output. Data can be saved in excel format for further analysis.The GC content estimates the amount of the guanine-cytosine in the nucleotide sequences. The GC3 infers the frequency of either G orC nucleotides present in the third position of the synonymous codon. This does not apply to methionine, tryptophan and the termination codon. These values have a direct correlation with Nc. It measures the synonymous codon usage of genes and its value ranges from 20-62(9).

$$\mathrm{Nc} = 2 + \mathrm{S} + \left(\frac{29}{\mathrm{S}^2 + [1 - \mathrm{S}^2]}\right)$$

The frequency of optimal codons*(Fop)* is the fraction of synonymous codons that are optimally used. It is given by (*Fop) =Noc/ Nsc* where N represents the frequency of each codon, *Noc* and *Nsc* represent optimal codons and synonymous codons respectively. The *Fop* values range from 0 to 1. If the value of *Fop* is 1, it shows the usage of all optimal codons.

GRAVY scores determine the hydropathic indices of amino acids (Peden, 1999). A positive score indicates the hydrophobic nature and a negative score shows the hydrophilic nature of aminoacids.

RSCU-Relative synonymous codon usage

The Relative Synonymous Codon Usage (RSCU) values for the genes are calculated to understand the characteristics of synonymous codon usage without the confounding influence of the amino acid composition of different gene samples (9). The codons with RSCU values >1.0 have positive codon usage bias (abundant codons), while those with RSCU values <1.0 have negative codon usage bias (less-abundant codons); and when the RSCU values are 1.0, it means that these codons are chosen equally or randomly, indicates lack of bias (9). The RSCU is the observed frequency of a codon divided by the frequency expected if all synonymous codons for that amino acidare used equally. The synonymous codons with RSCU more than 1.6 are thought to be over-represented (Fig 1), while the synonymous codons with RSCU less than 0.6 are regarded as under-represented (9). The RSCU values are particularly useful in comparing codon usage between genes that differ in size and amino acid composition.

AA	Codon	N	RSCU	AA	Codon	N	RSCU
Phe	UUU	1478	0.15	**Ser**	UCU	1478	0.26
	UUC	18,420	1.85		**UCC**	9110	1.59
Leu	UUA	439	0.05		UCA	2445	0.43
	UUG	6377	0.76		**UCG**	11,497	2.01
	CUU	6380	0.52		AGU	1944	0.34
	CUC	19,480	2.31		**AGC**	7792	1.36
	CUA	897	0.11	**Cys**	UGU	1176	0.36
	CUG	19,096	2.26		**UGC**	5387	1.64
Tyr	UAU	5356	0.70	**Pro**	CCU	2239	0.33
	UAC	10,055	1.30		CCC	6481	0.95
His	CAU	7382	0.99		CCA	3221	0.47
	CAC	7523	1.01		**CCG**	15,453	2.26
Gln	CAA	3912	0.37	**Arg**	**CGU**	7844	1.24
	CAG	17,336	1.63		**CGC**	18,196	2.87
Ile	AUU	7265	0.71		CGA	2919	0.46
	AUC	21,263	2.07		CGG	5063	0.80
	AUA	2230	0.22		AGA	1534	0.24
Asn	AAU	6393	0.64		AGG	2415	0.38
	AAC	13,665	1.36	**Thr**	ACU	2018	0.24
Lys	AAA	4807	0.47		**ACC**	15,580	1.84
	AAG	15,864	1.53		ACA	3548	0.42
Val	GUU	3108	0.27		**ACG**	12,781	1.51
	GUC	13,246	1.17	**Ala**	GCU	3800	0.25
	GUA	2313	0.20		**GCC**	26,109	1.71
	GUG	26,589	2.35		GCA	10,012	0.65
Asp	GAU	13,045	0.71		**GCG**	21,326	1.39
	GAC	23,821	1.29	**Gly**	GGU	7659	0.68
Glu	GAA	12,723	0.73		**GGC**	26,407	2.35
	GAG	22,073	1.27		GGA	5299	0.47
					GGG	5499	0.49

Fig 1: A representation of RSCU table

The CODON W software is used to calculate the correspondence analysis of codon count and amino acid frequencies. The file containing gene sequences is loaded in CODON W. For calculating the correspondence analysis option 5 is selected. Run the program and the output 'genes.coa' is selected for downstream processing.

$$\text{RSCU} = \frac{\text{Frequency of codon}}{\text{Expected frequency of codon (if codon usage is uniform)}}$$

CAI-Codon Adaptation Index

Codon adaptation index is a widely used index for studying gene expression in general and efficiency of translation in particular.CAI has been used extensively in biological research. It has been used to study functional conservation of gene expression across different microbial species (10), to predict protein production (11, 12), and to optimize DNA vaccines (13). CAI has recently been used for detecting lateral gene transfer (14).

The cai program in EMBOSS (15), typically referred to as the EMBOSS.cai program is most popularly used. Software for computing CAI is a web application called CAI Calculator 2 (16). The improved CAI is implemented as a new function in DAMBE (17, freely available at http://dambe.bio.uottawa.ca/dambe.asp), which uses a windowed user interface. DAMBE can read 20 standard sequence file-formats including files in the simple FASTA format and the more involved GenBank format or trace files from automatic sequencers. The CAI function can be accessed by clicking 'Seq. Analysis|Codonusage|CAI'. The ensuing dialog box is self-explanatory, except that, for species without a reference set of highly expressed genes, a codon table based on tRNA anticodon can be used by clicking the alternative option button. CAI values vary from 0 to 1 and higher CAI values indicate that the gene of interest has a codon usage pattern more similar to the highly expressed genes.

Codon usage bias (CUB) is usually defined as a species-specific deviation from uniform codon usage in the coding regions of genomic sequences. This bias is possible due to the redundancy of the genetic code, which allows differential use of synonymous codons.The particular pattern of bias observed in a given species is thought to be the product of drift and selection pressures acting on several parameters, but mainly on tRNA gene copy number and genomic %GC content. CUB is therefore a strong species-specific statistic with numerous applications, such as gene prediction or the identification of laterally transferred genes.

Statistical analysis

All the statistical analysis can be carried out using IBM SPSS Statistics 21 software.The Pearson and the Spearman rank correlation coefficients and their corresponding *P*-values can be computed integrally using Microsoft Excel. The Pearson correlations may be computed using built-in Excel functions. The Spearman rank correlations can be computed using the statistical function set of WimGielis. Throughout the manuscript, the Pearson correlation coefficients are denoted by *r* and the Spearman rank correlation coefficients by ρ. The asterisk rating system is used for correlation *P*-values [single asterisk (*), $P < 0.05$–0.01; double asterisks (**), $P < 0.01$–0.001; triple asterisks (***), $P < 0.001$]. *P*-values are relative to a two-tailed Student's *t*-test on the null hypothesis (no correlation). For multiple correlations, we report the mean and standard deviation of the correlation coefficient and the largest *P*-value among the correlations.

Protein energetic cost

It can be defined as the energy consumed for the synthesis of an amino acid encoded by a specific functional codon.Mostly, the energy cost of potentially highly expressed genes is lower than the energy budget of the rest of the proteome. But it cannot be applied to all organisms. In the case of actinobacteria, it has been shown that energy cost varies with its niche (18)

CAI is a major index to measure the m RNA expression level.Generally,genes with10% of the highest and lowest CAI value are chosen as potentially highly expressed and potentially lowly expressed genes respectively. The remaining genes are considered as potentially medially expressed genes.DAMBE software calculates the EC(Dambe ver.6.4.81).The EC values can be analyzed statistically using ANOVA test,F-test and t-test. Heat maps are generated using R statistical software.

tRNA Adaptation index

Estimation of t RNA usage by the coding sequences of a genome is termed as t RNA Adaptation index (tAI). It measures the availability of t RNAsfor every codon of a coding sequence and estimates the level of adaptation between a coding sequence and estimates the level of adaptation between a coding sequenceand the corresponding tRNA pool of the cell. tAI is estimated using the codonR scripts downloaded from http://people.cryst.bbk.ac.uk/*fdosr01/tAI/. it uses the formula

$$tAI_g = \left(\prod_{k=1}^{l_g} w_{ikg}\right)$$

l_g=the length the gene in codons; w_{ikg}= relative adaptability of the codon defined by the k-th triplet in the gene.

Blast Matrix

Blast matrix (BM) is the visual representation of the pair-wise alignment of sequences using the BLAST algorithm with 50/50 rule (5) (Fig 2).The genomic and proteomic sequences of the genomes are downloaded from IMG database. Coding sequences with 300 nucleotides or more and with proper initiation and termination codons are taken for this analysis. If 50% of the alignment is identical with the

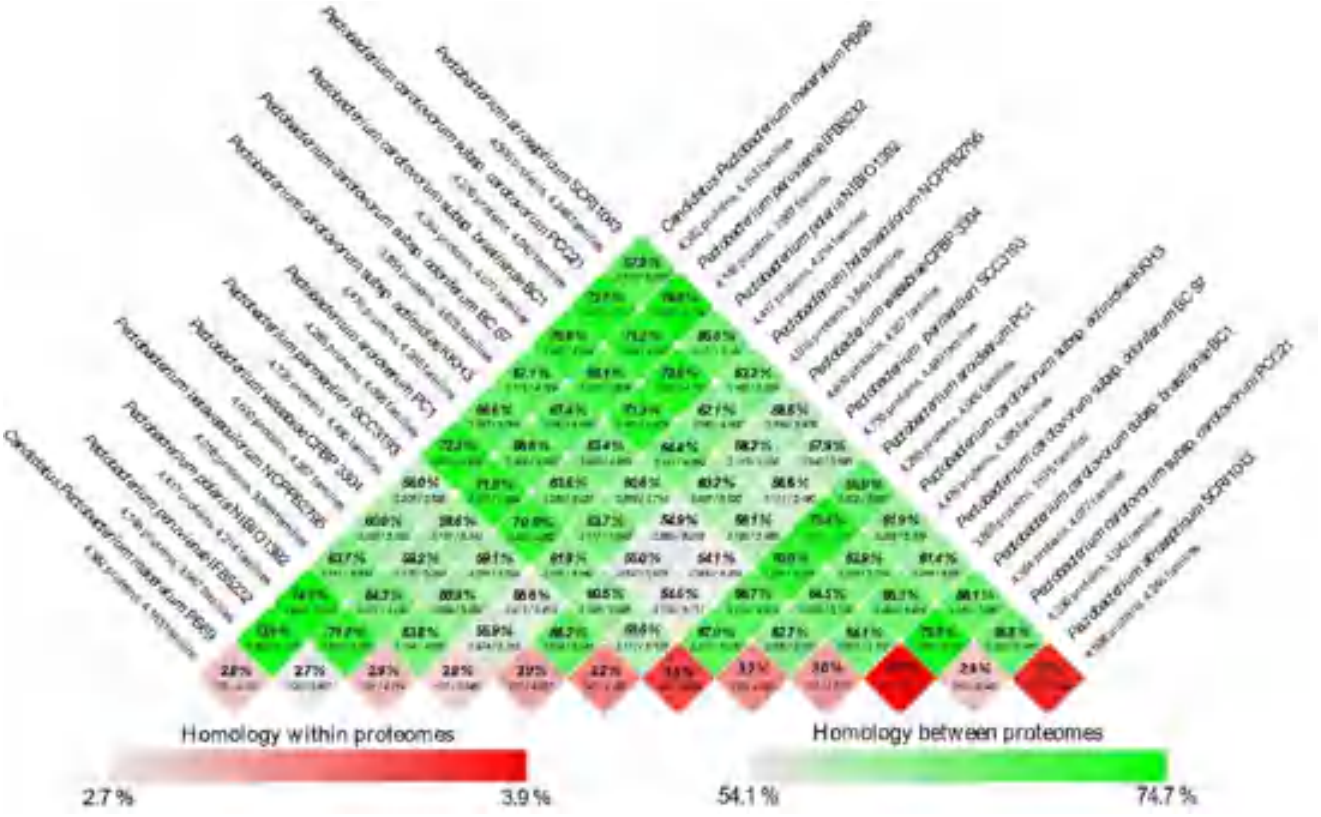

Fig 2: A representation of Blast Matrix

longest protein in the comparative study the BLAST hit is significant. The two sequences are assigned to a “protein family” if they share a similar cut-off value. The amount of shared proteins between the two proteomes is indicated as shaded green in the blast result. In BM, the homolog proteins within the proteome are shown in shaded redcolor at the bottom of the matrix. The color scales are automatically set from highest to lowest value observed. Two programs namely “matrix_createconfig”and “matrix” implemented in CMG Biotools software are used for creating BM (5).

Pan-Core genome plot

Pan-genome of a bacterial species refers to the gene families of all concerned strains of interest, representing a particular species (5) (Fig. 3). The core genome is defined as the conserved pool of genes shared between all strains of a particular species. The homology of genes within and across the genomes is identified based on sequence similarity. The genes are translated into amino acid sequences and aligned all against all using BLASTP. The two genes are considered as gene pair if the alignment follows '50/50' rule,i.e.; if the amino acid sequences are more than 50%identical over more than the length of their length.The common genes of all gene pairs are compiled into a gene family of a genome.

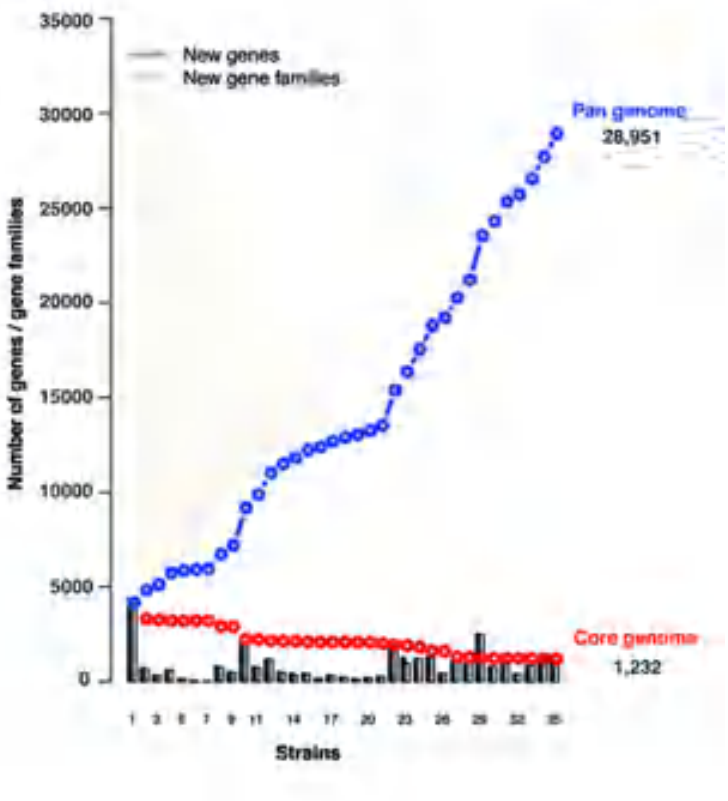

Fig 3: A representation of pan core plot analysis

Two programs namely, "pancoreplot_createConfig"and "pancoreplot" present in CMG Biotool software is adopted for creating the pan-core genome plot and identifying the core genomes among the strains under study.

Metagenomics for the uncultivable microbes

Uncultured microorganisms are mostly responsible for natural biodiversity on Earth. Typically, more than 99% of microorganisms from natural ecosystems are uncultured under laboratory conditions. Therefore,

there is a demand for “culture-independent” approaches for identification and characterization of such microorganisms to uncover their ecological roles in the biosphere. Metagenomics is culture-independent, sequencing-based and/or function-based analysis of the collective genome of a microbial community, enabling us for collection of essential information about community structure and genetic and metabolic potential of the members. This provides insights into the biology of these microorganisms. Another culture-independent method is single-cell genomics, which obtains information about microbial population based on the isolation and genome sequencing of a single cell.

Phylogeny and evolutionary analysis

Phylogenetic tree can be generated based on 16S, 23S, concatenated housekeeping genes and whole genomes(1)(Fig. 4). Most used algorithm in the field of Bioinformatics is Neighbor-joining phylogenetic tree (based on the Nucleotide: Maximum Composite Likelihood method) generated using Mega6.0 software (Tamura et al. 2013). Recently we have developed another method for generating phylogenetic tree and that is binary tree based on the present and absent of phylogeny. The presence and absence of a domain can be designated by 1 and 0 respectively. NTsys software may be used to build the phylogenetic tree (Sarkar et al. 2019).

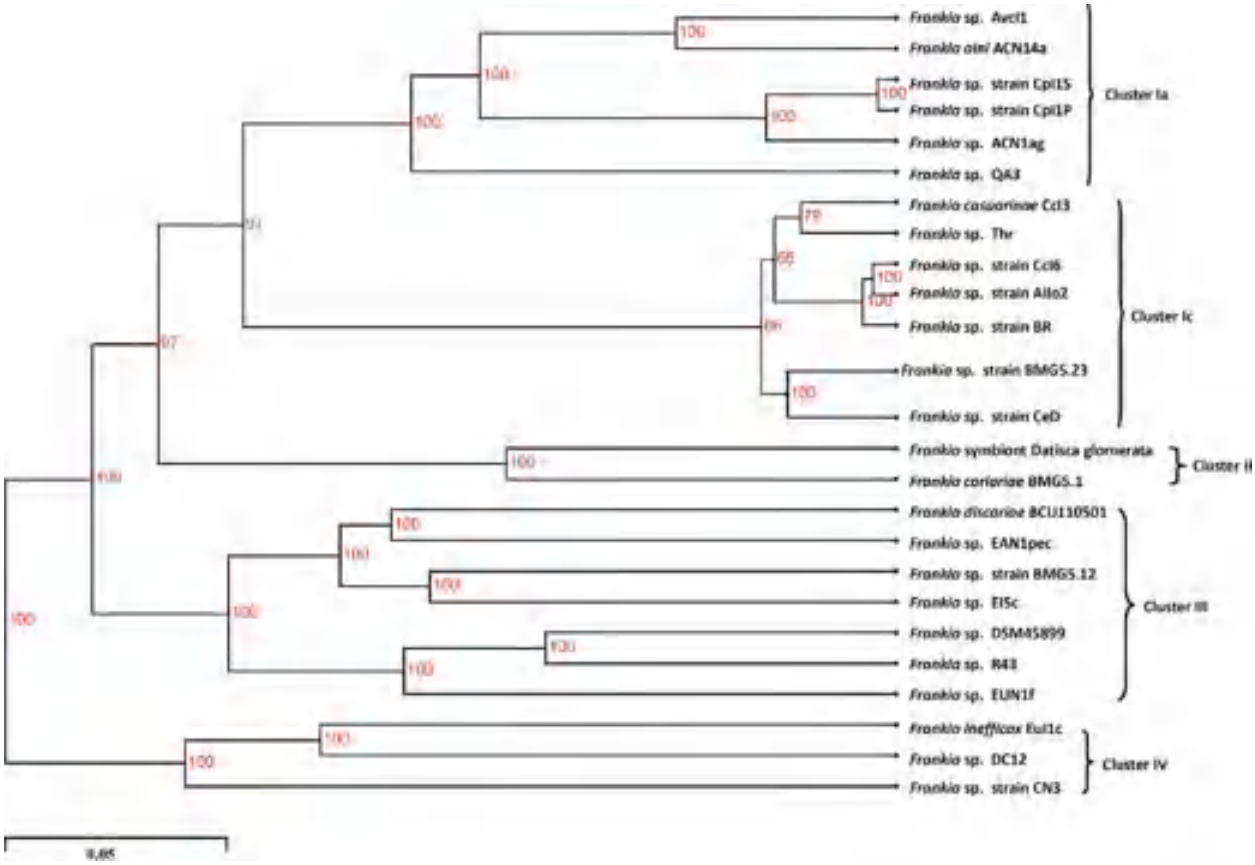

Fig 4: A representation of MLSA based phylogeny of *Frankia*

Kimura two parameter (K2P) model can be employed to generate a distance matrix based on the relative distances of the various clades that resulted from the phylogenetic tree and the corresponding K2P distances can beinvestigated for statistical analysis. The clades (groups) generated from the phylogenetic tree can be treated separately and the outgroup member in every clade should be considered as the reference strain against which the orthologous genes have to be screened in the other strains of that particular clade. Orthologous sequences can be identified using Reciprocal Best Blast Hit approach keeping an identity level of C50 %, an E-value of 1e-10 and at least 50 % region of alignment, using the local BLASTP program (http://www.ncbi.nlm.nlm.nih.gov/BLAST/down load.shtml).

The ratio (ω) of rate of non-synonymous substitutions per non-synonymous site (Ka) to rate of synonymous substitutions per synonymous site (Ks) has been an excellent estimator of the evolutionary selection constraint on a protein-coding gene. ω<1 signifies positive (diversifying) Darwinian selection whereas ω>1 symbolizes purifying (refining) selection. At neutral evolutionary stage, ω = 1, i.e., the rate of synonymous and non-synonymous substitutions are equal. The evolutionary rates of the orthologous protein coding genes can be calculated using Codeml program in the PAML software package (ver. 4.5) (http://abacus.gene.ucl.ac.uk/software/paml.html) with runmode = -2 and CodonFreq = 1. A BioPerl script, developed by us, can also be used along with the Codeml package that translated the cDNAs into proteins and aligned them accordingly. The protein alignments are then projected back into cDNA coordinates that should be used by the PAML package to perform the evolutionary rate analysis employing maximum likelihood method.

Our group	Target genome	dn/ds				
		PHX	S	CAZyme	S	PLX
Frankia alni ACN14a (C-Ia)	*Frankia* sp. AvcL1	0.19	>	0.14	<	0.26
Frankia sp. QA3 (C-Ia)	*Frankia* sp. ACN1ag	0.22	>	0.12	<	0.29
	Frankia sp. CpI1-P	0.24	>	0.11	<	0.35
	Frankia sp. CpI1-S	0.20	>	0.16	<	0.34
Frankia sp. Thr (C-Ic)	*Frankia casuarinae* Ccl3	0.20	>	0.15	<	0.23
Frankia sp. BR (C-Ic)	*Frankia* sp. Allo2	0.24	>	0.15	<	0.26
	Frankia sp. Ccl6	0.18	>	0.09	<	0.22
Frankia sp. CeD (C-Ic)	*Frankia* sp. BMG5.23	0.22	>	0.12	<	0.24
Frankia coriariae BMG5.1 (C-II)	*Frankia* Datisca glomerata Dg1	0.13	>	0.05	<	0.17
Frankia sp. EAN1pec (C-III)	*Frankia discariae* BCU110501	0.15	>	0.09	<	0.22
Frankia sp. EI5c (C-III)	*Frankia* sp. BMG5.12	0.14	>	0.05	<	0.19
Frankia sp. EUN1f (C-III)	*Frankia* sp. DSM 45899	0.14	>	0.07	<	0.22
	Frankia sp. R43	0.17	>	0.09	<	0.23
Frankia sp. CN3 (C-IV)	*Frankia* sp. DC12	0.18	N	0.17	<	0.24
	Frankia inefficax EuIIc	0.16	N	0.15	<	0.22

S Statistically significant ($p < 0.001$)

N Not statistically significant

Fig 4: A representative table for evolutionary analysis based on dN/dS score

Pairs of sequences with Ka and Ks values, evocative of saturation, should not be considered for further analysis. Evolutionary selection pressure among the differentially expressed genes can also be estimated to decipher the varying tendencies of evolution among studied genes. Evolutionary rates of the genes transcribed from the complimentary strands of replication (leading and lagging strands) can also be assessed separately for all the genomes under analysis.

Conclusion

In this book chapter, we have discussed some of the popular bioinformatics techniques used for downstream analysis of post-genomic era. All these techniques if, used in appropriate way will lead researchers to statistical results with proper visualization of data. Codon Usage, Amino acid usage, energy cost analysis along with phylogeny and evolutionary analysis will gather knowledge on culturable strains whose whole genome sequence is available. On contrary, 16s metagenomics will help us in identifying the unculturable micro-organisms present in specific niche. Thus, through combination of all these approaches, a holistic idea about Actinobacterial life style can be studied.

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