STUDIES ON THE ANTIFOULING BIOACTIVE COMPOUNDS ASSOCIATED WITH ACTINOMYCETES FROM NORTHERN COAST OF KERALA

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Declaration

I hereby declare that the work presented in this thesis entitled **"Studies on the Antifouling Bioactive Compounds Associated with Actinomycetes from Northern coast of Kerala."** is based on the original research work done by me under the guidance of **Dr. Ranjeet K**, Associate Professor, Department of Aquatic Environment Management, Kerala University of Fisheries and Ocean Studies Panangad 682506, Kerala, India and **Dr. M. RaziaBeevi,** Associate Professor and Head of Department, Post Graduate Department and Research Centre of Aquaculture & Fishery Microbiology, M.E.S. Ponnani College, Ponnani, Malappuram - 679 586 and that no part of this work has previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Certificate

This is to certify that the research work presented in this thesis entitled " **"Studies on the Antifouling bioactive Compounds Associated with Actinomycetes from Northern Coast of Kerala."** is based on the original work done by **Mrs. Reyhanath P.V.** under our guidance at Post Graduate Department and Research Centre of Aquaculture & Fishery Microbiology, M.E.S. Ponnani College, Ponnani, Malappuram District, Kerala, India 679 586, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and that no part of this work has previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Dedicated to my Dear Parents

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Reyhanath P.V.

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ABBREVIATIONS USED

et al.	:	*et alli* (Latin word, meaning 'and others')
CO_2	:	Carbon dioxide
GHGs	:	Greenhouse gases
Sox	:	Sulphur oxides
PM	:	Particulate matter
DNV	:	Det Norske Veritas
IMO	:	International Maritime Organization
AF	:	Antifouling
TBT	:	Tributyltin
MDRS	:	multidrug-resistant strains
UTIs	:	Urinary tract infections
TBTO	:	Tri-butyl tin oxide
TBTF	:	Tri-butyl tin fluoride
TPLA	:	Tri-phenyl tin-lead acetate
Cl	:	Chlorine
Br	:	Bromine
NPA	:	Natural Product Antifouling
SPC	:	Self Polishing Copolymer
CDP	:	Control Depletion Polymer
FR	:	Fire Retardant
STCs	:	Surface Treated Composites
PVC	:	Poly Vinyle Chloride
pH	:	Power of Hydrogen
BOD	:	Biological Oxygen Demand
ULT	:	Ultra low temperature
%	:	Percentage
^{0}C	:	Degree celsius
ppt	:	Parts per thousand
mg/L	:	Milligram per litre
µg/L	:	Microgram per litre

UV	:	Ultra Violet
SCA	:	Starch CaseinAgar
µg/ml	:	microgram per milliliter.
ISP2	:	International Streptomyces Project2
DNA	:	Deoxyribonucleic acid
DAP	:	Diaminopimelic acid
MR	:	Methyle Red
VP	:	Voges-Proskaeur
rRNA	:	ribosomal Ribonucleic acid
OTUs	:	Operational taxonomic units
PRIMER	:	Plymouth Routines in MultivariateEcological Research
ANOVA	:	Analysis of Variance
SPSS	:	Statistical Package for the Social Sciences
PCA	:	Principal Component Analysis
PCoA	:	Principal Coordinate Analysis
CAP	:	Canonical Analysis of Principal Coordinates
PERMANOVA	:	Permutational Multivariate Analysis of Variance
AvTD	:	Δ+Average taxonomic distinctness
VarTD	:	Variation in taxonomic distinctness
CCA	:	Canonical Correspondence Analysis
PAST	:	Paleontological Statistics Software
ppt	:	Parts per thousand
MDS	:	Multidimensional Scaling
mg/g	:	Milligram per gram
WSC	:	Water-Soluble Carbon
EPS	:	Extracellular polymeric substances
CRA	:	Congo Red Agar
PBS	:	Phosphate buffer saline
OD	:	Optical density
EPS	:	Exopolysaccharides

PCR	:	Polymerase chain reaction
ML	:	Maximum Likelihood
NCBI	:	National Center for Biotechnology Information
CFU		Colony forming unit
MEPC	:	Marine Environmental Protection Committee
DMSO	:	DimethyleSulfoxide
GC-MS	:	Gas Chromatography-Mass Spectroscopy
HPLC	:	High-Performance Liquid Chromatography
MIC	:	Minimum inhibitory concentration

Chapter 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 Microorganisms in the marine environment

Microorganisms are one of the most diverse and successful groups that have thrived on planet earth (Roychowdhury *et al*., 2017). They are widely distributed in - every part of the world and are regarded as pioneers in adapting to changing environments and establishing in any habitats. Being one of the most diverse ecosystems on earth, the marine biosphere is home to some of the unique habitats represented by a plethora of organisms, including the most versatile groups, namely the microbes. Their innate ability to evolve successfully in even extreme habitats is often associated with their ability to produce various biologically active compounds that have immense potential as pharmaceutical agents (Rasool and Srinivasan, 2017a; Dhevagi and Poorani, 2006; Bernan *et al*.,2004). The marine biome hosts a wide variety of microbes that contribute significantly to all global cycles of matter and energy (Baharum *et al*., 2010). Early life forms that originated in the sea have evolved for billions of years as bacteria, archaea, viruses, fungi and protists (including microalgae) and still dominate the living biomass. Recent rapid developments in molecular ecology, metagenomics and ecological modelling illustrate that microbes represent the most important biological group on Earth regarding phylogenetic and functional diversity (Glockner, 2011).

Marine microorganisms are the dwellers of the oceanic waters and exist not only on the surface of the sea but also in the lower and abyssal depths from coastal to

offshore regions. They span their territory from the general oceanic waters to more specialized niches like blue waters of coral reefs or black smokers of hot thermal vents on the seafloor. They extend their presence in all possible habitats, including estuaries and lagoons, mangroves and coral reefs, the deep sea and the seafloor (Quasim, 1999). Although microorganisms are seldom perceivable in natural habitats, their contribution amounts to half the world's biomass (Whitman *et al*., 1998). Unlike plant and animal diversity, microbial communities are more widely exposed to adverse environments, which have attracted many researchers to study their diverse physiologic lifestyle and metabolic capabilities. Microbial diversity is not only typological, but it is more incorporating and comprehensive compared to plant and animal domain and as well as having immense potential in the production of enzymes, antitumor compounds, and certain antibiotics (Roychowdhury *et al*., 2017). As a general trend, studies on microbial biodiversity help in understanding the ecological dynamics more in-depth, helping to explore different dimensions of their adaptability to a wide range of environments, the exogenous compounds they generate for their survival and futuristic use of these bioactive compounds for drug discovery (Bharum *et al*., 2010). The field of 'Blue biotechnology' is an offshoot of this property, which the researchers have started to explore for the identification and isolation of biocatalysts and secondary metabolites produced by marine organisms and their application in modern science (Collins *et al*., 2018). Marine habitats represent a prolific source for molecules of biotechnological interest. In particular, marine bacteria have attracted attention and were successfully exploited for industrial applications (Bollinger *et al*., 2018). With a vast area and unexplored microhabitats available in the coastal regions around the world, our present knowledge on the use of biomolecules from microbes is just the tip of the iceberg. In

this context, studies on microbial diversity and secondary metabolites from new strains of organisms pose greater significance in modern biotechnology.

1.1.2 Marine Actinomycetes

Actinobacteria, considered as connecting link between fungus and bacteria, form a vibrant living community in terrestrial and marine sediment. Being one of the first forms of life in this world, they have survived well throughout the geological time span and still thrive efficiently in almost all environments on our planet (Armbrust and Palumbi, 2015; Tully *et al*., 2018). Actinomycetes are a class of gram-positive bacteria with cosmopolitan distribution and being next to bacteria in abundance, carrying various physiological roles in their environment. They are involved in organic matter decomposition, biological N_2 fixation, nitrification, denitrification, and phosphate solubilization. They have a key role in the cycle of major and minor nutrients in various marine habitats. Their abundance and diversity have traditionally been monitored as a clue for ecological homeostasis. However, their importance to mankind was understood only after unravelling their role in producing a diverse range of secondary metabolites such as antibiotics, anticancer and antitumor agents, enzymes, and other important products. Marine actinomycetes, among various other microbes, have played an important role in being the greatest source of novel compounds (Deepa *et al*., 2013 Paneerselvam, 2013; Silambarasan *et al*., 2012). The diversity of enzymatic products obtained from different strains of actinomycetes, especially of the genus *Streptomyces*, have compelled researchers to link them to the environment where they are found. Speculation regarding the existence of indigenous populations of marine actinomycetes, therefore, arises because these bacteria produce resistant spores that are known to be transported from land into the sea where they can remain viable but dormant for many

years (Bull *et al*., 2000 and Cross, 1981). Thus, it has been frequently assumed that actinomycetes isolated from a particular region would have the capability to produce different forms of secondary molecules that the same species may not produce otherwise. In this pretext, exploring them in all possible ecosystems can result in the isolation of novel biomolecules, which signifies their diversity and association in sediments or other substrates.

In the marine realm, as has been demonstrated, many forms of actinomycetes prevail (Jensen *et al*., 2005b; Fenical and Jensen, 2006). However, their distribution varies drastically, and likewise, their diversity in intertidal zones (Goodfellow and Williams, 1983), seawater (Ramesh *et al*., 2006), associated fauna (Ramesh and Mathivanan, 2009), flora (Castillo *et al*., 2005), sponges (Zhang *et al*., 2008; Sun *et al*., 2010), and in ocean sediments (Jensen *et al*., 2005a; Das *et al*., 2008; Thornburg *et al.*, 2010; Xiao *et al*., 2011) are different. Some of the rare marine actinomycetes are found in specific microhabitats not recorded elsewhere (Maldonado *et al.*, 2005b; Jensen *et al*., 2005b). This unique adaptation of actinomycetes in the marine environment is a source of interesting research for new species and a promising source of pharmaceutically important compounds (Fenical and Jensen, 2006). Despite this promise, relatively little work has been done on marine actinomycetes, and only a tiny fraction of that has been directed at examining metabolite profiles (Lam 2006; Bull and Stach 2007). Since environmental conditions of the sea are extremely different from terrestrial conditions (Carte 1996; Kijjoa and Sawangwong, 2004), it is felt that marine actinomycetes may have different characteristics from terrestrial actinomycetes and therefore might produce much more novel bioactive compounds and antibiotics (Ellaiah and Reddy, 1987; Ramesh and Mathivanan, 2009). The research to date supports this hypothesis, and it

has been shown that marine actinomycetes produce novel types of new secondary metabolites (Lam 2006; Fenical and Jensen 2006). Many of these metabolites possess novel biological activities and have the potential to be developed as therapeutic agents (Feling *et al*., 2003; Maldonado *et al*., 2005 a). The actinomycetes are active components of marine microbial communities (Jensen *et al.*, 2005b) and form stable, persistent populations in various marine ecosystems (Das *et al*., 2006). The discovery of several new marine actinomycete taxa with unique metabolic activity in their natural environments (Fenical and Jensen, 2006) and their ability to form stable populations in different habitats and produce novel compounds with various biological activities (Magarvey *et al*., 2004; Jensen *et al*., 2005b, 2007; Lam 2006; Prudhomme *et al*., 2008; Olano *et al*., 2009; Asolkar *et al*., 2010; Rahman *et al.* 2010) clearly illustrate that indigenous marine actinomycetes indeed exist in the oceans and are an important source of novel secondary metabolites. Mangroves in India account for about 7 per cent of the global mangrove vegetation and are spread over 6740 sq. km. (Krishnakumar *et al*., 2012). With a coastal line of about 560 km and 41 rivers emptying into the Lakshadweep Sea, Kerala forms a congenial and convenient environment for the growth and development of mangroves (Neethu and Harilal, 2015). Kerala exhibit luxuriant mangrove vegetation, and the mangroves are distributed in all the coastal districts of Kerala. Among them, Malappuram and Kozhikode districts in Northern Kerala have a diverse path of mangroves and have very seldom been screened for potential marine actinomycetes.

1.1.2.1 Actinomycetes in the Mangrove ecosystems

Unexplored marine environments are now a popular research area due to the potentially huge resources present within them, and one among them is the mangrove

rhizosphere environment (Ryandini *et al.*, 2018). The mangrove ecosystem harbours a variety of microbes, including the actinomycetes. Mangrove actinomycetes are versatile in terms of their bioactivity, which motivates us to intrinsically assess their bioactive potentiality (Basha and Rao, 2017). Mangroves are a unique woody plant community of intertidal coasts in tropical and subtropical zones, located at the transition area between the land and the sea (Holguin *et al*., 2001; Kathireshan and Bingum, 2001).). This community represents a unique coastal habitat fringing the tropical and subtropical intertidal estuarine zones (Ancheeva *et al*., 2018), often exposed to high anthropogenic influence, including pollution. The ecosystem is unique and endowed with various ecological conditions, which reflects its dynamicity. The unique ecological conditions of mangroves include relatively high tidal range, high average temperature with little seasonal fluctuation, high salinity, strong winds, and muddy anaerobic or sandy soil (Tomilson, 2016; Wu *et al*., 2008; Saenger, 2002), low oxygen and high organic matter content (Loganathachetti *et al*., 2017 and Mendes and Tsai, 2014). Mangroves constitute a complex environment with high interaction between the communities viz., plants, animals and microorganisms, and with important features such as anaerobic and highly reducing sediment (Yan *et al.*, 2006; Lyimo *et al*., 2009), creating a hostile environment for many species. Mangrove is also a rich and underexploited ecosystem with great microbial diversity that have well adapted to this adversity (Li *et al*., 2019a). In mangrove sediments, microorganisms play an important role in productivity, conservation, and environmental recovery. They participate in the biogeochemical cycles and supply plant and animals with primary nutritional sources (Holguin *et al*., 2001; Andreote *et al*., 2012). The ecosystem has recently been demonstrated to be one with many unique forms of actinomycetes due to its sediment properties of anaerobic

condition and due to being rich in sulphide, with high salinity and organic matter. Because the mangrove soil conditions are extremely different from common terrestrial conditions, microorganisms, especially actinobacteria in mangrove soil, have distinctive adaptation characteristics and have the potential to produce novel bioactive metabolites (Manivasagan *et al*., 2014). Many investigations in various countries indicated that mangrove actinobacteria have rich diversity and various biological activities(Sangkanu *et al*., 2017; Kumar *et al*., 2018; Azman *et al*., 2015; Hong *et al*., 2009). World over researchers has been attracted to study microbial diversity in the mangrove sediments and their implications in the dynamics of the ecology (Simoes *et al*., 2015; Lognathachetti *et al*., 2017, Mendes and Tsai, 2014; Basak *et al*., 2016; Liang *et al*., 2007). of late, more impetus has been given to study actinomycetes capable of producing primary and secondary metabolites (Srivibool and Sukchrotiratana, 2006; Amrita *et al*., 2012).

1.1.2.2 Actinomycetes in the marine seaweeds.

Seaweed, known as macroalgae, are among the most important primary producers and act as ecological engineers on rocky coasts of the world's oceans. They are primary producers; provide shelter, nursery grounds and food source for various marine organisms. Seaweeds are of high ecological and of great economic importance (Doss and Rukshana 2016). Seaweeds grow abundantly along the Indian coastline, particularly in rocky shore regions; rich seaweed beds occur around Visakhapatnam in the eastern coast, Mahabalipuram, Gulf of Mannar, Tiruchendur, Tuticorin and Kerala in the southern coast; Veraval and Gulf of Kutch in the western coast; Andaman and Nicobar Islands and Lakshadweep (Sahoo, 2001; Rao, 1967; Silva *et al*., 1996). Seaweeds thrive in one of the harshest environments, including high surface

temperature, continuous-wave movement, and high salinity. The ecosystem consists of various forms of life that allow interactions and associations between themselves (Bull *et al*., 2000). The most frequent associations are between eukaryotic cells and microorganisms (Egan *et al*., 2008). Seaweed surface supplies protected and nutrient-rich conditions for bacterial growth (Armstrong *et al*., 2000c). So, compared to other multicellular organisms, seaweed has a rich diversity of associated microorganisms; these microorganisms may be beneficial or harmful to the seaweeds. In particular, epiphytic bacterial communities have been reported as vital for the morphological development of seaweeds, and bacteria with antibacterial properties are thought to protect the seaweeds from pathogens and surface colonization of other competition organisms (Devi *et al*., 2013). Some bacterial species show host specificity and bactericidal activity against specific pathogens; this specificity engages complex biochemical interactions between seaweed and bacteria (Strobel, 2003). These symbionts could produce compounds that resemble those produced by their host (Kelecom, 2002). Generally, the symbiosis of marine bacteria and marine organisms has a greater probability of producing chemical compounds than marine bacteria, which live freely (Mearns-Spragg *et al*., 1998). Various novel compounds with biological activity have already been identified from seaweed-associated bacteria. Many bioactive compounds such as antibiotics, antitumor, antifouling or antioxidant from the marine microorganism identified are due to the involvement of specific species function in their respective hosts (El-Shatoury *et al*., 2009). Some studies have demonstrated the presence of antibacterial activity from seaweed associated actinomycetes.

Kerala has a coastline of about 590 km, which includes nine districts and supports a large number of marine flora and fauna, including a rich diversity of

seaweeds. The estimated standing stock of seaweeds in Kerala is 1000 tonnes (wet wt.), of which about 150 tonnes are economically important seaweeds (Chennubhotla *et al*., 2011). Since there have been studies that have focused on seaweed associated actinobacteria for the production of bioactive compounds, especially those with antifouling potential, a detailed analysis of actinomycetes from the Northern coast of Kerala has immense potential.

1.1.3 Marine actinomycetes as a source of secondary metabolites

Actinomycetes are one the most economically and biotechnologically beneficial microorganisms to be studied since they have contributed to nearly two-thirds of the known antibiotics presently in clinical uses (Baltz, 2007; Naine *et al*., 2011; Raja and Prabakaran, 2011). They have produced a wide range of secondary metabolites of various medical and industrial importance, such as antibiotics, antifungal, antifouling, antiprotozoal, antiviral, anticholesterol, antihelminth, anticancer, and immunosuppressant. (Adegboye and Babalola, 2013). Actinomycetes are gram-positive bacteria of the order Actinomycetales; they are characterized by filamentous morphology. Their DNA has a high G+C content, LL-Diaminopimelic acid (LL-DAP), and the presence or absence of characteristic sugars in the cell wall. They are ubiquitous and form a stable and persistent population in various ecosystems. Actinomycetes are prolific producers of secondary metabolites with biological activities (Marinelli and Marcone, 2011). Secondary metabolites are metabolic products that are not essential for the vegetative growth of the producing organisms. However, they are considered differentiation compounds conferring adaptive roles, for example, by functioning as defence compounds or signalling molecules in ecological interactions. They are produced at the end of the exponential growth phase, and their syntheses greatly depend

on the growth conditions. They are structurally diverse, and most of them are endowed with biological activities, such as antimicrobial agents, toxins, pesticides, ionophores, bioregulators, and quorum signalling. These bioactive metabolites are profoundly used as antimicrobial agents for the treatment of diverse ailments (Vaishnav and Demain, 2011). Previous studies (Doroghazi and Metcalf, 2013; Bitok *et al*., 2017; Vila-Farres *et al*., 2017) showed that the gene cluster responsible for the production of secondary metabolites is not found in all bacteria and even in those present, it is not uniformly distributed among them (Arias *et al.,* 2011). Hence, the actinomycetes are considered the most potent source for producing secondary metabolites, antibiotics, and other bioactive compounds. It is well established that each actinomycetes strain has probably the genetic potential to produce 10–20 secondary metabolites (Bentley *et al*., 2002; Sosio *et al*., 2000). Streptomyces has been reported to contribute nearly 70% of metabolites described under actinobacteria (Zengler *et al*., 2005), and these Streptomycetes and related actinomycetes continue to be valuable sources of novel secondary metabolites with a range of biological activities that may ultimately find wide applications as anti-infectives, anticancer agents, or other pharmaceutically useful compounds (Bibb, 2005). From a biotechnological perspective, microorganisms, particularly actinobacteria, are an exploitable source of antifouling compounds (Manikandan and Vijayakumar, 2016). Hence a study on the synergistic activity of marine actinomycetes with their antibacterial property against biofouling bacteria is worth investigating.

1.1.4 Marine Biofouling

Marine fouling is a common phenomenon to indicate the accumulation of marine life forms on wetted surfaces. Fouling, technically and economically, causes

severe problems to shipping, offshore aquaculture and coastal industries around the world (Gopikrishnan *et al*., 2019). It is also a problem for underwater structures, such as pipelines, cables, fishing nets, and bridge pillars (Shan *et al.*, 2011). The succession of fouling states is generally considered in five main stages. In the first event, the adsorption of organic and inorganic macromolecules immediately after immersion, forming the primary film and then the transport of microbial cells to the surface, and the immobilization of bacteria on the surface. Then in the third stage, the bacterial attachment to the substratum is consolidated through extracellular polymer production, forming a microbial film on the surface. This microbial film formation corresponds to the development of a more complex community with the presence of multicellular species, microalgae, debris, sediments, etc., on the surface. In the last stage, the attachment of larger marine invertebrates such as barnacles, mussels and macro-algae forms macrofouling. The accumulation of biofouling organisms negatively affects unprotected, ocean-submerged surfaces, including shipping and leisure vessels, naval flees, and heat exchangers in power plants. Biofouling on vessels increases hull roughness and hydrodynamic drag (Schultz, 2007). This leads to decreases in speed and manoeuvrability, an increase in fossil fuel consumption, and as a result, increased emission of greenhouse gases (Poloczanska and Butler, 2010). All extents of biofouling have shown to have significant impacts on the powering of a ship. Relatively light biofouling of diatom slimes resulted in 10–16% powering penalties, whereas heavy calcareous fouling at full cruising speed resulted in power penalties of up to 86% (Schultz, 2007). Sea chest and piping fouling can significantly reduce the performance of machinery by interfering with their operation and causing premature wear and

damage. In addition, micro fouling and macrofouling induce or accelerate various forms of corrosion (Jones, 1996).

Biofouling also has a detrimental side effect on the environment. The increase in fuel consumption due to the drag penalty induced by fouling leads to increased emission of carbon dioxide (CO_2) and other greenhouse gases (GHGs). Other airborne emissions, such as sulphur oxides (SOx), nitrous oxides (NOx), and particulate matter (PM), are also increased due to fouling. Moreover, these emissions have a severe impact on the environment and human health. In recognition of the shipping industry's impact on global pollution, International maritime oranization (IMO) has introduced regulations for the control and progressive reduction of emissions from ships (IMO, 2015). Furthermore, fouling attached to the hull can be the source for translocation of invasive aquatic species (Hellio and Yebra, 2009). When introduced into a new environment, these marine organisms can pose a threat to the local ecosystem and human health. They can also have a negative economic and social impact by proving to be harmful to fishery, aquaculture, and infrastructures. Most importantly, the effects of invasive species on marine environments are almost always irreversible. The international maritime community has long recognized the potential for species transferred through biofouling to cause harm (IMO, 2011).

In marine aquaculture, biofouling is a specific problem where both the target culture species and infrastructure are exposed to various fouling organisms, leading to significant production impacts. In shellfish aquaculture, direct fouling of stocks causes physical damage, mechanical interference, biological competition and environmental modification, while infrastructure is also impacted. In finfish aquaculture, fouling of infrastructure, which restricts water exchange, increases disease risk and causes

deformation of cages and structures. Steps to curb biofouling have been attempted for a long time now, including mechanical removal, which is quite laborious. The use of antifouling paints and emulsions are a recent trend. Modern antifouling (AF) coatings are divided into two main categories, biocidal and non-biocidal. The former includes coatings whose AF mechanism is based on the release of biocides in seawater from a water-soluble matrix to exterminate fouling organisms or prevent their settlement. After the ban of tributyltin (TBT), copper has become the predominant AF biocide, while additional 'booster biocides' are utilized to target copper tolerant marine species (Dafforn *et al*., 2011). The three leading biocidal antifouling technologies currently available are Control Depletion Polymers, Self-Polishing Copolymers, and Hybrid or Self-Polishing AF coatings. Their main difference is the type of soluble acid binder that enables biocide leaching. The non-biocidal group includes Fouling Release coatings and Hard Inert coatings. These coatings are regarded to be non-toxic in terms of not containing biocides to control fouling. Many other non-toxic strategies are under development and testing or at an initial application stage. According to Gittens *et al*., 2013, current research into AF coatings is broadly divided into two areas, which are "the modification of the surface physical and chemical properties to deter permanent attachment of fouling organisms" and "the incorporation of biologically-derived or inspired components." Polymer brushes, sol-gel coatings, zwitterionic surfaces, amphiphilic surfaces, superhydrophobic surfaces and superhydrophilic surfaces are some of the AF mechanisms included in the first group (Scardino and de Nys, 2011; Gittens *et al*., 2013). The second group refers to biomimetic strategies, including coatings with bio-inspired surface microtopography (Almeida *et al*., 2007; Scardino and de Nys, 2011; Lejars *et al*., 2012), and coatings based on natural products. Such natural

compounds are various enzymes, bacterial products, algal products and terrestrial natural products (Almeida *et al*., 2007; Qian *et al*., 2010; Dafforn *et al*., 2011; Lejars *et al*., 2012). Pharmaceuticals are also potential candidates as AF compounds (Qian *et al*., 2010). A combination of technologies is also feasible (Scardino and de Nys, 2011), such as the combination of sol-gel technology with biological AF components (Gittens *et al*., 2013) or the combination of copper silyl acrylate and advanced hydro-gel in one product (RINA, 2013). Another ecological alternative to conventional coatings is the use of fibre coatings (Chambers *et al*., 2006; Almeida *et al*., 2007; Lewis, 2009) which are manufactured with the process of surface flocking.

To reduce the problems associated with fouling, metal-based or synthetic organic agents are used as antifouling coatings (Callow and Callow, 2011 and Wang *et al*., 2007). However, these antifoulants are found to be toxic and lethal to non-target life forms. In addition, the antifoulants could pollute the marine environment, due to which the usage of these chemical-based compounds has been strictly regulated. This followed the International Maritime Organization (IMO) banning organotins in antifouling coatings in 2008. As an alternate industry opined development of novel eco-friendly antifouling compounds (Gallo and Tosti, 2015). Research on antifouling metabolites from natural resources has since then increased. Notably, marine organisms were found to be among promising resources for the production of non-toxic antifoulants. The marine environment's special physical and chemical conditions favour the diverse biological groups that possess a variety of structurally unique molecules with pharmacological and other biological activities (Satheesh *et al*., 2016). Previous studies have reported that several natural antifoulants could be obtained from marine organisms, especially from invertebrates (Limnamol *et al*., 2009; Qian *et al*., 2013). In

addition, marine microbes have also gained much attention and are being explored as potential sources for producing environmentally friendly antifouling metabolites. In comparison to marine invertebrates, marine microbes, especially marine actinomycetes, are the real sources for natural products, including antifoulants, since they can be easily cultivated in a controlled environment and could be used for mass production of antifouling agents (Jin *et al*., 2016). For this reason, there is a need for the development of ‘‘environmentally-friendly’’ antifoulants. Marine actinomycetes have the potential to produce non-toxic antifouling compounds as they can produce substances that inhibit not only the attachment and/or growth of microorganisms but also the settlement of invertebrate larvae and macroalgal spores. Hence, the present study was carried out to unravel the diversity of marine actinomycetes from a less explored northern Kerala to screen potential antifouling bioactive compounds.

1.2 OBJECTIVES

- To study the diversity and distribution of actinomycetes from marine sediments and seaweeds of the Northern coast of Kerala.
- To identify and isolate marine actinomycetes having antifouling potential from the coastal regions of Northern Kerala.
- To isolate potential biomolecules from selected strains of marine actinomycetes and test their efficacy as an antifouling agent.

Chapter 2

REVIEW OF LITERATURE

Marine ecosystems are embodied with a diverse assemblage of microbes that have continuously evolved with geological time and successfully adapted to a wide array of habitats through their intrinsic physiological processes as well as extrinsic interventions that conducively modify their immediate surroundings to facilitate better growth and survival. The latter is through the production of various secondary metabolites, whose exploitation by humans has resulted in developing a new stream of science, namely Marine Bioprospecting. Exploring microbes that have immense potential for the production of extracellular metabolites has become a serious topic of research lately to isolate compounds that have medical relevance in humans. In the last decade, the rate of discovery of new compounds from terrestrial sources has decreased, whereas the rate of re-isolation of known compounds from the marine realm has significantly increased. With the available drugs turning less effective against present pathogenic microbes, there is a pressing need to develop a new drug for coping with infection against Multidrug-resistant strains of pathogens and drug-resistant cancers. This has resulted in researchers focusing on marine habitats, which have been less explored for such microbes. In this regard, actinomycetes have been one of the most widely studied microbes due to their innate capacity to survive in wide climatic conditions, spore-forming characteristics, easiness to culture in lab conditions, and secondary metabolite quality produced. They also have revealed to be widespread symbionts of eukaryotes and produce antibacterial compounds. Studies of actinomycetes as defensive mutualists have led to the discovery of new antibiotics with

potential pharmaceutical applications, renewing recognition of the value in understanding the ecology of Actinobacteria for drug discovery. Studies have shown that various habitats differ in composition as well as an abundance of actinobacteria. The combination of multiple species in each habitat is defined by their capabilities to coexist with other fauna and flora and the prevailing environmental parameters. This section discusses the habitat-wise distribution of actinomycetes, the bioactive compounds produced by them, especially those pertaining to antimicrobial or antifouling nature and the future possibility of these compounds for their commercial application.

2.1 Distribution of Actinomycetes

2.1.1 Mangrove sediment

Mangroves are keystone ecosystems providing numerous environmental services and critical ecological functions. They thrive along the coastlines in most of the tropical and sub-tropical regions. These ecosystems are highly productive and rich in floral and faunal biodiversity (Vinod *et al*., 2018). Mangrove is a rich and underexploited ecosystem with great microbial diversity to discover novel and chemically diverse antimicrobial compounds (Feina *et al*., 2019). Recent studies have focused on moderately or extremely halophilic microorganisms, but very little information is available concerning the taxonomic distribution of actinomycetes from mangrove environment (Ravikumar *et al*., 2011). Ravikumar *et al*. (2011) revealed that the diversity of actinomycetes is found maximum in the rhizosphere soil than in the non-rhizosphere. The diversity of actinomycetes is found maximum between the soil depth of 10-20 cm. Incidentally, their population in surface and subsurface depths (0-10 cm) was less despite high nutrients levels. This indicates that the physico-chemical

parameters, especially temperature and soil pH, significantly affect their distribution in mangrove regions. It was reported that in the mangrove rhizosphere, Actinobacterial genera such as Streptomyces, Micromonospora, and Nocardioform are abundant (Tan *et al*., 2009). Many actinomycetes isolated from mangroves have shown better antagonistic features against Multi-Drugs Resistant (MDR) bacterial strains of *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus cloacae and Enterobacter sp.* (Ryandini *et al*., 2018). According to Gnanam *et al*. (2013), factors like sediment structure, nutrient availability, total organic carbon, and total nitrogen significantly influence the distribution of actinobacteria. It is known that sediment rich in humus or organic matter and clay type of sediment is an excellent source of Actinobacteria population than sandy sediments. As clay sediments can retain more nutrients which can be available for microorganisms than sandy sediments, and thus they support more actinobacteria population (Dhevendaran *et al*., 2004).

Depth-wise analysis has shown that Streptomyces spp. has a higher percentage distribution in the nearshore area than the offshore region. It was assumed that marine actinobacteria are washed into the ocean from the terrestrial environment through continuous run-offs and other processes and adapted to marine conditions (Jensen *et al*., 1991). Predomination of streptomycetes at shallow depths was reported by Takizawa *et al*. (1993) from the Chesapeake Bay. Miyake *et al*. (2003) isolated Actinobacteria from the shallow layer of sediment (0–1 cm) and found that the isolates from the deep layer (9–10 cm) mostly belonged to phylum Firmicutes. Walker *et al*. (1975) observed that 98% of the streptomycetes observed were from water less than 3m deep, and also, the abundance decreased quickly with an increase in water depth. In peninsular India, the

prevailing monsoon has a direct role in the distribution of actinomycetes. Shirodhkar *et al*. (2009) and Sundaramanikam *et al.* (2008) correlated the seasonality and variations in nutrients on actinomycete in intertidal regions and described the importance of monsoon season in its distribution. The pre-monsoon season showed the most remarkable diversity, and during the southwest and northeast monsoon, due to the fluctuations in salinity, the diversity of actinomycetes was greatly hampered (Priya *et al*., 2014). Actinobacteria are an excellent source for various enzymes, and their metabolic diversity makes them the major producers of antibiotics and a great deal of other bioactive metabolites (Alderson *et al*., 1993, Sanglier *et al*., 1993). They can utilize and decompose a wide diversity of compounds, including polymers and xenobiotic compounds (Crawford, 1978; Goodfellow and Simpson, 1987; Warren, 1996; Schrijver and Mot, 1999). Basha and Rao (2017) reviewed that mangroves actinomycetes bioactivity is versatile in the way of its industrial enzymes production, antitumour, antimicrobial, antiangiogenesis, enzyme inhibition, probiotics and other notable bioactive metabolites. By far, the most extensively studied of the actinomycetes is Streptomyces, which has contributed to over 70% of presently available antibiotics. Hence most of the research has focused exclusively on this group in India. The first report on the diversity studies of *Streptomyces sp*. from the Gilakaladindi mangrove ecosystem was conducted by Naradala *et al*. (2016), who, through antimicrobial assay, reported seven strains of *Streptomyces sp*. to be active against the test microorganisms such as gram-positive bacteria, gram-negative bacteria and fungi. Karthikeyan *et al*. (2013) brought out the information about the diversity of actinomycetes from soil samples collected from Mamallapuram, Tamilnadu, mangrove ecosystem. Their study isolated twenty different actinomycetes and clearly showed the relationship between

physico-chemical parameters and the distribution of actinomycetes. All these studies have univocally reported that the marine environment provides a rich and exciting diversity of actinomycetes before concluding that these marine actinomycetes provide a new source for the efficient production of antibiotics or enzyme for industrial applications. Lakshmanaperumalsamy (1978) isolated 518 Streptomyces strains from the sediments of estuarine, backwater, marine, freshwater and mangrove environment of Porto Novo. Kala and Chandrika (1995) used different media for isolating and maintaining actinobacteria collected from mangrove sediments. Sediments of mangrove, estuary, sand dune and industrially polluted marine environment of Cuddalore have also shown good diversity of actinomycetes (Kathiresan *et al*., 2005). Other works from the east coast have covered the Pitchavaram mangrove ecosystem (Sivakumar *et al*., 2005), Thondi and Karankadu of the Palk Strait region (Ravikumar and Suganthi, 2011) and coastal mangroves of southern Tamilnadu coast (Rajesh *et al*., 2011). From the Southwest coast works of Das *et al*. (2014), who isolated actinomycetes capable of antimicrobial activity from the sediment samples of Valappattanam mangrove, Kerala is a major study. Similarly, in their study, Rosmine and Varghese (2016) reported that two isolates of the actinomycetes belonging to the genus *Streptomyces*, collected from mangrove and estuarine sediments of Cochin, showed higher antimicrobial activity against human and fish pathogens tested, with a maximum inhibition zone of 21.0 mm. Compared to the east coast of India, the isolation of actinomycetes from the sediments of mangrove ecosystems from the west coast are limited, and hence there is the immense possibility to isolate novel strains of these microbes from the region.

2.1.2 Coastal sediment

Coastal sediments harbour a rich diversity of actinomycetes throughout the world. Coastal intertidal sediments are exposed to wide fluctuations in the environmental variables and are regularly immersed and exposed to the vagaries of the climatic regime. Unfortunately, these are also an environment that represents an underexplored biological niche that can potentially discover novel antimicrobials strains (Jose and Jha, 2017). Being primarily originating from terrestrial habitats, actinomycetes have long been associated with soil fertility and biogeochemical cycles. However, in aquatic ecosystems, their importance as facilitators of ecological stability, especially retaining nutrient load, is well studied (Ozcan *et al*., 2013). Of late, there has been a concerted attempt from researchers worldwide to screen marine actinomycetes since they possess a rich diversity and have alternate pathways to produce secondary metabolites that endure the microbes in extreme climatic conditions. The genus *Streptomyces* has been one of the most widely researched groups as they contribute extensively to the production of new antimicrobial compounds (Bull and Stach, 2007). The diversity of these actinomycetes have been related to their counterparts from the terrestrial environment. However, a closer analysis shows that the marine actinobacteria vary in colony morphology and biochemical characteristics depending on the environment and aquatic habitat they occupy. Leena and Raju (2019), in their study along the coast of Visakhapatnam and Chirala in the Bay of Bengal, isolated several strains of actinomycetes that showed good antibacterial activity against six bacterial test organisms. Their study also emphasized that *Streptomyces* is one of the most easily cultivable genus and hence has immense potential for scaling up their production in a commercial scenario. In this regard, the selection and optimization of culture media

have to be very specific. Ozcan *et al*. (2013) reported a great diversity of actinomycetes from coastal sites surrounding the Anatolian peninsula of Turkey. They also reported that one-fourth of the isolated strains could be effectively used against at least one antibiotic-resistant microorganism, indicating their potential for future drug production. It was also observed that with an extensive screening of the extracts, they could isolate strains that showed high antimicrobial activities indicating the potential to produce antibiotics. This study then led Pereira *et al*. (2020) to isolate novel antifungal agent *Napyradiomycin* derivatives from *Streptomyces aculeolatus* from ocean sediments off the Madeira Archipelago. This discovery was a significant study since this compound could inhibit more than 80% of the marine biofilm-forming bacteria, as well as the settlement of larvae of invasive *Mytilus galloprovincialis*. Young and Sug K (2012), in their study, reported an antifouling diterpene *Lobocompactol* isolated from *Streptomyces cinnabrinus.* In an Indian scenario, the study of Bavya *et al*. (2011) revealed that the bioactive compounds extracts from *Streptomyces filamentosus* showed maximum inhibition against biofouling bacteria.

Although genus *Streptomyces* has been widely screened for antifouling compounds, other genera of actinomycetes have also shown promising results. Eccleston *et al*. (2008) revealed that bioactive compound produced from a species of *Micromonosporae* isolated from the aquatic habitats of the Sunshine Coast region in Australia showed good antifouling characteristics. Similarly, Pan *et al*. (2015) reported that two species of genus *Nocardiopsis*, namely *Nocardiopsis oceani* and *N. nanhaiensis,* isolated from marine sediment samples of the South China Sea produced antifouling compounds. Ruttanasutja and Pathom-aree (2015) observed that 209 actinomycetes isolates belonging to eight known actinomycetes genera, namely,

Curtobacterium, Dermacoccus, Micromonospora, Microbispora, Pseudonocardia, Rhodococcus, Streptomyces and Tsukamurella isolated from coastal marine sediments of Thailand, showed good production of secondary metabolites, many of which had antimicrobial potential. Specificity of the activity of these antimicrobial compounds have been a matter of concern among researchers; while some compounds have an antagonistic action against certain bacteria and fungus, some do not inhibit their growth considerably (Sinimol *et al*., 2016). Some are specific to a particular pathogen, as reported by Alfisyahri *et al*. (2017) from the coastal sediments of Jakarta, Indonesia. The result showed that some isolates inhibited *Staphylococcus aureus*, while others inhibited *Kocuria rhizophila, Candida albicans*, and *Saccharomyces cerevisiae*. Interestingly, none of these isolates showed inhibitory activity against *Escherichia coli*. A similar result was observed by Abdelfattah *et al*. (2016) and Patin *et al*. (2016), who reported such variability in the antimicrobial activity of genus *Salinispora* isolated from two separate regions along the Red Sea coast in Egypt. This indicates that though the diversity in the actinomycetes may vary with region and season, their antagonistic activity solely depends on the secondary metabolites they produce. Hence signifying the importance of studying each actinomycetes group more closely to screen specific bioactive compounds that are pathogen-specific. In such a case, for producing antifouling metabolites, it becomes even more relevant to isolate locally available actinomycetes that have the potential to cease or retard the growth of biofilm-forming bacteria.

2.1.3 Seaweeds

Seaweeds are considered an ecologically and biologically important component in marine ecosystems. Seaweeds substantially contribute to marine primary production

and provide habitat for nearshore benthic communities (Williams and Smith., 2007). Along with the functional and regulatory services that these faunas provide to the coastal community, they are also important substrates harbouring a wide range of microbes that have considerable importance in drug formation. Susilowatia *et al*. (2015) have shown that among the different seaweeds, brown algae have shown a better epiphytic bacterial population. Their study with three species of brown algae (*Sargassum polycystum, S. duplicatum and S. echinocarphum*) reported 23 marine bacterial strains that had good antibacterial activity against methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis.* Similarly, the growth of biofilm-producing bacterial strains of *Bacillus subtilis* has been effectively countered by many actinomycetes that have been isolated from seaweeds (Burgess *et al*., 2003). Another group of algae that have shown promising result in this regard is the red algae, *Gelidiella acerosa.* Ulfah *et al*. (2017) conducted several bioactivities and genetic screening test of actinobacteria associated with this red algae to discover new antibacterial compounds against *Vibrio alginolyticus*. An added feature of seaweeds as a suitable substrate for these antifouling microbes is their inherent capacity to coexist with symbiotic groups. Singh and Reddy (2014) reported that red algae live as host for diverse and abundant symbiotic microorganisms. These bacteria protect the host from predators or other harmful compounds excreted by boring invertebrates on these rocky shores as chemicals for settlement. There is also a counteraction by these symbionts to ward away unwanted intruders on these seaweeds. These usually are chemical signals, and as Matsuo *et al*. (2011) in their study has reported *Streptomyces sp*. associated with brown algae *Analipus japonicus* produced *Streptobactin*, which was later found to have significant application as an iron-chelating agent for treatment in various human

diseases. According to Piel *et al*. (2004) seaweed associated microorganisms participate in the biosynthesis of the bioactive compound, which can be used more effectively as a source of novel chemical compound. Moreover, their implications are far-reaching than its host due to the short life cycle of actinobacteria and the ability to be cultured in vitro. According to Naikpatil and Rathod (2011), approximately 80% of antibiotics are being produced by actinobacteria, especially genus *Streptomyces* and *Micromonospora.* Most of the recently developed drugs viz., *Abysomicin C,Diazepinomicin, and Salinoporamide A* have shown promising results against a broad spectrum of pathogens discovered from marine actinomycetes (Lam, 2006). Among them, seaweed-associated groups' contribution is also worth mentioning (Devi *et al*., 2013).

Some bacterial species have reported host specificity and bactericidal activity against specific pathogens; this specificity engages complex biochemical interactions between seaweed and bacteria (Strobel, 2003). Fajar *et al*. (2016), in their study, revealed that actinomycetes symbiont with three different seaweeds have antibacterial activity against *E. coli*, *Proteus sp*., and *Enterobacter sp.* and results also showed that actinomycetes symbiont with *Halimeda macroloba* were highly potent. Rajivgandhi *et al*. (2018) isolated 20 strains of endophytic actinomycetes from marine algae *Caulerpa taxifolia*, of which five strains had better antibacterial activity against multidrug-resistant uropathogens. An earlier study by a similar group had reported the significance of *Nocardiopsis sp* associated with brown algae to have better antimicrobial activity against multidrug-resistant strains (MDRS) on urinary tract infections (UTIs) in human (Rajivegandhi *et al*., 2016). Hence, a review clearly describes the significance of seaweed-associated actinomycetes to potentially act effectively against a broader group of pathogens. Since the secondary metabolites formed by these actinomycetes are

shown to vary with the habitat and seaweed on which they are associated, the screening for bioactive compounds must be specific. Therefore, it is of prime importance to understand the diversity of such actinomycetes in seaweeds and their potential to produce antimicrobial compounds beneficial for developing antifouling derivatives.

2.2 Bioactive compounds from marine actinomycetes.

So far, we have known the importance of marine actinomycetes as a goldmine for a plethora of novel bioactive secondary metabolites with antimicrobial activities. With the discovery of a new strain of such actinomycetes, their potential to produce new molecules has enthralled researchers to explore more on these inexhaustive resources (Adegboye and Babalola, 2013). Hence, modern medicine has now looked up to these groups to develop antimicrobial drugs, especially those potent against pathogenic organisms. Microbial secondary metabolites, especially those from actinomycetes, have been a phenomenal success in discovering novel drugs. They produce a wide range of secondary metabolites, and more than 70% of the naturally-derived antibiotics are currently in clinical use. The ability of these microorganisms to produce such a diverse array of drugs is attributed to their genetic structure. Arias *et al*. (2011) reported that *Streptomyces coelicolor* possesses more than 20 gene clusters while *S. avermitilis* possesses 30 gene clusters to synthesize secondary metabolites. The formation of gene clusters is basically dependant on the adaptive feature that the actinomycetes evolve during a period to adapt to the different environmental conditions to which they are exposed. This may be why certain groups of actinomycetes such as *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora and Actinoplane*s produce different forms of compounds that are presently commercially exploited (Solanki *et al.*, 2008). In their study, Fiedler *et al*. (2005) isolated 600 actinomycetes strains from

marine sediments from various sites in the Pacific and Atlantic Oceans and screened them for the production of bioactive secondary metabolites.

Marine *Streptomycetes* strains were producers of well-known chemically diverse antibiotics than those isolated from their terrestrial counterparts, as in the case of marine *Micromonospora*. This, therefore, indicates that though most of these actinomycetes have their origin from the terrestrial environment, compared to their predecessors, the ones in marine have evolved rapidly and hence have adapted to a wide range of habitats. This has enabled them to establish themselves in hostile environments and changed their genetic structure, resulting in the production of secondary metabolites to conducively modify their immediate environment. As pioneers to any habitat, they have an innate characteristic to establish through biochemical means. These 'outputs' have been beneficial to human as they have shown better antimicrobial, antifungal and generally antipathogenic activities (Saraswathi *et al*., 2015). Man has utilized this feature, and through further purification of these crude compounds, industrial production has been attempted. Most of the works that have resulted in characterizing any bioactive compound effective against a specific pathogen has used the crude extracts from the actinomycetes. Though the *in vitro* culture potential of these specific strains are an added advantage in their commercial exploitation, the secondary metabolites produced or the rate at which they are produced in a laboratory condition usually do not meet the standards as exploited from natural habitats. This has resulted in spending a lot of time and effort in standardizing their media to get optimum results. The industry often relies on developing synthetic compounds resembling the native bioactive compound to scale up their commercial production. Hence it is of utmost importance to understand how such metabolites are produced in a realistic scenario before replicating them for

industrial production. Onaka (2017) reported that many methods had been developed to activate the actinobacterial secondary metabolism, including combined cultures and the use of Goadsporins. In their study, Janardhan *et al*. (2014) isolated an actinomycete strain, *Nocardiopsis alba,* isolated from mangrove soil collected from the Nellore region of Andhra Pradesh, India. The bioactive compound produced by this strain was purified through column chromatography and subjected to HPLC for further purification. This is a normal method that has been followed, generally using liquid solvents for extraction. However, recently freeze-drying techniques have been used to improve the yield of bioactive compounds (Mangamuri *et al*., 2016). The significance of actinomycetes based drugs is increasing, with various synthetic analogues falling short in quality (Thakur *et al*., 2009).

Despite the vast advances in pharmacological science, the world has not developed an effective multi-role antipathogenic drug other than those exploited from natural fauna, especially from the genus *Streptomyces*. Hopwood (2007) has reported that *Streptomyces* have yielded many therapeutic agents, including antibacterial such as *Tetracyclines*, antifungal such as *Amphotericin*, and anticancer drugs exemplified by *Adriamycin* and the immunosuppressant *Tacrolimus.* According to Krzesniak *et al*. (2018), actinobacteria continue to be an important source of novel secondary metabolites in drug application among the natural producers of bioactive substances. Among them, *Arenimycin C, Chromopeptide lactone RSP 01, Kkocurin, Macrolactins A1* and *B1*, *Chaxamycin D*, and *Anthracimycin* are regarded as the most effective compounds with antibacterial activity. Similarly, the highest potency among available antifungal compounds is exhibited by *Enduspeptide B, Neomaclafungins A-I* and *Kribelloside D*. Simultaneously, *Ahmpatinin* IBU, *Antimycin A1a*, and *Pentapeptide*

4862F are recognized as the strongest antiviral agents. With the development of the latest technologies, there has been a surge in understanding the action of these drugs on the target organisms. Hence today, the -omics" technologies (genomics, transcriptomics, proteomics and metabolomics) together with bioinformatics are extensively used tools in inducing the overproduction of actinomycetes secondary metabolites (Krzesniak *et al*., 2018). Through the advent of these technologies, the researcher can now know the origin of the gene responsible for producing these secondary metabolites; through gene transfer using plasmids, this identified gene can now be incorporated in other bacterial strains for scaling up the laboratory production of novel drugs. Igarashi *et al*. (2012) found that the newly discovered *Spirotetronate* antibiotic owes to its polyketide origin from *Nomimicin*, which was isolated from the culture extract of *Actinomadura sp*. Although there have been significant works pertaining to the antibacterial activity of specific strains of actinomycetes on selected bacteria, there has also been a demand from the scientific community to both identify drugs that have a broad application as well as those that are specific in their activity (Attimarad *et al*., 2012; Subramani and Sipkema, 2019). Hence depending on the need today, a huge database of actinomycetes and their genetic structure has been maintained for future references and development of specific antimicrobial drugs.

Though the majority of the traditionally isolated actinomycetes were under the genus *Streptomyces,* basically due to their ubiquitous distribution, better adaptability to a wide array of habitats, faster mutation rate compared to other actinomycetes enabling the formation of different gene clusters, possibility to culture in controlled environments, success in the standardization of media and inherent spore-forming capability in adverse conditions, recently there have been other groups which have

shown resilience and equally good resources of novel drugs. Most of the compounds produced by these rare marine actinomycetes have antibacterial, antifungal, antiparasitic, anticancer or antimalarial activities. The highest numbers of natural products derived from them include the genera *Nocardiopsis, Micromonospora, Salinispora* and *Pseudonocardia*. Among the genus *Micromonospora* has revealed to be the richest source of chemically diverse and unique bioactive natural products. Hence, in our quest to discover new drugs, a new group of actinomycetes needs to be explored, and steps are taken to standardize their culture and commercial production.

2.3 Biofouling and antifouling technologies

Marine biofouling has been described as the undesired growth of marine organisms such as microorganisms, barnacles and seaweeds on submerged surfaces (Callow and Callow, 2011). Biofouling is one of the deterioration processes that affect all stationary and moving engineered structures irrespective of the nature and type of material employed. Biofouling, therefore, is a global problem for maritime industries, such as shipping, fishing, mariculture, tourism, underwater installations in desalination plants, oil industry, heat exchangers, oceanographic sensors, oceanariums and other civil constructions. They also drain vast amounts from the exchequer for maintenance and require exorbitant costs to repair these underwater structures. Traditionally biofouling of structures in the sea has been divided into several phases viz., the occurrence of the biofilm, followed by molecular fouling, formation of primary film formation and slime layer by microorganisms such as bacteria, diatoms, blue-green algae, fungi, actinomycetes, protozoa and algal spores. This is followed by the settlement of secondary invaders such as macroalgae, barnacles, hydroids, serpulids, and finally by tertiary settlers such as mussels, ascidians and sponges. These are

arbitrary sequences that can vary dramatically by season and with geographical location. The success in controlling fouling depends upon the generation of basic information on species composition, growth, biomass build-up, diversity, seasonality, succession, and distribution of the organisms concerned to estimate the fouling potential of a given area. Fouling is characterized by continuous changes in species composition in relation to different biotic and abiotic factors over a time period, which is referred to as fouling community development (Greene and Schoener, 1982). Fouling composition varies temporally as well as spatially (Brown and Swearingen, 1998). Therefore, understanding the formation of biofouling communities specific to a region and their development through time is an essential prerequisite for economic design and construction of marine structures and establishing appropriate cleaning programs. At the same time, knowledge of their taxonomic diversity at the species level is also a prerequisite to understanding the functioning of a community because each species is characterized by an independent ecological role (Maggiore & Keppel, 2007).

The extent of damage varies with the intensity of fouling. Schultz (2007) has reported that increased roughness presented by a heavily fouled ship hull can result in powering penalties of up to 86% at cruising speed; even relatively light fouling by diatom 'slimes' can generate a 10–16% penalty. The process of fouling starts the moment the structure is in contact with water. When a clean surface is immersed in natural seawater, it immediately starts to adsorb a molecular 'conditioning' film primarily consisting of dissolved organic material (Jain and Bhosle, 2009). Many studies have reviewed biofouling more closely and have reported the extent of the economic loss incurred by the maritime industry (Lakshmi and Akondi, 2015). Their observation shows that fouling organisms can cause extensive damage to commercially

important marine structures resulting in increased drag of marine surface and submarine vessels incurring increased fuel loss. It is also seen that biofouling in freshwater systems is less pronounced than seawater, which has high salt content and forms a complicated solution containing the majority of the known elements (Callow and Callow, 2002). Similarly, the prevalence of marine biofouling is naturally higher in the shallower water along the coast. Dhana *et al*. (2005) reported that bioforms adapt and tolerate wide fluctuations in environmental conditions such as temperature, water flow, and salinity become the dominant foulers on ship surfaces. There has been a renewed interest among researchers to study the role of substrate specificity in biofouling. The surface pH has proved to be one of the most decisive parameters in determining material bioreceptivity. A decrease in surface pH by carbonation can promote algal development significantly, with colonization beginning much earlier and happening much faster. A precursor to visible macrofouling is microfouling caused by bacteria, fungi and other microscopic organisms. About 5000 biological species have been listed as involved in the fouling of structures exposed to or immersed in water. The composition and community assemblages of these species show wide variations from site to site. Once attached to a surface, bacteria rapidly divide and form a slime film. Mould and fungi communities develop along with a variety of single-celled algae and multi-celled seaweeds that live as large filamentous or branching plants. At first, an algal film is formed on the submerged surface, known as the primary film. This is an essential requisite for many foulers as the larvae of these settle on them. These films are composed of diatoms and algal spores with relatively small proportions of bacteria. They serve as the food of the larvae and also as the foothold on the substratum.

This understanding that modifying the surface of the structure can retard the assemblage of biofilm-forming groups leads to rampant use of antifouling paints that contained Organotin compounds like Tributyltin (TBT), tri-butyl tin oxide (TBTO), tri-butyl tin fluoride (TBTF), tri-phenyl tin-lead acetate (TPLA), and copper and zinc-based other metallic species. However, it was only later that studies showed the negative impact of these compounds on aquatic ecosystems (Chambers *et al*., 2006; Douglas-Helders *et al*., 2003; Omae, 2003). Coating the surface with antifouling paints released toxic elements such as copper oxidizing biocides like Cl, Br or peroxidases into the water column resulting in environmental pollution. More interestingly, the target groups for which such chemicals were used developed strategies to protect themselves from fouling (Armstrong *et al*., 2000a). A noteworthy observation by Douglas- Helders *et al.* (2003) and Longeon *et al*. (2004) was that despite heavy fouling, certain regions in the immersed structures had little or no biofouling impact. They understood that naturally, certain bacteria had developed a 'chemical weapon' to ward away these fouling agents. They isolated two proteins with molecular masses of approximately 100 and 190 kDa from the green pigmented bacteria, *Pseudoalteromonas tunicata* attached to these surfaces and found that it had immense antifouling activity. This leads the scientific community to look for alternate biological agents that could alternatively be used as chemicals, primarily derived from microbes, which produced secondary metabolites and other physical and mechanical strategies to remove the foulers. The use of the secondary metabolites from bacteria also exhibited antifouling activity against invertebrate macrofoulers like *Balanus amphitrite* and *Ciona intestinalis.* A number of groups have tried the approach of incorporating natural products into antifouling paints. The coatings exhibited significant protection against marine bacteria, barnacle larvae

and algal spores, revealing their application potential. These studies have opened up the immense scope of using the microbial biofilms or bacteria immobilized in hydrogels for controlling biofouling (Armstrong *et al*., 2000a; Peppiatt *et al*., 2000; Prochnow *et al*., 2004).

Several authors reviewed that the microbial approach is an inexpensive way to produce large amounts of potentially active biological antifouling agents. The Natural Product Antifouling agent (NPA) active ingredients characterized are forming the basis of further biotechnological research for heterologous production. A great deal of attention is currently focused on identifying non-toxic, environmentally friendly modes of their extraction and application. This has, therefore, paved the way for a new field of science, namely the long term green technology alternative (Douglas-Helders *et al*., 2003; Burgess *et al*., 2003; Peppiatt *et al*., 2000; Pettitt *et al*., 2004), where natural biocide paint or coating contains natural substances as the biocide to prevent fouling or hinder the fouling process. The combination of copper with the natural compound *quebracho tannin* has lowered the copper content in paint formulations by a factor of 40 when compared to that found in cuprous oxide paints. Today novel enzymes such as *Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases, Ligases* from the microbes form the future of producing antifouling agents (Aldred *et al*., 2008; Kristensen *et al*., 2008). Though these isolated compounds, both purified and crude, were effective in laboratory conditions to inhibit the growth of bacteria, their on-field trials received little success. A major breakthrough was attained by Persson *et al*. (2011), who reported that a secondary metabolite isolated from the red algae, *Bonnemaisonia hamifera* altered the composition and decreased the density of bacterial colonies when tested at various concentrations on submerged field panels.

Concordantly, Raveendran *et al*. (2011) demonstrated that the crude extract from eight coral species collected from Indian waters exhibited a reduction in settlement of larvae of Balanus. From these extracts, five natural antifoulants were identified and produced via synthetic routes.

Most of the antifoulants available to date have a biological origin. *Oroidin*, a natural antifoulant, was isolated from sponges of the family *Agelasidae* and has served as the template for creating over 50 synthetic analogues. Structure-activity relationship analysis has been widely used to design small molecules such as *Dihydrooroidin*, which has shown inhibitory activity against the formation of bacterial biofilms. These small molecules are incorporated in marine paint compositions as an alternative to metallic analogues (Melander *et al*., 2009). Similarly, *Furanones* secondary metabolites extracted from the surface of the red alga *Delisea pulchra* have been shown to deter fouling at natural concentrations both in the laboratory and in field testing (Dworjanyn *et al*., 2006). It is reported that *Furanones* inhibit bacterial colonization and biofilm formation and also repel larval attachment of the bryozoan *Bugula neritina* and the polychaetes (Steinberg *et al*., 1997; Lowery *et al*., 2009; Dobretsov *et al*., 2007). According to Michelis and Gougoulidis (2015), modern antifouling (AF) coatings are divided into two main categories, biocidal and non-biocidal. Dafforn *et al*. (2011) have reported that after the tributyltin (TBT) ban, copper has become the predominant AF biocide while additional 'booster biocides' are utilized to target copper tolerant marine species. Control Depletion Polymer (CDP) coatings are the most economical AF option offered in the market; however, they have certain deficiencies. They are less effective than the other biocidal coatings. Thus, they are only suitable for use in areas with lower fouling activity (Yebra *et al*., 2004; Almeida *et al*., 2007). In shipbuilding, Rosin based

paints which are high in silicates and ferric oxides, were conventionally used. Rosin based AF coatings may suffer physical degradation if they experience prolonged exposure to the atmosphere during drydocking or along the waterline of vessels (Lewis, 1998; Yebra *et al*., 2004). With the advent of Self Polishing Copolymer (SPC) paints, the earlier technologies, which were rather cumbersome, paved the way (Bressy *et al*., 2009). However, SPC coatings can display steady AF performance only until the entire paint film has been polished away. Thus the rate of biocide leaching is constant over time and controlled by the degree of polymerization and the hydrophilic character of the copolymer.

More resilient coating that is a hybrid of CDP and SPC coatings has yielded better results since their biocide-releasing mechanism is a mixture of hydrolysis and hydration (Lejars *et al*., 2012). It is also reported that the antifouling ability of the fouling release coatings is based on the low-friction and ultra smoothness of the surface they provide at a molecular level, which prevents fouling adherence. Their low surface energy degrades an organism's ability to generate a strong interfacial bond with the surface. The main types of Fire Retardant (FR) coatings currently in the market are silicone, fluoropolymer, hybrid and hydrogel silicone (Townsin and Anderson, 2009; Dafforn *et al*., 2011). Their reported lifetime in service is typically five to ten years (Lejars *et al*., 2012). Hard inert coatings are offered in the market as "high performance abrasion-resistant anticorrosive coatings" (Lewis, 2009). These coatings possess hard inert coatings such as epoxies, glass flake reinforced epoxies or polyesters, glass flake reinforced vinyl ester resins, surface treated composites (STCs), and ceramic epoxies. Although the durability of these products is beyond doubt proven, as witnessed in their wide-scale use in structures that are continuously in contact with water, such as tanks,

pipes etc., they still are ineffective to curtail the formation of biofilms and require serious maintenance quite often.

Many inherent issues potentially attributed to the vast diversity of biofilm-forming agents available have made progress in developing AF coatings based on natural biocides slow. Some of the difficulties faced by researchers regarding the incorporation of natural AF compounds into coatings is the identification of naturally produced materials that offer a broad-spectrum protection (Yebra *et al*., 2004; Chambers *et al*., 2006; Cao *et al*., 2011) and adequate lifespan (Cao *et al*., 2011), as well as their successful integration into a coating (Dafforn *et al.,* 2011). Moreover, the challenge for the coating industry is to source or produce natural AF compounds in sufficient quantities and at a reasonable cost while meeting the requirements of environmental regulation agencies (Qian *et al.,* 2010; Dafforn *et al.,* 2011). Regardless, biocidal and FR coatings alternatives have to compete against highly efficient and reasonably valued products and their applicability. Owing to the huge economic burden that it may pose on the paint industry, many producers are reluctant to depend on the biocidal agents in paints. A difference could be made if any short, easily synthesizable and sturdy biomolecule to act on a broad range of biofilm-forming bacteria is discovered. Natural antifouling strategies based on chemicals produced by new strains of actinomycetes have immense potential to be characterized and synthesized as biocidal components in the production of AF paints. Due to increasingly restrictive regulations on the polluting antifouling compounds, there is a growing need for other methods to prevent marine biofouling. For this reason, there is a need for the development of ‘‘environmentally-friendly’’ antifoulants. Since marine actinomycetes have the potential to produce non-toxic antifouling compounds as they can produce

substances that inhibit not only the attachment and/or growth of microorganisms but also the settlement of invertebrate larvae and macroalgal spore. Under the above background, a study was conducted to unravel the diversity of marine actinomycetes from a less explored Northern coast of Kerala for screening potential antifouling bioactive compounds. It is envisaged that through the exploratory surveys in different habitats of this region, a rich diversity of marine actinomycetes could be isolated, which can later be screened for characterizing potential antifouling agents.

Chapter 3

ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FROM NORTHERN COAST OF KERALA

3.1 Introduction

Being one of the most diverse ecosystems on earth, the marine biosphere is home to some of the unique habitats represented by many organisms, including the most versatile groups, namely the microbes. The importance of marine microbes is ever increasing as they are rapidly evolving as important and novel sources of various biologically active compounds and are receiving significant interest for their considerable benefits (Rasool and Srinivasan, 2017b). Marine microorganisms, therefore, have a cosmopolitan distribution dwelling not only in the surface waters of the sea but also in the lower and abyssal depths from coastal to the offshore regions, and from the general oceanic to the specialized niches including oceans, estuaries and lagoons, mangroves and coral reefs, deep-sea and the seafloor (Quasim, 1999). Among these microbes, Actinomycetes are one of the most efficient groups of secondary metabolite producers and are very important from an industrial point of view. They are classified as a group of gram-positive bacteria unique for their spore-forming abilities and formation of mycelia structures. These bacteria have been noted to serve as rich reservoirs of medicinal antibiotics and are extremely relevant to scientists, pharmaceutical industries and agricultural industries (Kumar *et al.*, 2010). The importance of Actinomycetes as prolific producers of novel antimicrobial agents has made them one of the most sought after groups of organisms in biopharmaceutical

research (Atta *et al*., 2010). Marine actinomycetes, among various other microbes, have played an important role in being the greatest source of novel compounds (Silambarasan *et al.*, 2012; Deepa *et al*., 2013), and they show a range of biological activities including antibacterial, antifungal, anticancer, insecticidal and enzyme inhibition. The number and types of actinomycetes present in a particular soil would be significantly influenced by geographical location, soil temperature, soil type, soil pH, organic matter content, cultivation, aeration, and moisture content (Arifuzzaman *et al*., 2010). The diversity of terrestrial Actinomycetes are of extraordinary significance in several areas of science and medicine, particularly in antibiotic production (Magarvey *et al*., 2004). Representative genera of actinomycetes that have shown promising results include *Streptomyces*, *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Frankia*, *Micrococcus*, *Micromonospora* etc. (Solanki *et al.,* 2008).

The diversity of actinomycetes in the marine habitat has also been related to the substrate where they are found (Goodfellow and Hyness, 1984). Seaweeds and coastal mangrove patches are a haven for a wide variety of actinomycetes, and a number of novel and chemically diverse antimicrobial compounds have been discovered from them (Srivibool and Sukchrotiratana 2006; Amrita *et al*., 2012). The mangrove environment in particular functions as a source of microorganisms, including actinomycetes capable of producing primary and secondary metabolites and the actinobacteria here have rich diversity and display various biological activities (Hong *et al.,* 2009; Azman *et al*., 2015; Sangkanu *et al*., 2017; Kumar *et al*., 2018). Seaweeds (macroalgae) support a unique association with the marine actinomycetes, usually which is symbiotic (Egan *et al.* 2008). Generally, the symbiosis of marine bacteria and marine organisms has a greater probability of producing chemical compounds than marine bacteria, which live

freely (Mearns-Spragg *et al*., 1998). Various novel compounds have already been identified from seaweed associated bacteria, especially actinobacteria. Many bioactive compounds that act either as antibiotics, antitumor, antifouling or antioxidant agents have been discovered from these marine microorganisms (El-Shatoury *et al*., 2009; Mastuo *et al.,* 2011).

Kerala, a Southwestern coastal state of India, has a coastline of about 590 km bestowed with rich and varied marine biodiversity. The variation in the coastal habitats supported by the outflow of 41 rivers in the Arabian Sea makes it one of the most productive fishery regions in the country. The Northern part of Kerala includes four coastal districts, namely Kasaragod, Kannur, Kozhikode and Malappuram. The region is relatively undisturbed and supports many marine flora and fauna, owing to its various habitats such as beaches, backwaters, estuaries, cliffs, lagoons, mangroves and coral reefs. Thus it forms an integral part of the marine biodiversity of India (Yadav *et al.,* 2015). Kerala has only less than 1% of India's total mangrove ecosystem majority of which is in the Northern part (Radhakrishnan *et al*., 2016). The minimal extent of mangroves is disturbed in discrete and isolated patches, mostly confined to the small flats of the delta, on the faces of estuaries and embayment margins of the coast. All along the Kerala coast, there are a good number of small mangrove stands, though mostly in isolated patches, fringing the estuaries and backwaters and around islets or along river margins in the coastline stretches. The seaweed diversity of Kerala is also high (Krishnamurthy *et al*., 1982). The northern parts of the Kerala coast are mainly sandy with scattered rocks and bedrocks that support the maximum seaweed numbers (Yadav *et al.,* 2015). The rich diversity and luxuriant growth of seaweeds were recorded

from Puduponnani, Beypore, Kappad, Thikkodi, Mahe, Thalassery, Ezhimala and Kappil beach of Northern Kerala.

Mangrove ecosystems are one of the most productive ecosystems, and under unique environmental conditions, the mangrove habitat contains abundant and characteristic microbial resources. Mangroves thrive in extreme climatic conditions, and hence the microorganisms associated with them too have capabilities to withstand this wide variation in environmental parameters. This often acts as a limiting factor for their growth, but to tide over unfavourable conditions, they produce metabolites that may have additional biochemical properties. Therefore, the only groups of microorganism equipped with highly complex survival mechanisms can grow and survive in this area, and one of these is the Actinomycetes. Actinomycetes are a subgroup of bacteria equipped with filament and have a high G+C content in their genetic materials (Katili and Retnowati, 2017). In the marine environment, bacteria are the most common colonizers on the surface of macroalgae (Armstrong *et al.,* 2000b), and seaweed-associated bacteria can synthesize biologically active, beneficial compounds that help the host organisms to survive under varied environmental conditions too (Sing *et al*., 2011). Seaweed surface supplies protected and nutrient-rich conditions for bacterial growth. Along with the different types of marine bacteria, actinomycetes are considered the most potent source for secondary metabolites (Janardhan *et al.*, 2014). Seaweeds can host verdant varieties of heterotrophic bacteria, and many of these bacteria play a cardinal role in maintaining the health of the host organism (Bolinches *et al.*, 1988) by producing unique bioactive secondary metabolites. Hence, epibiotic bacteria become an incredible bioactive compound and biocontrol agent source (Holmstrom and Kjelleberg, 1999). Since the quality and quantity of bioactive compounds isolated from

actinomycetes are directly related to the habitat and environmental conditions, there is a greater diversity among these terrestrial microbes and the chemicals they produce. Despite the wide reputation for producing these secondary metabolites, one of the greatest challenges for researchers is standardizing the isolation and characterization of available biomolecules. Many epifaunal actinomycetes, therefore, have been less studied, especially from the northern Kerala coast. Under the above backdrop, a study was conducted for isolating, characterizing and identifying potent strains of Actinomycetes from the Northern coast of Kerala that has capabilities of producing secondary metabolites.

3.2 Materials and methods

3.2.1 Study Area

The present study was carried out along the coastal areas of Northern Kerala. The sampling area spanned three districts of Northern Kerala viz., Malappuram, Kozhikode and Kannur. Monthly sampling from six ecologically diverse sites viz., Ponnani (Malappuram district), Kadalundi, Kotooli, Thikkodi and Payyoli (Kozhikode district) and Dharmadam (Kannur district) were carried out. The stations were so selected based on the variations in the habitats. Sampling was done seasonally for the Pre-monsoon, Monsoon, and Post monsoon period during 2016-17.

Care was taken to include mangroves, nearshore muddy and Intertidal rocky regions as stations to ascertain adequate representation of various ecological systems and their combinations. The areas where anthropogenic interactions like tourism, fishing activities, and waste disposal are experienced were also considered while fixing the stations.

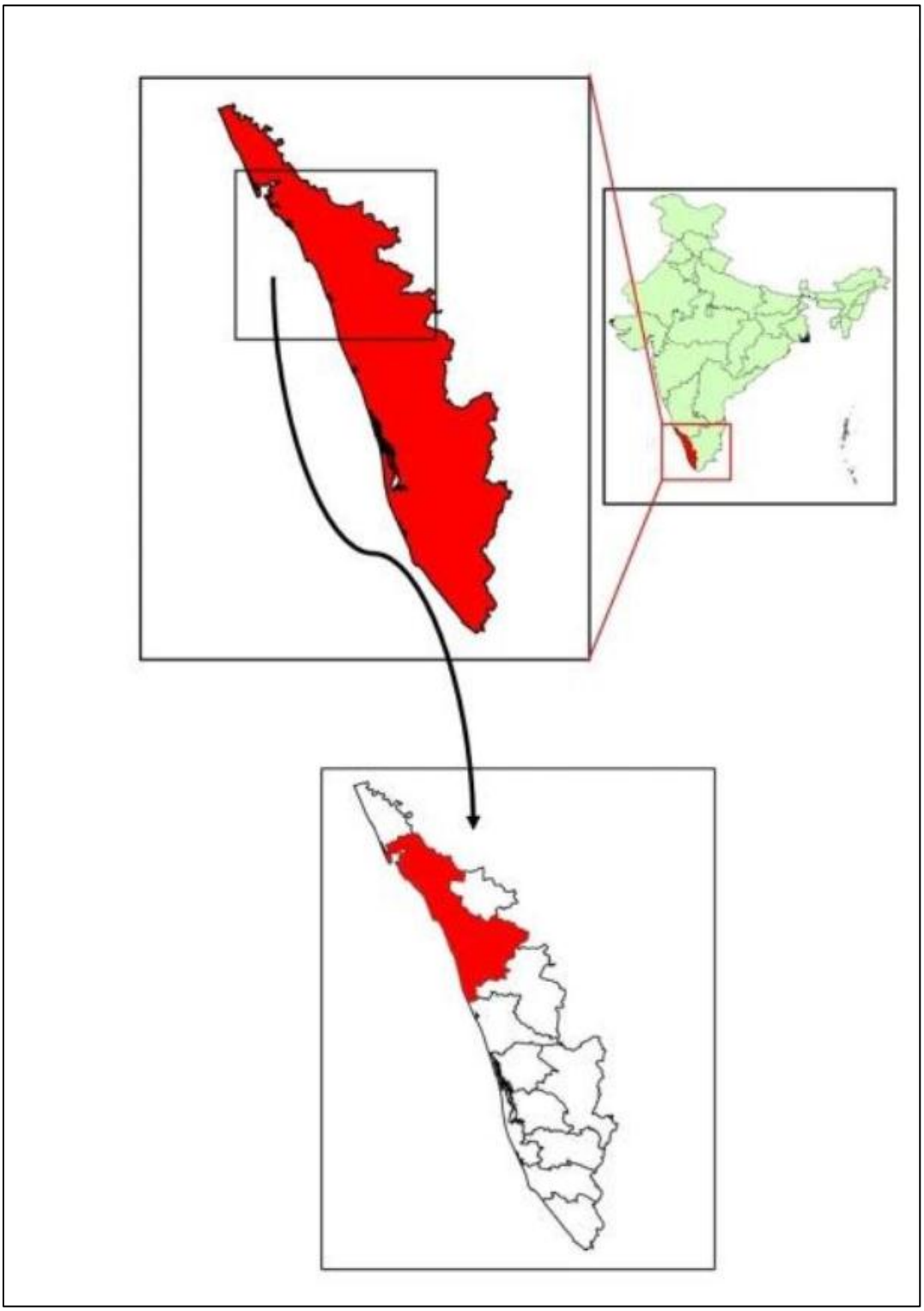


Figure 3.1 Map showing the study area in the South-west coast of India

Figure 3.2 Location map of six sampling station in the study area of Northern Kerala.

Ponnani, a coastal town in the Malappuram district, is one of the most populated regions in Northern Kerala. The region is supported by one of the largest estuarine systems (Lat 10° 46' and 10° 48' N and Long 75° 54' to 75° 56' E), which is an open estuary drained by a tributary of the Bharathappuzha River, the largest river of South India, and that drains into the Arabian Sea at this region. It is known for its fringing mangroves patches supporting a wide variety of fishes and birds.

Kadaludi mangrove ecosystems (Lat 11^0 07' and 11^0 13' N and Long 75^0 49' and 75^0 81' E). which stands as Kadalundi – Vallikkunnu Community Reserve located within Kozhikode and Malappuram districts, is also the first community reserve of Kerala, spreading across 1.5 sq. km. The area is a blend of various microhabitats, including patches of mangroves and a discontinuous estuarine system. Although human

intervention here has been limited to fishing, oyster and mussel collection, due to sand bars forming at the river's mouth, the area is also witnessing a gradual death of the mangrove vegetation (Rahees *et al*., 2014).

Kottoli (Lat 11^0 27' N and Long 75^0 79' E) and Payyoli (Lat 11^0 53' N and Long 75^0 62' E), a popular tourist place located in the Kozhikode district, are rich, exquisite shallow beaches and long stretches of silver sands. Being predominantly sandy, this region supports a diverse epibenthic fauna. The high saline soils without canopy cover were expected to display a different diversity of actinomycetes when compared to the mangrove regions. Hence this station was selected for the collection of typical coastal sediment based Actinomycetes of Northern Kerala.

Thikkodi (Lat 11^0 46'N and Long 75^0 64' E) is located in the Kozhikode district. Thikkodi coast has a long and wide rocky coast with black and clayish sand. The natural granite rocks and the artificially laid stones provide suitable substratum for the growth of many seaweed. Being one of the regions facing extreme environmental conditions, and since a fair portion of the coast was exposed during low tide periods, this intertidal region was selected for isolating different forms of actinomycetes capable of withstanding the climatic vagaries and those that close association with the seaweeds for its sustenance.

Dharmadom (Lat 11^0 46' N and Long 75^0 27' E) is a station in the vicinity of Dharmadom estuary in Kannur district, which has a different faunal assemblage with respect to seaweed diversity. Being a rocky shore, the region has a connection with the estuary at two locations, and the intertidal region is a mixture of the rocky and sandy substrate, providing a favourable habitat for the occurrence of diverse actinomycetes

species. The area is dominant with some seaweed species like *Gracilaria corticata* and subdominant species like *Chaetomorpha antenna*, *Bryopsis plumose*, *Grateloupia filicina* and *G. lithophil*e, *Hypnea valentine* etc.(Ushakiran *et al*., 2017). All the stations were easily accessible, and sampling was conducted based on the lunar periodicity as per the tide table. A detailed record of the associated flora and fauna from each station was also recorded during the surveys.

3.2.2 Sampling Procedure

Mangrove sediment samples were aseptically collected in sterile polyethelene bags from Ponnani and Kadalundi mangrove areas, especially from the rhizosphere region of the soil using sterile PVC pipe at a depth of 5-15 cm. Likewise, typical coastal sediment samples were also aseptically collected from a depth of 10-20 cm, mainly from the nearshore regions in sterile polyethelene bags from coastal areas of Kottoli and Payyoli stations. Seaweed samples were collected from the rocky intertidal shores of Thikkodi and Dharmadam during low tide periods. The seaweed samples were separately collected using a sterile spatula in sterile ziplock bags and marked accordingly. Sampling was conducted thrice in a year (representing the three-season) during pre-monsoon season (February to May), monsoon season (June to September) and post-monsoon season (October to January). For each sampling site, sub-samples of sediments were collected, pooled together and homogenized to obtain a representative sample. All the collected samples were placed in an icebox and transferred aseptically to the laboratory. Water samples were also collected using clean plastic bottles for estimating water quality parameters that required laboratory analysis.

3.2.3 Physico-chemical Parameters

Water and sediment samples were analyzed from all the stations and during all seasons to interrelate their variations, if any, with the diversity and distribution pattern of actinomycetes. The sampling was done during 2016-17, and multiple samplings were done routinely every season to have a more comprehensive representation of water quality parameters and actinomycetes in the samples collected. The physico-chemical parameters analyzed at the station included water temperature, water pH, salinity and soil pH. In contrast, the dissolved oxygen, nutrients (nitrate and phosphate) and organic carbon were analyzed in the laboratory after acid fixing the samples. The samples for the determination of dissolved oxygen were collected in glass stoppered BOD bottles. For analyzing nutrients, samples were collected in 1L plastic bottles and stored in a deep freezer (Arctiko ULT Freezer) until analysis. The methods and instruments used to determine parameters were as per following (Table 3.1).

Table 3.1 Instruments and analytical methods followed for water and sediment analysis

Sl. No.	Parameter	Unit	Method	Instrument
1	Temperature	^{0}C	Thermometry (APHA, 2005)	Mercury bulb thermometer
2	Salinity	ppt	-	Salino-refracto meter
3	Dissolved oxygen	mg/L	Modified Winkler Method	-
4	Soil pH	-		Portable Hand Soil pH meter
5	Water pH	-	Electrometry (APHA, 2005)	Portable digital pH meter
6	Nitrate	mg/L	Grasshoff's method	UV-Vis Spectrophotometer
7	Phosphate	µg/L	Grasshoff's method	UV-Vis Spectrophotometer
8	Organic carbon	%	Walkley-Black method	-

3.2.4 Isolation of Actinomycetes

3.2.4.1 Pretreatment of sediment samples

Sediment collected from mangrove and nearshore regions were air-dried at room temperature for a week and then transferred to sterile petriplates and kept at 55^0 C for 10 minutes to retard the growth of slime forming bacteria (Pisano *et al*., 1986).

3.2.4.2 Isolation of Actinomycetes from sediment

Isolation of actinomycetes was done by the pour plate technique (Gopikrishnan *et al*., 2013). Ten grams of pretreated sediment samples were taken and ground in a sterile mortar with 90 ml of sterile distilled water and serially diluted up to 10^{-5} dilutions. Starch Casein Agar (SCA) medium was prepared in 50% seawater and isolated culturable actinomycetes. The SCA medium was supplemented with Nalidixic acid (20 µg/ml) and Cycloheximide (50 µg/ml) to retard the growth of bacteria (other than actinomycetes) and fungi, respectively. About 1 ml of an aliquot from each dilution were transferred into an SCA medium, and isolation was done by the pour plate technique. All the plates were incubated at 28 ± 2^oC and observed from the 5th day onwards for one month. The same procedure was followed for all the samples from all the stations. Colonies with suspected actinomycetes morphology were enumerated, selected and purified by quadrant streaking method using yeast extract –malt extract agar medium (Shirling and Gottileb, 1966). The pure cultures of the actinomycetes were maintained as slant stock on ISP2 agar as well as in 30% glycerol broth at 4^0C, as mentioned by Bavya *et al.,* 2011.

3.2.4.3 Isolation of Actinomycetes from seaweeds

The collected seaweed samples were repeatedly rinsed with sterile seawater to remove any associated epiphytes, salt, sand particles, microbes, debris and other

suspended materials. About 10g of the cleaned thallus of each seaweed were cut and separately added in 90ml of sterile buffer and serially diluted up to 10^{-5} dilution. About1 ml of aliquots from each dilution were transferred to Starch Casein Agar medium by pour plate technique. After sterilizing media, the media supplemented with 25 µg/ml Ketoconazole and 25 µg/ml Ciprofloxacin to eliminate fungal contamination and fast-growing bacteria (Ulfa *et al*., 2017). The plates were incubated at 30^{o}C and observed from the 5th day onwards for one month. Based on colonial morphologies, different bacteria were chosen and purified by using the streak plates method (Pikoli *et al*., 2000) on the ISP2 agar medium. These purified cultures were also maintained as a slant stock culture on the ISP2 agar slant in 30% glycerol broth at 4^{0}C.

3.2.5 Characterization of Actinomycetes

All the isolated actinomycetes from sediment and seaweeds samples were subjected to morphological or cultural, biochemical and physiological characterization.

3.2.5.1 Morphological and Cultural Characterization

Morphology

Morphological and physiological properties of the strains were studied as per the International Streptomyces Project (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989). Morphological characters of the selected isolates were studied by inoculating the isolates onto different sterile media such as

1) Starch Casein Agar (Starch 10.0 g; Casein 1.0 g; K_2HPO_4 0.7 g; KH_2PO_4 0.3 g; $MgSO_4.7H_2O$ 0.5 g; $FeSO_4.7H_2O$ 0.01 g; $ZnSO_4$ 0.001 g; Agar 20.0 g; seawater 1L; pH 7.2)

2) Yeast Extract-Malt Extract Agar (ISP 2) (Yeast extract 4.0 g; Malt extract 10.0 g; Dextrose 4.0 g; Agar 20.0 g; seawater 1L; pH 7.2)

3) Glycerol Asparagine Agar (ISP 5) (L- Asparagine 1.0 g; Glycerol 10.0 g; K_2HPO_4 1 g; Agar 20.0 g; seawater 1 L; pH 7.2) and

4) Nutrient Agar (Peptone 0.5 g; Beef extract 0.3 g; Agar 2 g; seawater 100 ml)

The media was sterilized and poured into sterile Petri dishes. After solidification of the media, isolates were streaked aseptically and incubated at 30°C for seven days. Morphological characters such as colony characteristics (size, shape, colour, consistency), pigment production, absence or presence of aerial and substrate mycelium were observed under the Stereomicroscope (Tresner *et al*., 1961)

Coverslip culture technique

The arrangement of spores and sporulating structures of Actinomycetes was studied microscopically using the coverslip culture method. The inoculation of isolates was carried out in Starch Casein Broth media and was incubated for 1-2 days at 28°C. Four sterile coverslips were inserted at an angle of 45° into the Starch Casein Agar medium (Cross and Williams, 1971). A loopful of spore suspension of actinomycete was dispensed at the intersection of the medium and coverslip. The plates were then incubated at 28°C for seven days. The coverslip was carefully removed using sterile forceps, placed upward on a clean glass slide, and observed under high power and oil immersion objectives.

3.2.5.2 Biochemical and Physiological Characterization

All the pure cultures of actinomycetes strains isolated from sediment and seaweeds were subjected to biochemical characterization since it is very important to characterize the actinomycetes isolates to understand the basic physiology of

Actinomycetes. The physiological and biochemical tests for characterization of aerobic sporogenous actinomycetes was done as per International Streptomyces Project (Shirling and Gottlieb, 1966), Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989), UK standards for microbiology investigations and Berd (1973), with slight modifications.

Oxidase Test

Oxidase reaction was carried out by using oxidase discs (HiMedia). Actinomycetes spore mass were spread on the oxidase disc using sterilized wooden sticks. The reaction was observed within 5-10 seconds at 36±1.0ºC. A colour change from deep dark to blue is taken as a positive result. A change later than 10 seconds or no change at all was considered a negative reaction.

Indole Production

Peptone water (HiMedia; composition; Peptone 10 g; Sodium chloride 5g; Final pH (at 25°C) 7.2±0.2) was used for the Indole production study, and the presence of indole was demonstrated using Kovac's reagent (Himedia). About 5 ml of sterile Peptone water is inoculated with actinomycetes isolates in test tubes. The tubes were then incubated at 36 ± 1.0ºC for 24 - 48 hours and then added with 0.2 - 0.3 ml of Kovac's reagent. The appearance of a red coloured ring in the surface layer within 10 minutes is noted as a positive result.

Methyl-red test

In the Methyl Red (MR) test, MR-VP broth tubes (HiMedia; composition; Buffered peptone 7.0g; Dextrose (Glucose) 5.0g; Dipotassium phosphate 5.0g; Final pH (at 25°C) 6.9±0.2) were inoculated with the actinomycetes isolates. Incubated at 36

± 1.0ºC for 48 h, five drops of methyl red indicator (HiMedia) were added. A change in the colour to yellow is recorded as negative.

Voges-Proskauer test

In the Voges-Proskauer **(**VP) test, MR-VP broth tubes (HiMedia; composition; Buffered peptone 7.0g; Dextrose (Glucose) 5.0g; Dipotassium phosphate 5.0g; Final pH (at 25°C) 6.9±0.2) were inoculated with the actinomycetes isolates and incubated at 36 ± 1.0ºC for 48 h. This was added 12 drops of V-P reagent I and 2-3 drops of V-P reagent II (both HiMedia). No change in colouration in the inoculated broth was taken as a negative result.

Citrate utilization

Simmon's Citrate Agar medium (HiMedia) ($(NH_4)_2HPO_4$ 1.0 g; Sodium citrate 0.2 g; K_2HPO_4 1.0 g; $MgSO_4.7H_2O$ 0.02 g; NaCl 15.0 g; bromothymol blue 0.02 g; Agar 20 g; seawater 1 L; pH 6.9) was used for testing citrate utilization. The colour change from green to Prussian blue of the medium was recorded as a positive result.

Nitrate reduction

Nitrate broth supplemented with potassium nitrate (0.1%) was used for the nitrate reduction test. An inverted Durham's tube was introduced into the basal medium (Peptone 5.0 g; Potassium nitrate 1.0 g; Beef extract 3.0 g; seawater 1L; pH 7.2). The medium was inoculated with a spore suspension of isolates and incubated for one week with un-inoculated tubes as a negative control. Equal volumes of reagents A (α-naphthyl amine 0.5 g; acetic acid (5 N) 100 ml) and B (sulfanilic acid 0.8 g; acetic acid (5 N) 100 ml) was added to the inoculated tubes to detect Nitrate reduction. The red colour formation due to azo dye indicated nitrate reduction to be positive. If the suspension turned colourless after the addition of reagents, a small pinch of zinc dust was added to

the medium to find if the medium remained colourless for a positive result. Turning medium into pink after the addition of zinc powder was considered as a negative result.

Hydrogen sulfide production

Hydrogen sulphide production was detected along with the nitrate reduction test. Sterile lead acetate strips (5%) were inserted through the mouth of the test tube of the nitrate broth when the nitrate reduction test is carried out. A positive result is the blackening of the strips.

3.2.5.3 Enzyme production

The isolates were screened for the production of various hydrolytic enzymes, viz., Catalase, Urease, Amylase, Caseinase, Gelatinase, and Lipase.

Catalase

A catalase test was performed by adding 3% hydrogen peroxide solution (H_2O_2) to a heavy suspension of isolates on a microscopic glass slide. Within one minute after the addition of H_2O_2, Catalase positive cultures produced bubbles of oxygen. The release of free oxygen gas bubbles was considered a positive catalase test.

Urease

Christensen's Urea Agar medium (HiMedia; composition; Peptone 1g; Dextrose (Glucose) 1g; Sodium chloride 5g; Disodium hydrogen phosphate 1.20g; Potassium dihydrogen phosphate 0.8g; Phenol red 0.012g; Agar 15g; Distilled water 1L; Final pH (at 25°C) 6.8±0.2) was used to test the production of urease. The above ingredients except urea were dissolved in 950 ml of distilled seawater and autoclaved at 15 lbs for 15 minutes. Aseptically prepared 50 ml of 40% sterile urea solution was added to the basal medium and prepared agar slants (3 ml each). Cultures were

inoculated, and tubes were incubated at 36 ± 1.0ºC for 18-24 hours. A change of colour in the medium from yellow to pink was noted as positive for urea hydrolysis.

Amylase

Amylase activity was tested using starch (1%) supplemented nutrient agar medium (Agar 2 g; Beef extract 0.3 g; Peptone 0.5 g; seawater 100 ml; pH 7.0). Spot inoculation of the culture plates was followed for testing the activity of enzymes. The inoculated culture plates were incubated for seven days. Then, 2% potassium iodide solution was flooded on the culture plate. A clear zone around the culture spot was a positive result for amylase activity.

Caseinase

Screening of enzyme caseinase was done using Skim Milk Agar (Skim milk powder 10 g; Agar 2g; Distilled seawater 100ml; distilled water 100ml). Autoclaved 10 g of skim milk in 100 ml distilled water. Then two grams of agar was added to 100 ml of seawater and were autoclaved separately. Sterile skim milk and agar were mixed thoroughly and poured into Petri plates. Isolates were spot inoculated, and the plates were incubated at 36 ± 1.0ºC for 5-7 days. Decomposition of casein was observed by clearing skim milk agar around the colony and was recorded as positive.

Gelatinase

Nutrient agar medium (peptone 0.5 g; beef extract 0.3 g; agar 2 g; sea water 100 ml; pH 7.0) supplemented with gelatin (2%) were prepared for gelatinase assay. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 3 to5 days. Clearing around the growth after flooding with Frazier's reagent is recorded as positive results for gelatinase production.

Lipase

Lipase activity was tested using Nutrient agar medium (peptone 0.5 g; beef extract 0.3 g; agar 2 g; sea water 100 ml; pH 7.0) supplemented with Tributyrin (1%). Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 3 to 5 days, and observations were made. A clear zone around the colonies on Tributyrin agar for the production of lipase.

Carbon source utilization tests

The carbon source utilization test plays an important role in the taxonomic characterization of actinomycete strains (Pridham and Gottlieb, 1948). Fourteen different carbon sources like glucose, lactose, sucrose, xylose, dextrose, salicin, arabinose, rhamnose, raffinose, cellobiose, fructose, mannitol, inositol, and galactose were carried out. In the present study, carbohydrate differentiation discs (HiMedia) were used to differentiate bacteria based on their ability to ferment different carbohydrate sources except for glucose.

To examine the glucose fermentation ability of isolates typical microbiological method using peptone water broth base was employed. For this, the actinomycete isolates were inoculated in peptone water broth containing 1% solution of glucose (total amount of sugar was kept at 10%) and to which phenol red was added as an indicator of acid production. Inserted an inverted Durham's tube in the culture tubes and incubated at 36 ± 1.0ºC for 18-48 hours. Development of yellow colour and gas formation in Durham's tube and colour change was considered positive. For all other carbon sources, carbohydrate differentiation discs on phenol red agar base were used. Sterile plates containing the agar medium were surface seeded with actinomycetes isolates, and carbohydrate discs were placed and pressed gently on the surface of the plate at a

sufficient distance from each other. Incubation was carried out at 36 ± 1.0ºC for 18-48 hours, and results were recorded at 18 - 24 hours and again at 48 hours. The results were frequently observed since the reversal of fermentation reaction can take place. Agar plates fermentation is visualized by a change in colour around the disc. When a microorganism ferments a carbohydrate, the acid (or acid and gas) produced lowers the pH of the medium, and the indicator in the basal medium thus changes colour (e.g. phenol red changes from red to orange to yellow) and recorded as positive.

3.3 Results

3.3.1 Composition of Actinobacteria and general distribution

The present study results indicated that the region had a good diversity of actinomycetes and was represented by 529 isolates. All the isolates were subjected to morphological and biochemical characteristics. On further analysis, it was seen that they belonged to nine genera and seven families. The genus comprises *Streptomyces, Nocardiopsis, Kitasatospora, Streptosporangium, Micromonospora, Nocardia, Rhodococcus, Actinopolyspora and Actinomadura.*

Table 3.2. Biochemical characterization of actinomycetes isolated from Northern coast of Kerala

Characteristics	Representative Colonies with a positive result	Possible Species
Aerial mass colour		
White	94	SM, KS, ND, SP, AP, MM, AM
White change to Grey	75	SM, KS, NO, ND, SP, AP
Grey	82	SM, KS, NO,
White cream/Yellow	116	SM, AM, NO, SP
Olive Green	13	SM
White change to blue	12	SM, AM
White change to pink	18	SM, SP
Red/Orange/Pink	27	SM, RD, NO, MM, SP

Substrate Mycelium		
Substrate Mycelium	488	SM, KS, AP, SP, AM
Fragmented substrate Mycelium	21	NO
Sporophore morphology		
Sporangia formation	209	SM, SP, AM, AP
Straight	62	SM
Spiral	103	SM
Flexuous	84	SM
Retinaculum apertum	28	SM
Conidia formation	12	AM, AP
Pigment production		
Melanin	96	SM
Reverse colour	75	SM
Soluble colour	70	SM
Isolates showing pigmentation	91	SM
Carbohydrates utilized by the isolates		
Glucose	432	SM,KS,ND,SP, MM,RD,AP,AM
Lactose	312	SM,KS,ND,SP,MM,NO,RD
Sucrose	348	SM, KS, ND, SP, MM, NO, RD, AM
Xylose	423	SM, KS, SP, MM, NO, RD
Dextrose	198	SM, KS, ND, SP, NO, RD
Salicin	278	SM, KS, ND, SP, MM, NO, RD
Arabinose	410	SM, KS, ND, SP, MM, NO, AM
Rhamnose	255	SM, KS, ND, SP, MM, NO, ND, RD,AM
Raffinose	384	SM, KS, ND,MM, NO, ND, RD
Cellobiose	220	SM, KS, ND, MM, NO,ND,AM
Fructose	418	SM, KS, ND, MM, NO, ND, AM,AP
Mannitol	324	SM, KS, ND, MM, AM, AP, SP
Inositol	110	SM, ND, MM, AP, SP, NO,AM
Galactose	358	SM, ND,MM, NO,ND
Enzyme Activity		
Oxidase	144	SM, AM, AP, KS
Catalase	324	SM, KS, RD
Urease	360	SM, KS, MM, RD, SP
Amylase	270	SM, KS, MM, AM
Gelatinase	342	SM, KS, MM, AM, RD
Caseinase	324	SM, KS, MM, AM, RD, SP
Lipase	288	SM, KS, MM, AM, RD
Presence of Di Amino Pimelic Acid	276	SM

SM- *Streptomyces* spp.**; KS** – *Kitasatospora* spp.**; ND-** *Noccardiopsis* spp.**;**
SP – *Streptosporangium* spp.**; MM** – *Micromonospora* spp.**; NO -** *Nocardia* spp.**;**
RD – *Rhodococcus* spp.**, AP -** *Actinopolyspora* spp.**, AM -** *Actinomadura* spp.

Very few studies have been reported on the isolation and identification of Actinomycetes from mangrove sediment and seaweeds of the Northern coast of Kerala. In the present study, based on the results of colony morphology, biochemical and enzymatic characterization, the 529 isolates studied were grouped under 30 species falling under nine genera and seven families. On further analysis, it was seen that the majority of the actinomycetes belonged to the genera Streptomyces (53.9%) followed by *Kitasatospora* (9.1%), *Nocardiopsis* (8.7%), *Streptosporangium* (8.3%), *Micromonospora* (5.3%), *Nocardia* (4.9%), *Rhodococcus* (4.0%), *Actinopolyspora* (3.0%) and *Actinomadura* (2.8%). A total of 30 species were identified from the sediment and seaweed samples, and the species diversity was high for genus Streptomyces (15 species), followed by *Nocardiopsis* and *Kitasatospora* (3 each).

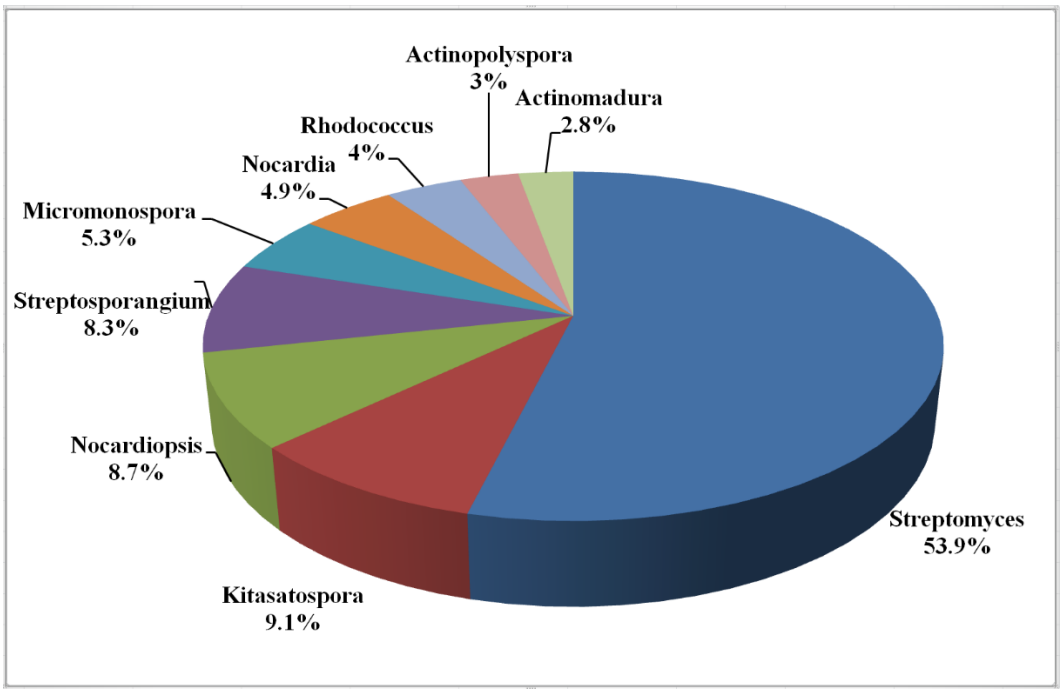


Figure 3.3. Percentage composition of Actinobacteria of Northern Kerala

Table 3.3 Abundance of Actinomycetes isolates from six stations and three seasons during 2016-17

	Ponnani			Kadalundi			Thikkodi			Payyoli			Dharmadam			Kotolli		
Species	**Pre**	**Monsoon**	**Post**	**Pre**	**Monsoon**	**Post**	**Pre**	**Monsoon**	**Post**	**Pre**	**Monsoon**	**Post**	**Pre**	**Monsoon**	**Post**	**Pre**	**Monsoon**	**Post**
Streptomyces sp1	0	0	1	4	1	2	0	0	0	3	1	1	0	0	0	0	0	0
Streptomyces sp.2	4	0	2	0	0	1	2	0	0	0	0	0	0	0	0	4	3	3
Streptomyces sp.3	0	0	0	3	2	3	0	0	0	2	0	0	2	2	1	0	0	1
Streptomyces sp.4	2	0	0	2	1	1	3	0	1	0	0	0	0	0	0	0	2	2
Streptomyces sp.5	3	3	5	0	0	1	0	0	0	4	3	4	0	0	0	0	1	0
Streptomyces sp.6	2	0	0	5	0	4	0	0	0	3	3	4	0	0	0	0	0	0
Streptomyces sp.7	3	2	0	5	2	1	2	1	1	0	0	1	0	0	0	1	2	1
Streptomyces sp.8	6	3	3	2	2	2	0	0	0	2	0	0	2	0	0	5	0	2
Streptomyces sp.9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	3	3
Streptomyces sp.10	7	5	6	4	3	3	0	0	1	0	0	0	0	0	0	0	0	0
Streptomyces sp.11	0	0	0	3	0	3	0	0	0	6	2	2	0	0	0	0	0	0
Streptomyces sp.12	0	0	1	4		1	0	0	0	0	0	1	1	0	0	1	0	1
Streptomyces sp.13	2	2	2	6	0	0	1	0	0	1	1	1	3	3	2	0	0	0
Streptomyces sp.14	1	1	2	5	1	3	2	1	1	5	3	3	0	0	0	2	0	0
Streptomyces sp.15	0	0	0	0	0	0	3	1	2	0	0	0	0	0	0	0	0	0
Nocardiopsis sp.1	0	0	0	1	0	0	0	0	0	4	0	3	0	0	0	0	0	2
Nocardiopsis sp.2	3	0	3	6	0	2	1	0	1	0	0	0	0	0	0	3	0	2
Nocardiopsis sp.3	2	0	0	2	2	2	0	0	0	1	0	0	0	0	0	3	2	1
Kitasatospora sp.1	4	4	0	0	0	0	0	3	0	3	1	1	0	0	0	3	0	3
Kitasatospora sp2	0	0	0	4	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Kitasatospora sp3	0	0	3	1	0	0	0	0	0	0	0	2	0	0	0	6	4	4
Micromonospora sp 1	0	0	0	0	0	0	0	0	0	0	0	0	4	2	3	2	0	1
Micromonospora sp.2	2	1	2	4	3	2	0	0	0	0	0	0	1	0	1	0	0	0
Streptosporangium sp.1	0	0	0	3	3	5	2	1	1	3	2	3	0	0	0	3	3	2
Streptosporangium sp .2	0	0	1	2	0	2	0	0	0	3	3	0	0	0	0	2	0	0
Nocardia sp.1	3	2	2	4	3	2	0	0	0	2	1	1	0	0	0	0	0	0
Nocardia sp.2	1	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	1
Actinomadura sp.1	0	0	0	0	0	0	5	3	4	1	0	2	0	0	0	0	0	0
Actinopolyspora sp.1	0	0	0	3	0	3	0	0	0	5	1	4	0	0	0	0	0	0
Rhodococcus sp.1	0	0	0	3	0	1	0	0	0	0	0	0	4	3	2	3		2

3.3.2 Morphological and Cultural characterization of Actinomycetes isolates

The actinomycete isolates exhibited good growth in the ISP medium (ISP 2) and starch casein agar. The majority of the isolates grew within 3-5 days, and sporulation was noticed after 5-7 days. The colour of mature sporulating aerial mycelium was recorded by visual observation as white/off the white, grey, olive green, brown, lemon yellow, yellow, blue, pink and orange. Various types of spore chain morphology were also noticed for actinomycetes grown in coverslip and observed under high power and oil immersion objectives. Typically four types of spore chain morphology were observed. The most prominent morphology was the spiral one (39%, mostly verticillate type), followed by 33% exhibiting rectiflexibiles (straight to flexuous) and 11% retinaculiaperti (open hooks, loops or spirals with one to two turns) spore chain morphology. The remaining 17% of the isolates exhibited a long chain of spores with zigzag fragmenting hyphae. Meanwhile, four types of spore surface morphology were observed, including smooth, spiny, warty and hairy (Nonomura, 1974). The isolated Actinomycetes were primarily grouped and identified based on their colony morphology (Table 3.4).

Table 3.4 Morphological characteristics of selected Actinomycetes isolates.

Species	Substrate mycelium	Aerial mycelium	Reverse side colour	Spore chain morphology	Microscopic morphology
Streptomyces sp.1	White, powdery, concentric	Ash/grey	Brown/yellowish brown	Spiral	Rod
Streptomyces sp.2	Off white	Olive green	_	Retinaculiaperti	Short rod
Streptomyces sp.3	White, powdery	White	Off white	Straight	Oval
Streptomyces sp.4	Off white, umbonate	Off white	-	Simple Spira	Long rod
Streptomyces sp.5	Off white, velvety	Dark yellow	Yellow	Rectiflexibiles	Rod
Streptomyces sp.6	White, small, concentric	Off white	-	Spiral	Cocci
Streptomyces sp.7	Slimy	White	-	Rectiflexibilis	Rod
Streptomyces sp.8	Pale yellow	Bluish white	-	Rectiflexibiles	Rod
Streptomyces sp.9	Off white, concentric	White,	-	Spiral	Cocci
Streptomyces sp.10	White	Yellowish white	Off white	Spiral	Rod
Streptomyces sp.11	Off white	White	Yellow	Rectiflexibiles	Globoid
Streptomyces sp.12	Dark brown	Pure white	-	Straight rod	Rod
Streptomyces sp.13	brownish	Pink	Dark brown	Long-chain of Spores	Long rods
Streptomyces sp.14	Grey	Greyish brown	Dark brown	Spiral	Rod
Streptomyces sp.15	Grey	Dark grey	Dark grey	Simple Spiral	Rod
Kitasatospora sp.1	Dark Brown	Dark grey	Brown	Rectiflexibiles	Cylindrical
Kitasatospora sp.2	Yellowish Brown	Grey	Brown	Rectiflexibiles	Cylindrical
Kitasatospora sp.3	Creamy white	Dull white	Creamy yellow	Rectiflexibiles	Cylindrical
Nocardiopsis sp.1	White	Grey, wrinkled	Greenish Brown	Zig-zag	Coccoid
Nocardiopsis sp.2	Grey	Grey	Dull Brown	Zig-zag	Coccoid
Nocardiopsis sp.3	Grey	White	Creamy yellow	Zig-zag	Coccoid
Micromonospora sp.1	Yellowish-brown	Absent	Pale yellow	Branched clusters	Dolifoform
Micromonospora sp.2	yellow	Lemon yellow	Pale yellow	Monopolar monotrichous	Oval
Streptosporangium sp.1	White	Brownish	Light yellow	Spiral	Globoid
Streptosporangium sp.2	Yellow	Pink or White	Creamy yellow	Spiral	Spherical
Nocardia sp.1	Light brown	Orange colour, Patchy	Fiant brown	Rectiflexibiles	Long rods
Nocardia sp.2	White	Ash, dry, chalky	Yellow	Rectiflexibiles	rods
Actinomadura	Creamy white	Blue	-	Irregular spiral	Long rods
Actinopolyspora	White	Chalky white	-	Straight to flexuous	Rod
Rhodococcus	Pale yellow	Orange slimy	Light brown	Spiral	Irregular rods and Cocci

3.3.3 Biochemical and Physiological Characterization of Actinomycetes isolates

All the isolated 529 actinomycetes were subjected to a series of biochemical characterization. Out of 529 isolates, Nitrate reduction was carried out by 337 (64%) isolates and 266 (50.2%) isolates that produced hydrogen sulphide as indicated by blackening of lead acetate strips. The result of hydrogen sulphide production and Nitrogen reduction of selected species of actinomycetes are given below (Table 3.5).

Table 3.5 Biochemical characterization of selected actinomycetes isolates

No	Isolates	Indole production	Methyl red	Voges Proskauer	Citrate utilization	H_2S production	Nitrate reduction
1	*Streptomyces sp.1*	+	+	+	-	-	+
2	*Streptomyces sp.2*	-	+	-	+	-	+
3	*Streptomyces sp.3*	-	+	-	+	+	+
4	*Streptomyces sp.4*	-	+	+	-	-	+
5	*Streptomyces sp.5*	+	-	+	-	+	-
6	*Streptomyces sp.6*	-	+	+	-	-	+
7	*Streptomyces sp.7*	-	+	+	-	-	+
8	*Streptomyces sp.8*	-	+	-	-	+	-
9	*Streptomyces sp.9*	+	+	+	-	-	+
10	*Streptomyces sp.10*	-	+	+	-	-	-
11	*Streptomyces sp.11*	-	+	-	-	-	+
12	*Streptomyces sp.12*	-	-	+	+	-	+
13	*Streptomyces sp.13*	+	+	-	-	-	+
14	*Streptomyces sp.14*	+	+	-	+	+	+
15	*Streptomyces sp.15*	-	+	-	-	+	+
16	*Nocardiopsis sp.1*	+	+	+	-	-	+
17	*Nocardiopsis sp.2*	+	+	+	-	-	+
18	*Nocardiopsis sp.3*	-	+	-	-	+	+
19	*Kitasatospora sp.1*	-	-	+	+	+	-
20	*Kitasatospora sp.2*	-	-	+	-	-	+
21	*Kitasatospora sp.3*	-	+	+	-	+	+
22	*Micromonospora sp.1*	+	+	-	-	+	-
23	*Micromonospora sp.2*	+	+	+	-	+	-
24	*Streptosporangium sp.1*	+	-	-	-	+	-
25	*Streptosporangium sp.2*	+	-	-	-	+	+
26	*Nocardia sp.1*	+	-	-	+	+	-
27	*Nocardia sp.2*	+		-	-	+	-
28	*Actinomadura sp.1*	+	-	+	-	-	-
29	*Actinopolyspora sp.1*	+	-	+	-	+	-
30	*Rhodococcus sp.1*	-	+	+	-	-	+

3.3.3.1 Enzyme activity of Actinomycetes isolates

Results of enzymatic activity on isolates of actinomycetes showed better production of various hydrolytic enzymes viz., Amylase, Gelatinase, Caseinase, Lipase, Oxidase, Catalase, Urease (Table 3.6).

Table 3.6 Enzyme production of selected Actinomycetes isolates.

No	Isolates	Oxidase	Catalase	Urease	Amylase	Gelatinase	Caseinase	Lipase
1	*Streptomyces sp. 1*	_	_	+	+	+	_	+
2	*Streptomyces sp. 2*	_	+	+	+	+	_	_
3	*Streptomyces sp. 3*	_	+	_	_	+	+	_
4	*Streptomyces SP. 4*	+	+	+	_	_	+	+
5	*Streptomyces sp. 5*	_	+	_	_	_	+	+
6	*Streptomyces sp. 6*	_	_	+	+	+	_	+
7	*Streptomyces sp. 7*	_	_	+	+	+	_	_
8	*Streptomyces sp. 8*	_	+	+	_	+	+	+
9	*Streptomyces sp. 9*	_	_	+	_	+	+	+
10	*Streptomyces sp. 10*	_	_	_	_	_	+	_
11	*Streptomyces sp. 11*	+	+	+	+	+	+	+
12	*Streptomyces sp. 12*	_	+	_	+	+	_	+
13	*Streptomyces sp. 13*	_	+	+	_	+	_	_
14	*Streptomyces sp. 14*	_	+	+	_	_	+	+
15	*Streptomyces sp. 15*	_	+	_	+	_	+	+
16	*Nocardiopsis sp. 1*	+	_	+	+	_	_	+
17	*Nocardiopsis sp. 2*	+	_	+	+	_	_	_
18	*Nocardiopsis sp. 3*	+	_	+	-	+	+	_
19	*Kitasatospora sp. 1*	_	_	+	+	+	_	+
20	*Kitasatospora sp. 2*	+	+	+	+	+	_	_
21	*Kitasatospora sp. 3*	_	+	+	+	+	+	+
22	*Micromonospora sp. 1*	_	+	+	_	_	+	_
23	*Micromonospora sp. 2*	_	+	+	+	+	+	+
24	*Streptosporangium sp. 1*	_	_	+	_	+	+	_
25	*Streptosporangium sp. 2*	_	_	_	_	+	_	_
26	*Nocardia sp. 1*	_	+	_	_	_	+	_
27	*Nocardia sp. 2*	_	+	+	_	+	+	_
28	*Actinomadura sp. 1*	+	_	_	+	+	+	+
29	*Actinopolyspora sp. 1*	+	+	_	+	_	_	_
30	*Rhodococcus sp. 1*	_	+	_	_	+	+	+

3.3.3.2 Carbon Source Utilization of Actinomycetes isolates

The carbohydrate fermentation ability of the selected Actinomycete strains is represented in Table 3.7 The result indicated that the isolates showed variation in their carbon source assimilation pattern.

Table 3.7 Carbon source utilization of selected Actinomycetes isolates.

No	Isolates	Glucose	Lactose	Sucrose	Xylose	Dextrose	Salicin	Arabinose	Rhamnose	Raffinose	Cellobiose	Fructose	Mannitol	Inositol	Galactose
1	*Streptomyces sp.1*	+	_	+	_	+	+	+	+	+	_	+	_	+	+
2	*Streptomyces sp.2*	+	+	_	_	+	+	_	+	+	_	_	_	+	+
3	*Streptomyces sp.3*	_	+	+	+	_	_	+	_	+	+	_	_	_	_
4	*Streptomyces sp.4*	+	_	+	+	+	_	_	_	_	+	_	_	+	+
5	*Streptomyces sp.5*	+	_	_	+	+	+	+	_	_	+	+	_	_	_
6	*Streptomyces sp.6*	+	_	+	+	_	+	+	+	+	_	+	_	+	+
7	*Streptomyces sp.7*	_	+	_	+	+	_	+	_	+	_	+	_	+	_
8	*Streptomyces sp.8*	+	_	_	_	+	+	+	+	_	+	_	+	_	+
9	*Streptomyces sp.9*	_	_	+	+	_	+	_	_	_	_	+	+	+	+
10	*Streptomyces sp.10*	_	_	+	_	_	_	_	_	_	+	_	_	_	_
11	*Streptomyces sp.11*	_	_	-	+	_	+	+	_	+	_	_	_	_	_
12	*Streptomyces sp.12*	+	+	+	_	_	+	+	_	+	+	_	_	_	+
13	*Streptomyces sp.13*	+	_	+	_	_	_	_	+	_	_	+	_	+	_
14	*Streptomyces sp.14*	_	_	+	_	_	+	+	+	+	+	+	_	+	+
15	*Streptomyces sp.15*	+	+	+	_	+	_	+	_	+	_	_	+	_	_
16	*Nocardiopsis sp.1*	+	+	+	_	+	+	+	_	_	_	+	+	+	+
17	*Nocardiopsis sp.2*	+	_	+	_	+	+	+	_	_	+	+	+	_	+

18	*Nocardiopsis sp.3*	+	+	+	_	+	_	_	+	+	_	+	+	+	_
19	*Kitasatospora sp.1*	+	+	+	+	-	_	+	+	+	+	+	_	_	+
20	*Kitasatospora sp.2*	+	+	+	+	_	+	+	+	_	_	+	_	_	_
21	*Kitasatospora sp.3*	+	_	+	_	+	+	_	+	_	_	+	+	_	+
22	*Micromonospora sp.1*	_	_	+	+	_	_	+	+	+	_	_	+	+	_
23	*Micromonospora sp.2*	+	_	_	+	_	+	_	+	_	+	+	_	_	+
24	*Streotosporangium sp.1*	+	_	_	_	_	_	+	+	_	_	_	+	_	_
25	*Streptosporangium sp.2*	+	+	+	+	+	+	_	_	_	_	+	+	+	_
26	*Nocardia sp.1*	_	_	_	+	+	_	+	_	+	_	+	_	+	+
27	*Nocardia sp.2*	_	+	+	_	_	+	+	_	_	+	_	_	_	_
28	*Actinomadura sp.1*	+	+	+	_	+	_	+	+	_	+	+	+	_	_
29	*Actinopolyspora sp.1*	+	_	_	_	_	+	_	+	_	_	+	+	+	_
30	*Rhodococcus sp.1*	+	_	+	+	+	+	_	+	+	_	_	_	_	_

Table 3.8 Taxonomic Classification of selected Actinomycetes isolates

Species	Family	Order	Class
Streptomyces sp.1	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.2	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.3	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.4	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.5	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.6	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.7	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.8	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.9	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.10	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.11	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.12	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.13	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.14	Streptomycetaceae	Actinomycetales	Actinobacteria
Streptomyces sp.15	Streptomycetaceae	Actinomycetales	Actinobacteria
Nocardiopsis sp.1	Noccardiopsaceae	Actinomycetales	Actinobacteria
Nocardiopsis sp.2	Noccardiopsaceae	Actinomycetales	Actinobacteria
Nocardiopsis sp.3	Noccardiopsaceae	Actinomycetales	Actinobacteria
Kitasatospora sp.1	Streptomycetaceae	Actinomycetales	Actinobacteria
Kitasatospora sp.2	Streptomycetaceae	Actinomycetales	Actinobacteria
Kitasatospora sp.3	Streptomycetaceae	Actinomycetales	Actinobacteria
Micromonospora sp.1	Micromonosporaceae	Actinomycetales	Actinobacteria
Micromonospora sp.2	Micromonosporaceae	Actinomycetales	Actinobacteria
Streptosporangium sp.1	Streptosporangeaceae	Actinomycetales	Actinobacteria
Streptosporangium sp.2	Streptosporangeaceae	Actinomycetales	Actinobacteria
Nocardia sp.1	Nocardiaceae	Actinomycetales	Actinobacteria
Nocardia sp.2	Noccardiaceae	Actinomycetales	Actinobacteria
Actinomadura sp.1	Thermomonosporaceae	Actinomycetales	Actinobacteria
Actinopolyspora sp.2	Actinopolysporaceae	Actinomycetales	Actinobacteria
Rhodococcus sp.1	Noccardiaceae	Actinomycetales	Actinobacteria

3.3.4 Station wise distribution of Actinomycetes

Out of the six sampling stations, the Kadalundi Mangrove region showed the greatest abundance represented by 150 isolates, while Dharmadam recorded the least number of isolates (36). Payyoli region stands as second in the abundance of actinomycetes isolates represented by 103 isolates followed by Ponnani mangrove (101), Kotoli (96) and Thikkodi (43). In all the stations, Streptomyces was the dominant genera. Based on the analysis, it was observed that the abundance of actinomycetes is not uniform throughout the study area, and a higher number of isolates were recorded from stations that were rather less impacted by anthropogenic activities such as pollution or sand mining. Hence compared to Ponnani, the abundance of actinomycetes in Kadalundi was much higher. In stations such as Payyoli and Kotolli, the diversity of actinomycetes was higher than that of Thikkodi and Dharmadam. A significant difference in these stations is the nature of the substrate. Hence, it is understood that the nutrient-rich sediments of mangrove have better actinomycetes representation than that of the nearshore regions, which is higher than the seaweeds found on rocky substrates. A detailed analysis of the different environmental parameters impacting the diversity and distribution of actinomycetes in Northern Kerala has been presented in Chapter 4.

A further assessment of the distribution pattern of actinomycetes among the different seaweed communities showed that a higher number of isolates (47) were collected from the red algae, followed by green algae (19) and brown algae (13). A genus-wise distribution of actinomycetes on the seaweeds showed that there is a good association of these isolates on *Grateloupia filicina.*

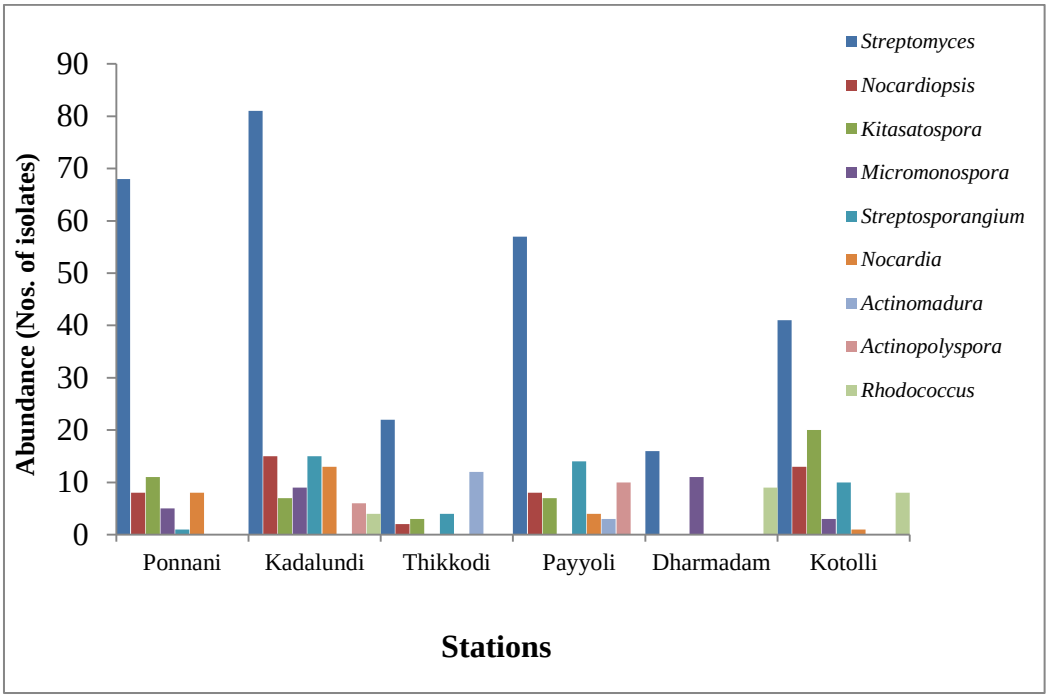


Figure 3.4 Station wise distribution of Actinomycetes from the Northern coast of Kerala.

Table 3.9 Genus wise distribution of actinomycetes on different seaweeds

Name of the seaweed	Number of Isolates	Possible
Red seaweeds		
Gateloupia filicina (Dharmadam)	25	SM, MM, RD
Chondracanthus acicularis(Thikkodi)	3	SM
Gracillaria corticata(Thikkodi)	19	SM, AM
Green seaweeds		
Cladophora sp (Dharmadam)	11	SM, MM
Ulva lactuca(Thikkodi)	4	SP.
Enteromorpha linza(Thikkodi)	4	SM
Brown seaweeds		
Padina tetrastomatica(Thikkodi)	6	SM, ND
Ectocarpus siliculosus(Thikkodi)	7	SP, KS

SM- *Streptomyces* spp.; KS – *Kitasatospora* spp.; ND- *Noccardiopsis* spp.;
SP – *Streptosporangium* spp.; MM – *Micromonospora* spp.; RD – *Rhodococcus* spp.;
AM- *Actinomadura* spp.

3.3.5 Season wise distribution of Actinomycetes

The occurrence of actinomycetes in the sediment and seaweeds greatly influenced by the season. In this study, it was clear that Pre-monsoon had the highest actinomycetes abundance represented by 250 isolates, while the monsoon had the least representation contributing 113 isolates (Fig.3.5). The trend was relatively uniform in all six stations. Detailed analysis on the seasonal variation in the diversity and distribution of actinomycetes is depicted in Chapter 4.

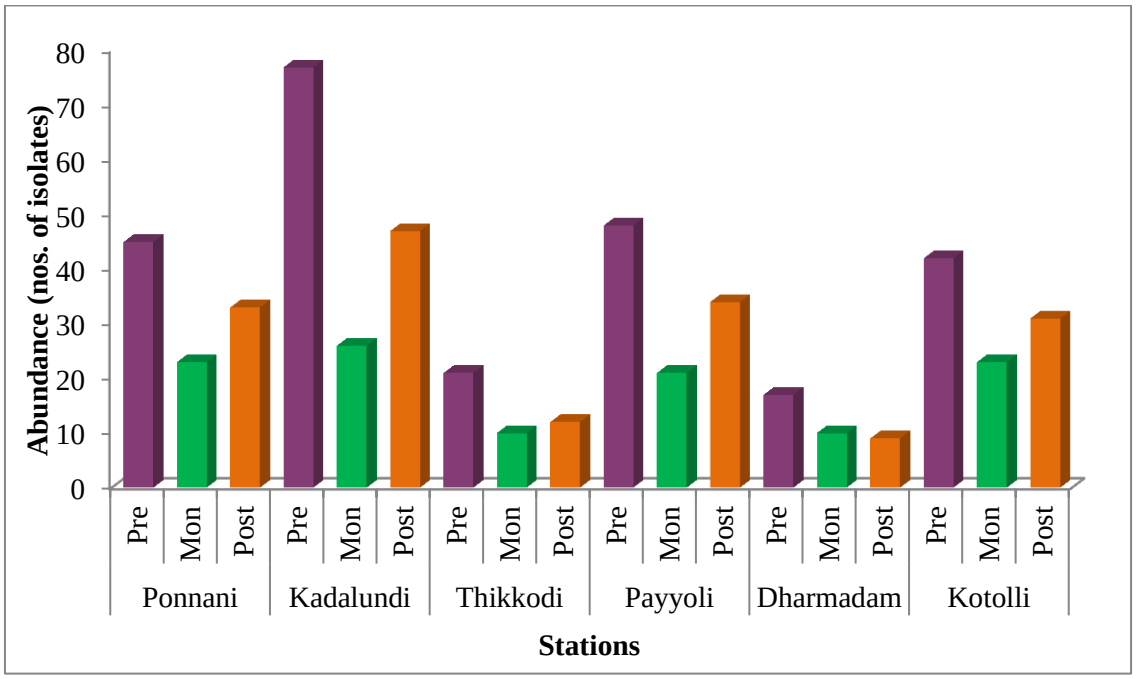


Figure 3.5 Season-wise variation in abundance of Actinomycetes of Northern Kerala

3.4 Discussion

Actinobacteria can be isolated from various environments, as they show adaptations to cope with terrestrial and aquatic conditions. Actinobacteria are considered indicators of the habitat, wide distribution of actinobacteria in aquatic habitats due to the washed in from the surrounding terrestrial habitats (Sykes and

Skinner, 1973, Prescott *et al*., 1993). Hence marine ecosystems are known to have immense microbial diversity, which has been increasingly explored for many novel chemical entities. As marine microorganisms, actinomycetes, have evolved for millions of years and well adapted to the present environment either through modifications of their genomic or metabolic activity, often producing novel secondary metabolites to ward away any unwanted ecological conditions (Lam, 2006). In the present study, isolation of Actinomycetes from the northern coast of Kerala was carried out. This is perhaps one of the pioneering studies that have covered a wide habitat with extreme environmental variations, which have not yet been screened for actinomycetes with potential bioactivity. Although prior to this study, efforts to isolate actinomycetes from sediments from specific regions have been attempted (Rosmine and Varghese, 2016; Chakraborty *et al*., 2015; Remya and Vijayakumar, 2008), very few reports are available about the isolation of actinomycetes from its Northern coast. The diversity of actinomycetes in the marine habitat has also been related to the substrate where they are found (Goodfellow and Haynes, 1984). Seaweeds and coastal mangrove patches are a haven for a wide variety of actinomycetes, and a number of novel and chemically diverse antimicrobial compounds have been discovered from them (Srivibool and Sukchrotiratana, 2006; Amrita *et al*., 2012). The mangrove environment in particular functions as a source of microorganisms, including actinomycetes capable of producing primary and secondary metabolites and the actinobacteria here have rich diversity and various biological activities (Hong *et al*., 2009; Azman *et al*., 2015; Sangkanu *et al*., 2017; Kumar *et al*., 2018). Seaweeds (macroalgae) support a unique association with the marine actinomycetes, usually which is symbiotic (Egan *et al*., 2008). Generally, the symbiosis of marine bacteria and marine organisms has a greater probability of

producing chemical compounds than marine bacteria, which live freely (Mearns-Spragg *et al*., 1998).

Seaweeds can act as a host for a variety of heterotrophic bacteria, and these bacteria have a crucial role in maintaining the health of the host organisms and can synthesize many bioactive secondary metabolites (Bolinches *et al.,* 1988). In this study, the prominent group of Actinomycetes encountered from Northern Kerala came under the genera *Streptomyces*. A similar pattern of distribution of actinomycetes isolated from eight different locations of Kerala was carried out by Remya and Vijayakumar (2008), and the majority of the isolates belonged to the genus Streptomyces. The coastline of Kerala shows a rich diversity of seaweeds. However, the distribution pattern of these seaweeds exhibits a wide range of variation in different areas. The northern parts of the Kerala coast are mainly sandy with scattered rocks and bedrocks which support the maximum numbers of seaweeds (Yadav *et al*., 2015). In this study, eight different types of seaweeds were collected from the rocky shores of Thikkodi and Dharmadam. They belonged to the family Rhodophyceae, Chlorophyceae and Phaeophyceae, which have been reported to harbour a good quantity of seaweed associated bacteria (Armstrong *et al*., 2000b, Weinberger *et al.,* 2007, Dimitrieva *et al*., 2006). Seaweeds are also a rich source of Actinomycetes with great species diversity. The study conducted by Ulfah *et al*. (2017) reported that the red algae *Gelidiella acerosa* to be rich sources of actinomycetes with good antibacterial activity. Cho *et al*. (2012) investigated the antifouling actinomycetes species, *Streptomyces praecox*, isolated from the Rhizosphere of the marine seaweed *Undaria pinnatifida.*

Among the media employed for the isolation of actinomycetes, starch-casein agar medium provided better recovery of actinomycetes. This medium was initially devised by Grein and Meyers, 1958 and recommended by Aaronson,1970, and Williams and Cross, 1971, has been widely used for the isolation of marine actinomycetes in particular (Walker and Colwell, 1975, Takizawa *et al*., 1993, Kala and Chandrika, 1993, Patil *et al*., 2001, Ellaiah *et al*., 2002, Kokare *et al*., 2004, Kathiresan *et al*., 2005, Peela *et al*., 2005, Sujatha *et al*., 2006, Saha *et al*., 2006, and Adinarayana *et al*., 2006). Findings from Kala and Chandrika (1993) emphasized that starch as a carbon source and casein as a nitrogen source facilitates the better growth of actinomycetes. In the present study, too, such a favourable condition for the development of actinomycetes was seen when Starch-Casein agar was used. Even for stations with comparatively lesser salinity, the medium provided ample condition for the growth of actinomycetes. The efficacy of the medium could be improved with slight modifications in the pretreatment as prescribed by Nolan and Cross (1988) and Kim *et al*. (1995), including drying and heating of sediments. Likewise, in the present study, the sediments collected from mangroves and nearshore regions were simultaneously subjected to pretreatment. The procedure followed by air drying of sediment samples at room temperature for a week. It was then transferred to sterile petriplates and kept at 55°C for 10 minutes to retard the growth of slime forming bacteria (Pisano *et al*., 1986). It was seen that heat pretreatment prior to actinomycete isolation reduces the numbers of gram-negative bacteria commonly found in the sediment samples and often overrunning isolation plates (Barcina *et al*., 1987; Jensen *et al*., 1991). Takizava *et al*. (1993) reported that heat treatment prior to isolation resulted in a marked decrease in antagonistic growth of

bacterial colonies that looked similar to actinomycetes and allowed the growth of heat resistant Actinomycetes spores.

Chromogenic of aerial mycelium is considered an important character for grouping actinomycetes (Pridham and Tresner, 1974). This study found that the actinomycetes cultures isolated from seaweeds and sediments of Northern Kerala were morphologically distinct based on spore mass colour, aerial mycelium, substrate mycelium pigmentation, spore chain morphology etc. The majority of isolates were white, followed by grey, olive green, lemon yellow, yellow, brown, pink, blue and orange spore mass. Out of the total isolates selected, 46.66% exhibited white/offwhite spore mass with dark colony reverse colour, grey spore mass (20%), olive green (6.66%), brown (6.66%), lemon yellow (6.66%), yellow (3.33%), blue (3.33%), pink (3.33%) and orange (3.33%). Similar colour series of actinomycetes were recorded by previous workers (Baskaran *et al*., 2011; Das *et al*., 2008; Patil *et al*., 2001; Sujatha *et al*., 2005). Lekshmi *et al*. (2014) isolated similar actinomycetes isolated from the sediments of the southern coast of Kerala. The actinomycetes colonies with different shades of colours pose a great advantage, especially in developing antifouling paints since the natural pigmentation could be retained during commercial production. A similar investigation on actinomycetes was described by Ruttanasutja and Pathom-aree (2015), whereby the distinct colour of actinomycetes colonies ranging from white to dark purple was used to synthesize natural pigments. It was also found that an array of the actinobacterial genus such as *Curtobacterium, Dermacoccus, Micromonospora, Microbispora, Pseudonocardia, Rhodococcus, Streptomyces and Tsukamurella* contributed exclusively to these colour producing colonies. Apart from the colour of the colonies, traditional methods such as spore chain morphology and spore surface

ornamentation, observed under light microscope and scanning electron microscope, are considered valuable tools in the characterization of actinomycetes. Especially, spore chain morphology is considered one of the important characteristics in identifying Streptomyces since it may greatly vary among the species (Tresner *et al*., 1961). It has been already reported that the majority of the marine Streptomycetes isolates produced aerial mycelia with coiled spiral spore chains (Das *et al*., 2008; Mukherjee and Sen, 2004; Peela *et al*., 2005; Roes and Meyer, 2005; Chacko *et al*., 2012) followed by rectiflexibiles spore morphology. In the present study, the spore chain morphology was either spiral, rectiflexibilis or rectinaculiaperty in most marine actinomycetes. No abnormal spore chain formation was witnessed in the study, and hence they could be grouped easily without anonymity. A preliminary understanding of the diversity of actinomycetes in the northern coastal regions of Kerala indicated a distinct diversity in their occurrence and abundance. Likewise, based on the season and substrate on which they were found, similar diversity could be observed in colony-forming units. Compared to seaweeds, sediment samples showed a greater load of actinomycetes. Earlier reports also confirm sediment samples to be good sources for enumerating the actinomycetes population (Ghanem *et al*., 2000; Karthik *et al*., 2010). Several studies reported that the abundance of actinomycetes depends on the ecological condition where they occur (Varghese *et al.,* 2012, Nasarbadi *et al.*, 2013, Das *et al.*, 2008). Premonsoon had the greater abundance compare to monsoon and post-monsoon season. A similar pattern of seasonal variation in abundance of actinomycetes isolates reported by Varghese *et al.* (2012). A load of actinomycetes was high during pre-monsoon followed by post-monsoon and monsoon seasons. The result indicates that Actinomycetes were most active during the pre-monsoon season because of better soil

temperature, favouring microbial activity (Chhonkar & Tarafdar, 1984). Bhatt & Pandya, 2006 reported that Actinomycetes load was lower in monsoon season, perhaps due to competition for nutrients by plants and protozoan predation decreases the number of microorganisms. Soil organic matter has a key role in beneficial biological processes and the chemical and physical properties of soils. It provides energy for the soil microbial community, increases cation exchange capacity and ameliorates soil aggregate and structure (Wolf and Wagner, 2005). In the above pretext, it was found pertinent to investigate the factors determining the distribution and diversity of actinomycetes on different substrates and seasons in the Northern coast of Kerala, considering the environmental variables and other physico-chemical factors. However, the significant results emerging from this chapter portray that the Northern coast of Kerala has a good assemblage of actinomycetes, and any information on their diversity from these lesser-studied regions have immense potential for the isolation of novel bioactive compounds. Since the study particularly focuses on identifying antifouling compounds, naturally pigmented actinomycetes colonies have an added advantage.

PLATE 3.1

Sampling Area

Station-1 Ponnani Mangrove

Station-2 Kadalundi Mangrove

Station-3 Kottoli Coast

PLATE 3.2


Station – 4 Payyoli Coast

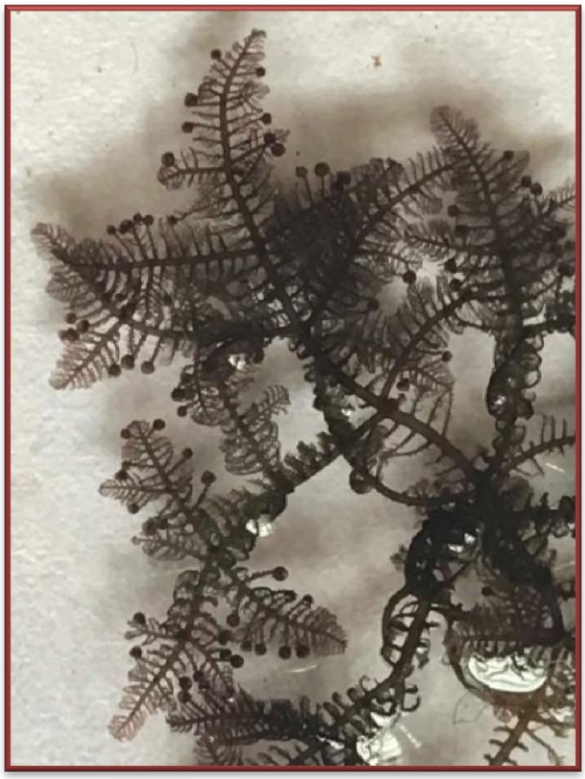

Station – 5 Thikkodi Rocky shore


Station – 6 Dharmadam Rocky Shore

PLATE 3.3

Seaweeds Collected for the isolation of Actinomycetes from Thikkodi and Dharmadam coast

Red Seaweeds



Grateloupia Filicina

Chondracanthus acicularis

Gracillaria corticata

PLATE 3.4

Green Seaweeds

Cladophora sp

Ulva lactuca

Enteromorpha linza

PLATE 3.5

Brown Seaweeds

Padina tetrastomatica

Ectocarpus siliculosus

PLATE 3.6

Utilization of Various Carbon Source

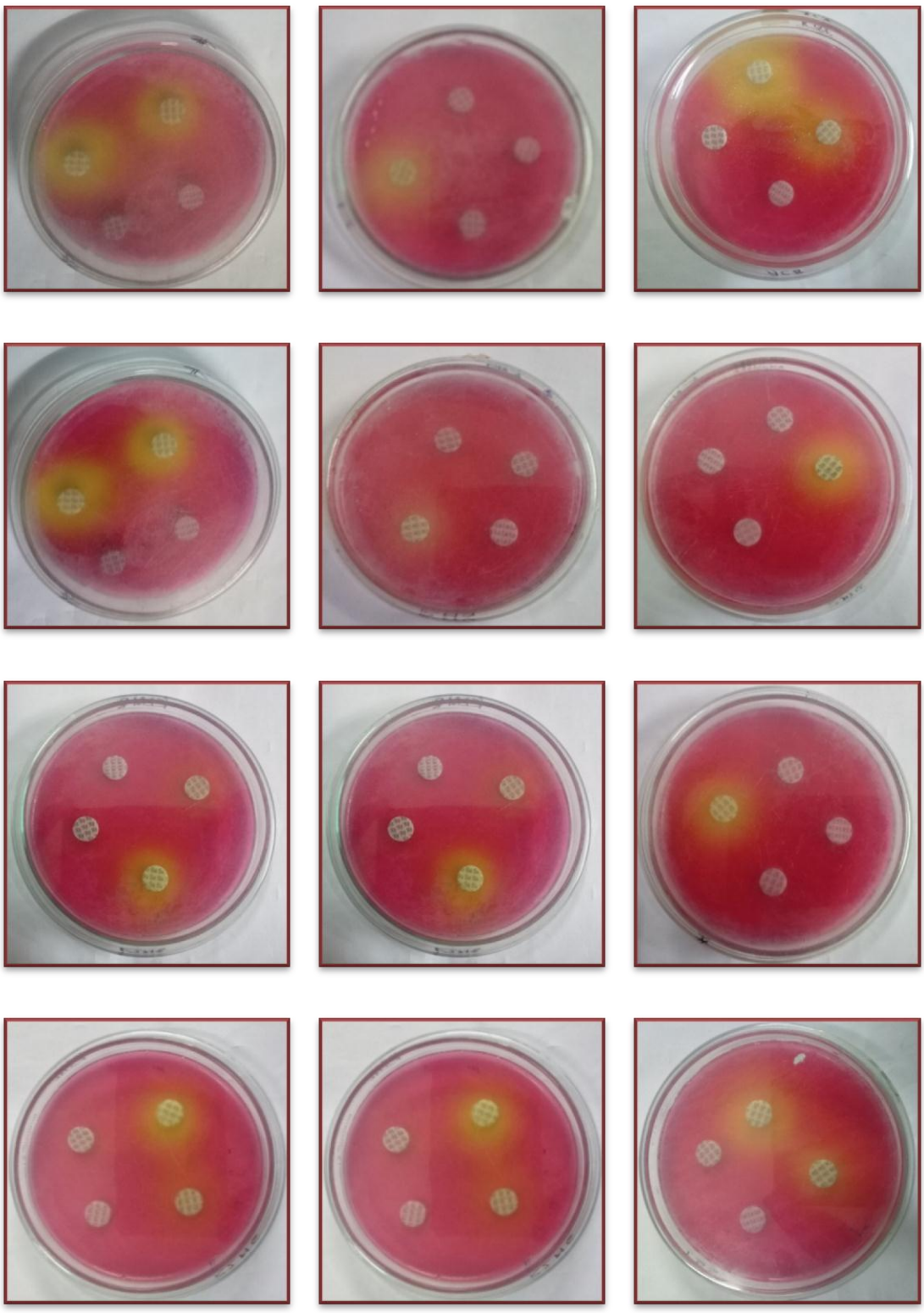


PLATE 3.7

Different species of Actinomycetes isolates selected during the study

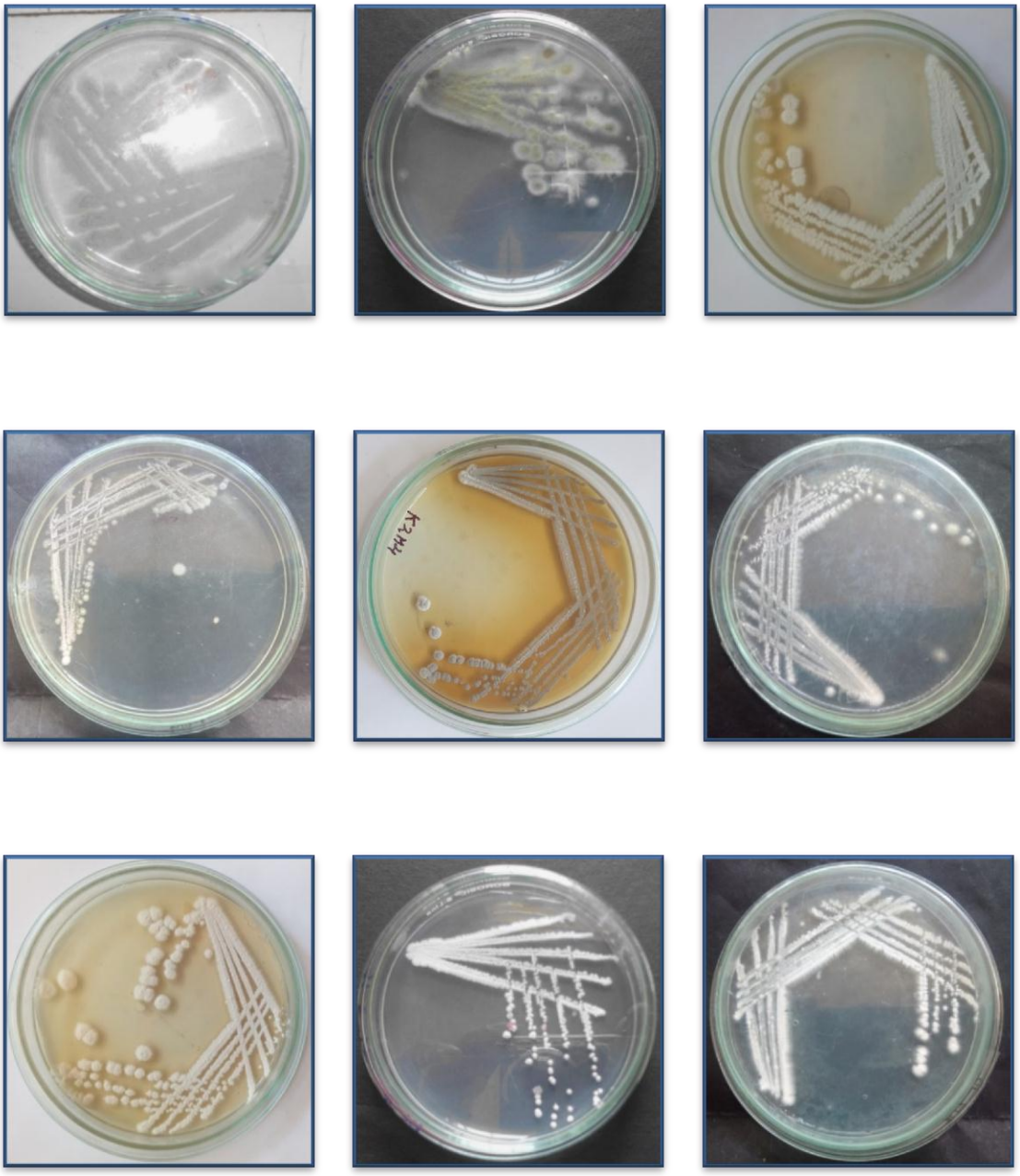


PLATE 3.8

Different species of Actinomycetes isolates selected during the study

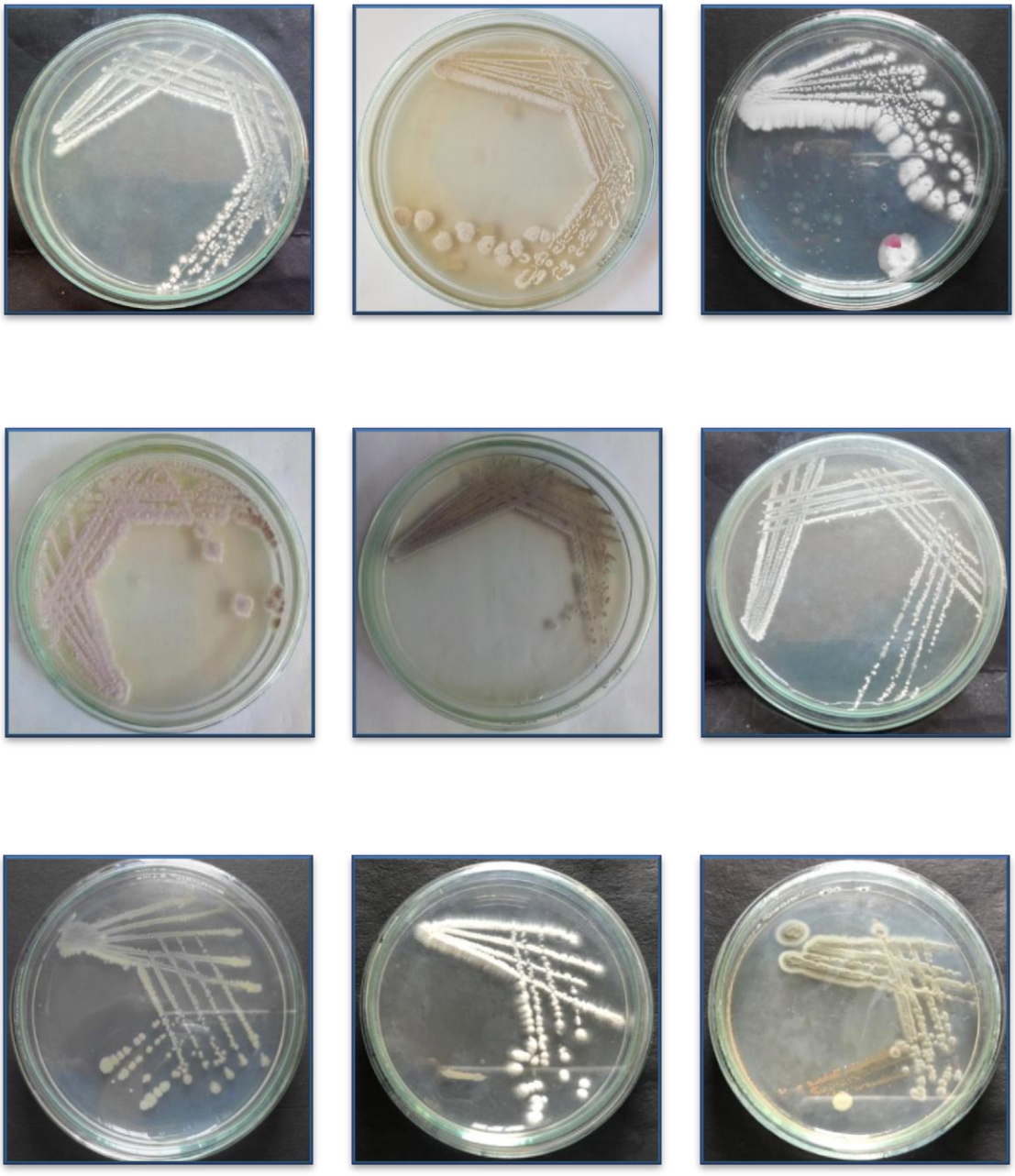
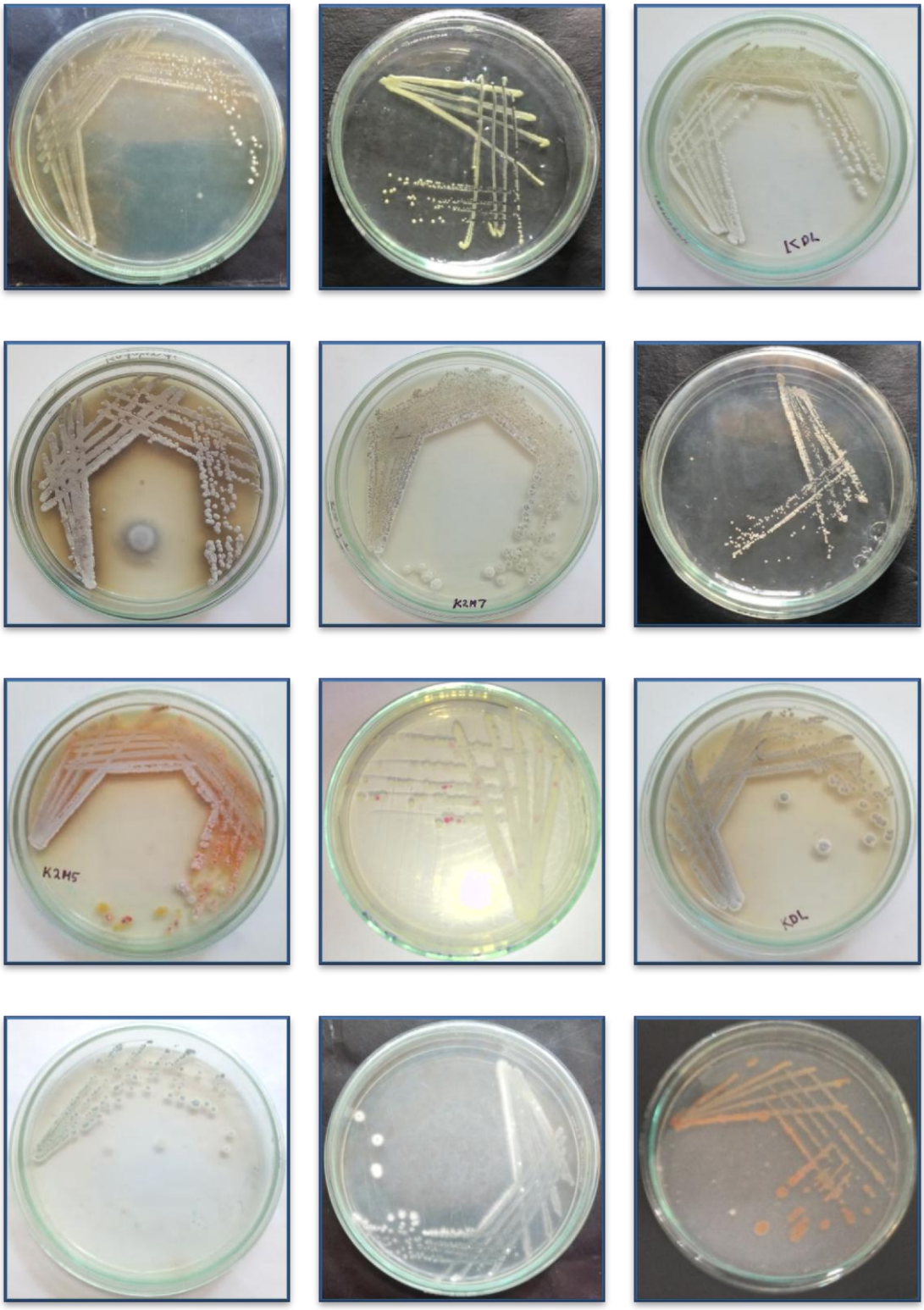


PLATE 3.9

Different species of Actinomycetes isolates selected during the study



Chapter 4

DIVERSITY AND DISTRIBUTION OF MARINE ACTINOMYCETES IN DIFFERENT HABITATS OF NORTHERN KERALA

4.1 Introduction

Microorganisms are ubiquitous in nature and function as an important entity in maintaining ecological homeostasis. The world ocean is the largest ecosystem on earth and has been used for a variety of purposes by man for millennia. However, the knowledge regarding marine microbial diversity is limited and primarily based on the cultured groups exploited for various industrial and commercial purposes (Das *et al*., 2006). Difficulties in sampling microorganisms from the sea has been a significant bottleneck in its exploration and utilization (Karl and Dore, 2001). Biological diversity means the variability among living organisms from all sources and the ecological complexes of which they are part, and it includes the diversity within species and between them and the ecosystems wherein they survive. Since diversity generally means "variation" or "differentiation", the term that usually goes well is "diversification", which in contrast to "uniformity" provides an assessment of the variety of species, their abundance and distribution in a particular habitat. When assessing biological systems, diversity may also be seen as "richness" (Kratochwil 1999), which embodies the numerical abundance of a particular species in a region at a given point in time. Due to their unique physiological and biological nature, marine systems differ from terrestrial systems in many ways and hence comparing them with any terrestrial systems may not be suitable for any ecological assessment (May, 1994; Gray, 1997). While the three-dimensional physical structure dictates the diversity in terrestrial ecosystems, its

counterpart in aquatic ecosystem relies on both physical and physico-chemical parameters, which makes them even more incongruent, especially when it comes to species assemblage (Raghukumar and Anil 2003). The diversity of microorganisms associated with water, soil and flora and fauna is therefore unbelievably rich. The world ocean has a coastline of 312,000 km and a volume of 1.46 x 10^9 km^3 with an average depth of 4000 m (Rumney 1968), making it the largest ecosystem on earth. In spite of its vastness, marine ecosystems have been relatively less studied, which may be why our knowledge of marine biodiversity falls far below that of the terrestrial systems (Ellingsen 2001, 2002; Ellingsen and Gray 2002). The study of marine microbial diversity is important in understanding the community structure and the pattern of distribution in the different niches of the marine environment. Nevertheless, microbial diversity is one of the problematic areas of biodiversity research (Watve *et al*., 1999), and India's microbial diversity is perhaps one of the most significant in the world (Budhiraja *et al*., 2002).

The marine environment is a virtually unexploited source of novel actinomycete diversity (Bull *et al*., 2005; Stach *et al*., 2003). The distribution of actinomycetes in the sea is largely unexplored, and the presence of indigenous marine actinomycetes in the oceans remains indefinable. Early evidence supporting the existence of marine actinomycetes came from the description of *Rhodococcus marinonascene*, the first marine actinomycete species to be characterized (Helmke and Weyland, 1984). Recent data from culture-dependent studies have shown that indigenous marine actinomycetes genuinely exist in the oceans and include the members of the genera *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia Maris and*

Verrucosispora (Bull *et al*., 2005; Jensen *et al*., 2005b; Magarvey *et al*., 2004; Stach *et al*., 2004). Among the early groups studied for novel compounds were the genus, *Salinispora* (formerly known as *Salinospora*) from the ocean sediments (Mincer *et al*., 2002; Mincer *et al*., 2005). Research has progressed much ahead now, with isolation of both culture-dependent and culture-independent methods widely being used to screen out novel actinomycetes from every part of the oceans, including the deep seafloor to the coral reef sediments to invertebrates and plants. Since actinomycetes are distributed in all depth ranges in the ocean realms, viz., from the surface of the oceans to hydrothermal vents and from the nearshore inter-tidal environments to the deep sea, studying their diversity has greater relevance in mining novel species (Madrid *et al*., 2001). Another advantage of studying these groups is that since they are found even in adverse environments, such as in marine organic aggregates and deep-sea gas hydrate reservoirs, they have shown to adapt to a wide array of habitats, which in itself is a field worth investigating. Studies have shown that they can withstand the adversities of the environment through the production of secondary metabolites (Jensen *et al*., 2005a).

Actinomycetes are primarily a diverse group of Gram-positive, saprophytic bacteria and contribute to nutrient turnover, using many available nutrient sources for their existence (Williams *et al*., 1983; Jensen *et al*., 1991; Stackenbrandt *et al*., 1997; Mincer *et al*., 2002). Many actinomycetes are an excellent source for producing secondary metabolites of economic importance (Lazzarini *et al*., 2000; Bull *et al*., 2000; Bull and Stach, 2007). During the last few decades, isolation of actinomycetes has been mainly performed as part of drug discovery programs and has focused on terrestrial sources, but recent efforts turned to marine environments because of the increasing discovery rate of new isolates and valuable bioactive compounds from these

ecosystems (Fenical 1993; Bull *et al*., 2000; Donadio *et al*., 2002; Lam, 2006). Some actinomycetes have been studied in detail due to their biotechnological importance; however, more comprehensive studies are needed to understand the diversity, distribution and ecology of the large majority of actinomycetes in marine habitats (Stach *et al*., 2003; Maldonado *et al*., 2005a; Bull and Stach, 2007). 16S rRNA gene sequences are a standard tool for assigning taxonomical hierarchies in actinomycetes, which are genetically closely aligned and perform quantitative analyses of the communities (Martin, 2002; Wang *et al*., 2007). Existing tools assign sequences to operational taxonomic units (OTUs) based on genetic distances between sequences, allowing the estimation of ecological parameters such as richness, diversity, and degree of similarity between microbial communities (Schloss and Handelsman, 2006).

Identification and classification are the difficult parts of traditional actinomycetes research. Several biochemical tests have been employed to characterize the species (Tresner *et al*., 1961; Shirling & Gottlieb, 1966, 1968a, 1968b, 1969a, 1969b) and Nonomura, 1974 the identification can be made. However, colony isolation is often the most frustrating and time-consuming task as it involves the examination of morphological characters. Hence, besides the traditional methods, the advanced method for the identification of actinomycetes through molecular 16S rRNA sequence analysis also helps to determine phylogenetic relationships and makes possible the recognition up to species level using sequence signatures followed by BLAST search (Magarvey *et al*., 2004). It has been shown that the presence, distribution and diversity of actinomycetes are associated with their different ecological habitats (marine, soil, etc.). They are universally widespread and constitute an essential part of the soil microflora (Kuster, 1968). In particular, ecological studies of actinomycetes have been widely

conducted in terrestrial environments (Alexander, 1978; Goodfellow and Williams, 1983). The distribution of microbes present in soil has been determined by several environmental factors like pH, moisture content, soil organic matter, etc. (Kennedy *et al*., 2005). The number and types of actinomycetes present in a particular soil would be significantly influenced by geographic location and physicochemical conditions such as temperature, type, pH, salinity, organic matter, cultivation, aeration and moisture content. Physicochemical properties of the soil may be varied among different locations and seasons. These variations mainly attributed to the topography and climate variations of the area (Varghese *et al*., 2012). Earlier studies have shown a strong relationship between topographic position and soil physicochemical characteristics and, in particular, the percentage of organic carbon in the sediment (Miller *et al*., 1988). Soil organic matter has a crucial role in beneficial biological processes and the chemical and physical properties of soils. It provides energy for the soil microbial community, increases cation exchange capacity and ameliorates soil aggregate and structure (Wolf and Wagner, 2005). Salinity usually acts as a limiting factor in most aquatic microbes, but not for actinomycetes that quickly adapt to diverse saline conditions (Nasrabadi *et al*., 2013).

The northern coast of Kerala has a wide variety of coastal and nearshore habitats, which have rarely been explored for microbial communities. The rich floral and faunal biodiversity of this region also is subjected to a range of environmental perturbations. Salinity, organic carbon and water temperature fluctuate well within the extremes, making the survival of many microbes hard. However, those who have adapted to these environments have also succeeded in establishing a robust community in the region. Therefore, it is envisaged that while understanding the community

assemblage of actinomycetes in these environments, the ecological dynamics of their abundance in relation to the prevailing environmental conditions or habitats can also be resolved. Under the above perspective, a study was conducted to correlate the community assemblage structure of actinomycetes in Northern Kerala, to understand their temporal and seasonal diversity in different habitats.

4.2 Materials and methods.

4.2.1 Sampling and assessment of environmental parameters.

Sediment and water samples were collected from the six sampling sites as given under Section 3.2.2 of Chapter 3. Physico-chemical parameters such as water temperature, salinity, water pH, and soil pH were measured using suitable instruments at the site (see Section 3.2.3), while for analyzing remaining parameters, samples were transported to the laboratory in cool aseptic conditions. Organic carbon in sediment was determined following El-Wakeel and Riley's method (El Wakeel and Riley, 1957). For hydrological analysis, one litre of water was used to estimate Dissolved oxygen (Winkler's method), Nitrate (Brucine method), and Phosphate following Grasshoff *et al*. (1999).

4.2.2 Data analysis.

Relative abundance (%) of actinomycetes species from each sampling site was calculated from the total of isolates. Various diversity indices are being widely used in ecology to compare communities on spatial and temporal scales as well as for the assessment of environmental quality. The discriminating ability of diversity indices varies, depending on the type of study being conducted and on other aspects of the samples. The total number of species and their relative abundance in the different stations and seasons were determined, and the diversity indices, viz., Shannon-Weiner

index (H'), Margalef's richness index (d), Pielou's evenness index (J') and Simpson diversity index (1- λ) were estimated from numerical abundance data using Plymouth Routines in Multivariate Ecological Research (PRIMER 6) software (Clarke and Warwick, 2001). Significant differences in diversity indices with regard to sampling zones and seasons were tested using one-way ANOVA in Statistical Package for the Social Sciences (SPSS) version 22.0. The similarity in assemblage structure of actinomycetes between the six sampling zones was estimated by Multidimensional scaling using Bray-Curtis similarity measure (Clarke and Ainsworth, 1993) and was statistically verified using cluster analysis in PRIMER 6. Multidimensional scaling is a visual representation of distances or dissimilarities between sets of objects. The non-parametric permutation-based Principal Component Analysis (PCA), which is the most fundamental, general-purpose multivariate data analysis method, was carried out to test changes in species assemblage structure between different sampling sites and seasons.

A Principal Coordinate Analysis (PCoA) and Canonical Analysis of Principal Coordinates (CAP) was performed to understand the association between environmental variables and actinomycetes assemblages. To ascertain the degree of difference in the species assemblage based on the physico-chemical parameters studied, the PERMANOVA (Permutational Multivariate Analysis of Variance) test was carried out for different stations and seasons. The average taxonomic distinctness (AvTD or Δ+) was estimated from presence or absence data (Clarke and Warwick, 1998), and funnel plots were constructed to measure the variation in the taxonomic distinctness (Λ+) at different sites. Since the variation in taxonomic distinctness (VarTD) measures the irregularities and divergence in the distribution of taxonomic groups within a sample, the value was taken to measure the taxonomic strength of the groups at a particular area.

Environmental influence on the distribution of actinomycetes was assessed with Canonical Correspondence Analysis (CCA) using log-transformed data (ter Braak, 1994). The CCA plot helped define which environmental factor influenced the distribution of the species. Monte Carlo permutation tests were conducted to identify the significance of each environmental parameter on Actinomycete assemblage on square-root transformed data using PAST ver. 3.26 (Paleontological Statistics Software).

4.3 Results

Mean values of the physicochemical parameters of the sediment and water collected from the six sampling stations during all seasons from 2016-17 are presented in Table 4.1. Salinity ranged from 0 to 35 ppt, and maximum salinity in the present study was recorded from Dharmadam during the pre-monsoon season, while salinity reduced significantly in mangrove regions during monsoon season. Sediment organic carbon levels were higher in samples collected from Ponnani mangrove during monsoon season (6.82 %), while the values were lowest in Dharmadam during the pre-monsoon season. Similarly, dissolved oxygen values were higher in Ponnani (8.67 mg/l) and lowered in Kadalundi during the pre-monsoon season (3.27). Variation in water temperature at the sampling sites during three seasons indicated the wide range of environmental regimes prevalent in the study area. While the temperature was relatively low during monsoon, they fluctuated between 32^{o}C to 34^{o}C during pre-monsoon and post-monsoon season. However, the soil and water pH did not vary much during the three seasons in these six sampling sites. Though their values were low during monsoon season in all locations, in stations such as Thikkodi, Ponnani and Kottoli, the values increased above the yearly mean during post and pre-monsoon seasons. Higher values for nitrite and nitrate were recorded from all stations except Ponnani and Kadalundi,

where the values were considerably low during monsoon months. Nitrate was relatively higher during the post-monsoon seasons, especially in Thikkodi (42.37) and Payyoli (42.31); however, an interesting observation was the reduced levels of nitrite during the same seasons in these stations (1.79 and 1.82). On the other hand, the high value of phosphate was observed from Dharmadam (15.02) followed by Kottoli (13.88) during the post-monsoon season. Organic carbon of sediments also showed a distinct pattern of variation among the stations. The stations associated with mangrove vegetation (Ponnani and Kadalundi) expectedly had a higher percentage of organic carbon (6.20 and 5.94 respectively) than other stations, and a seasonal variation incidentally showed the highest values during monsoon and least during post-monsoon seasons.

Table. 4.1 Mean values of different environmental variables during the study

Parameters	Ponnani			Kadalundi			Thikkodi			Payyoli			Dharmadam			Kottolli		
	PM	MO	PS	PM	MO	PS	PM	MO	PS	PM	MO	PS	PM	MO	PS	PM	MO	PS
Temperature(^{0}c)	33.4	27.0	32.0	34.0	26.0	33.0	33.5	27.1	30.7	32.4	27.0	30.4	32.5	28.0	30.4	32.0	27.0	30.5
Salinity (ppt)	34.0	0.0	7.0	34.0	0.0	8.0	34.0	26.0	27.0	33.0	26.0	28.0	35.0	24.0	27.0	35.0	25.0	27.0
Dissolved oxygen (mg/l)	4.88	8.7	5.2	3.3	5.7	4.5	5.2	6.8	4.4	5.1	6.6	4.4	4.4	5.2	3.9	4.4	5.1	3.8
Soil pH	7.6	8.1	9.8	9.8	8.7	10.9	8.2	9.1	9.6	8.0	9.5	9.7	8.4	8.7	10.1	8.3	8.7	10.0
Nitrite (mg/l)	0.8	0.7	1.0	0.6	0.5	0.9	0.6	3.8	1.8	0.6	3.5	1.8	0.6	4.1	1.6	0.6	4.2	1.7
Nitrate (mg/l)	3.5	7.6	5.9	2.1	8.7	5.6	5.2	23.9	42.4	5.0	23.0	42.3	1.2	29.2	11.3	1.3	29.1	11.3
Phosphate (µmol/l)	1.5	9.9	1.4	1.9	11.4	2.8	1.3	9.2	11.1	1.8	8.9	10.0	0.6	6.1	15.0	1.9	8.9	13.9
Water pH	8.0	7.5	7.9	7.6	6.3	7.2	8.1	6.5	7.1	7.6	6.5	7.4	8.0	6.3	7.1	8.0	6.1	7.4
Organic carbon (%)	6.0	6.8	5.4	4.5	5.9	2.3	2.4	1.2	2.0	2.9	2.1	2.3	1.3	0.5	1.0	2.1	0.7	1.9

PM: Premonsoon; MO: Monsoon; PS: Post monsoon

Table. 4.2 Variations in the physico-chemical parameters in the study area

Parameters		Sum of Squares	df	Mean Square	F	Sig.
Temperature	Between Groups	3.251	5	.650	.065	.996
	Within Groups	119.234	12	9.936		
	Total	122.485	17			
Salinity	Between Groups	910.444	5	182.089	1.495	.263
	Within Groups	1461.333	12	121.778		
	Total	2371.778	17			
Dissolved_ oxygen	Between Groups	8.232	5	1.646	1.038	.439
	Within Groups	19.030	12	1.586		
	Total	27.262	17			
Soil_pH	Between Groups	2.620	5	.524	.570	.722
	Within Groups	11.040	12	.920		
	Total	13.660	17			
Nitrite	Between Groups	6.984	5	1.397	.729	.615
	Within Groups	22.996	12	1.916		
	Total	29.980	17			
Nitrate	Between Groups	979.858	5	195.972	1.061	.428
	Within Groups	2215.827	12	184.652		
	Total	3195.685	17			
Phosphate	Between Groups	31.561	5	6.312	.201	.956
	Within Groups	375.987	12	31.332		
	Total	407.548	17			
Water_pH	Between Groups	1.135	5	.227	.443	.810
	Within Groups	6.142	12	.512		
	Total	7.277	17			
Organic_carbon	Between Groups	56.340	5	11.268	13.027	.000
	Within Groups	10.380	12	.865		
	Total	66.719	17			

While analyzing the variations in the mean values of observed physico-chemical parameters, it was seen that except for organic carbon, no other parameters varied significantly between the stations (Table 4.2)

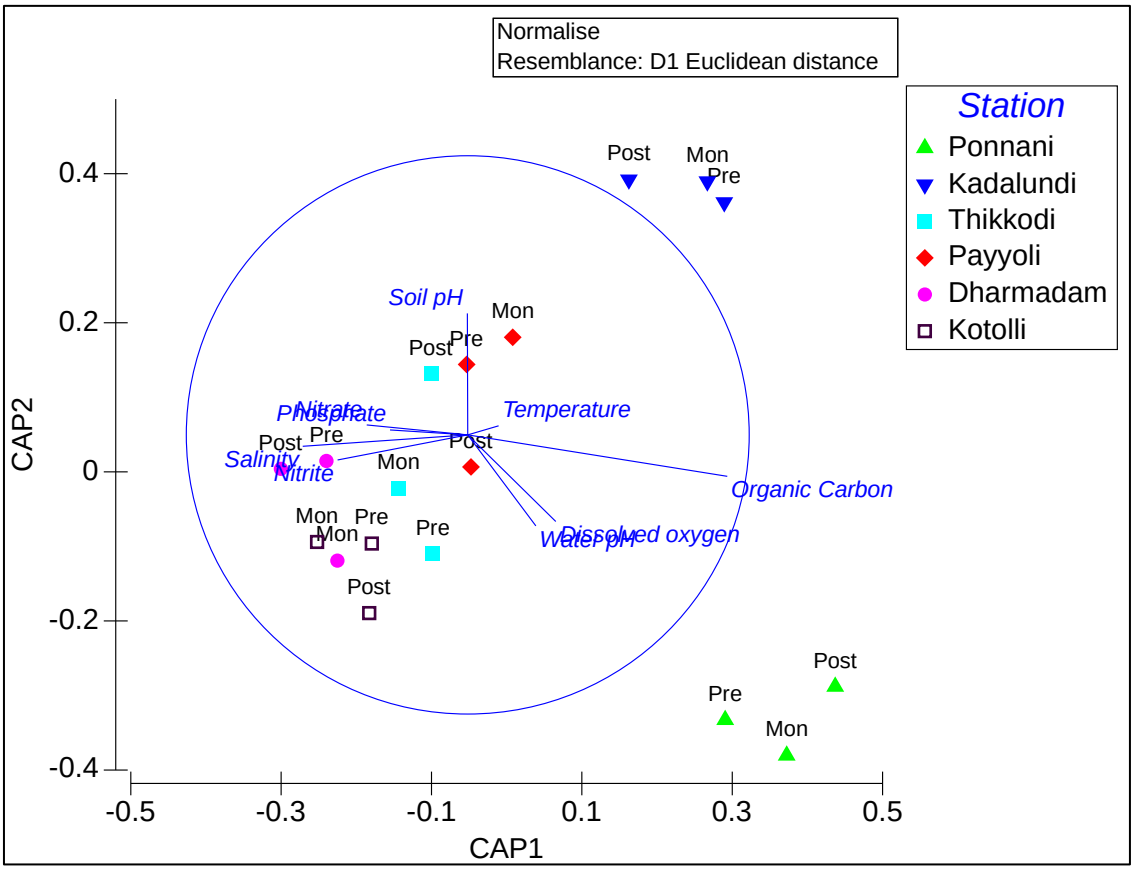


Figure 4.1 Results of Canonical analysis of principal coordinates (CAP).

Canonical analysis of principal coordinates (CAP) is an approved methodology for analyzing the distance or dissimilarity measure of variables, in the present study, environmental parameters to correlate their variation with different stations. Results showed that except for Ponnani and Kadalundi, all other stations followed a consistent trend in the variation in environmental parameters for all the season. However, in these stations, the environmental variables such as nutrients and salinity were the primary factor that altered significantly. In Ponnani, it was organic carbon, water pH and dissolved oxygen which turned out to be the significant variable, while in Kadalundi, it was the temperature and soil pH.

Table. 4.3 Results of PERMANOVA on Environmental Variables

Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms
Stations	5	42.867	8.5734	2.7893	0.007	999
Seasons	2	79.397	39.698	12.916	0.001	999
Residual	10	30.736	3.0736			
Total	17	153				

To further analyze the extent of variation in the environmental parameters from the six stations, the Permutational Multivariate Analysis of Variance (PERMANOVA) test was carried out. Results showed a high F value ($P<0.001$), indicating that the variables from each station and season were significantly diverse. It also indicated a significant difference ($P<0.01$) in the distribution of actinomycetes based on physicochemical parameters monitored within station and season.

Principal Coordinate Analysis (PCO) was carried out to perform multidimensional scaling of environmental parameters to determine if any particular parameter significantly contributed to the diversity as seen in PERMANOVA analysis. The results also provide the eigenvalues identifying the parameters or combination of parameters that contributed to both individuals and cumulative environmental variability. In the present study, the first three-axis were the most critical variables, the combination of which contributed to 83.28% of the total variability. Results indicated a set of environmental parameters that significantly contributed to the diversity, as seen in PERMANOVA analysis.

Table. 4.4 Principal Coordinate Analysis (PCO)

Axis	Eigenvalue	Individual%	Cumulative%
1	66.111	43.21	43.21
2	38.98	25.48	68.69
3	22.326	14.59	83.28
4	9.2317	6.03	89.31
5	7.0308	4.6	93.91
6	4.8197	3.15	97.06
7	2.834	1.85	98.91
8	1.0977	0.72	99.63
9	0.56928	0.37	100

As evidenced in Figure 4.2, a specific grouping of the seasons could be made based on the PCO analysis. This indicates that the environmental variables varied according to the season in all the stations. The correlation of these parameters further indicates that the impact of environmental parameters during different seasons and their distribution in each station was highly diverse, indicating that it was the accumulative impact of all these parameters that contributed to the diversity. However, certain environmental parameters had a more significant role in assigning the species assemblage specific to that station. Hence, a detailed analysis of the individual parameters with the season in different stations would better understand the ecological dynamics of that region.

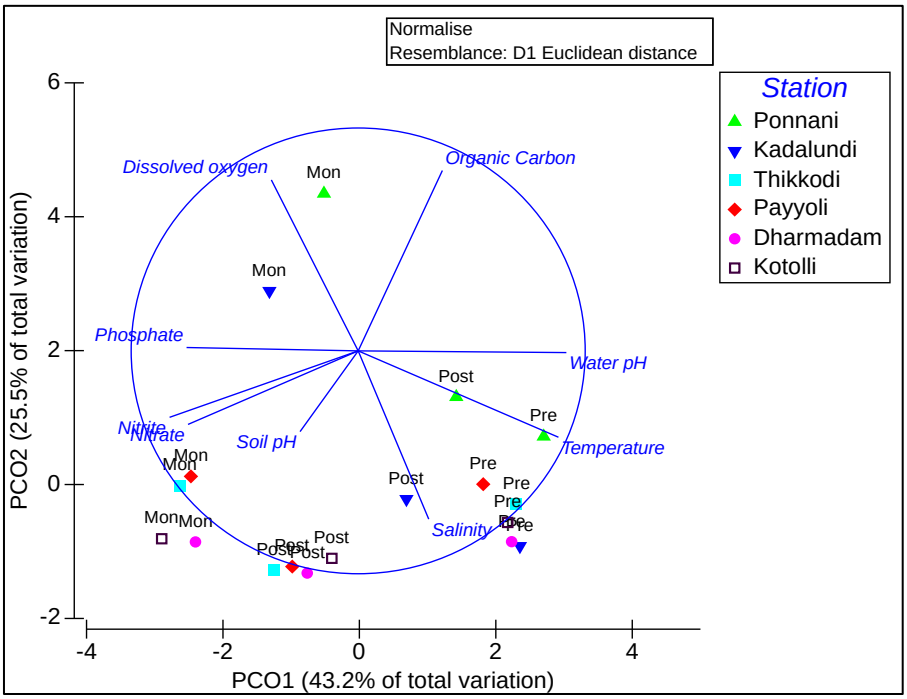


Figure 4.2 Principal Coordinate Analysis (PCO) of the variability of Environmental variables

Table 4.5 Results of Principal Component Analysis (PCA) to assess the cumulative influence of environmental parameters

PC	Eigenvalues	%Variation	Cum. %Variation
1	3.89	43.2	43.2
2	2.29	25.5	68.7
3	1.31	14.6	83.3
4	0.543	6	89.3
5	0.414	4.6	93.9
6	0.284	3.2	97.1
7	0.167	1.9	98.9
8	6.46E-02	0.7	99.6
9	3.35E-02	0.4	100

Principal Component Analysis (PCA) was conducted to group the variables responsible for showing the diversity among environmental parameters. The plot shows how strongly each characteristic influences as a principal component and which variables are most strongly correlated with each component, which in this case is the season. Similar

to PCO analysis, PC1 and PC2 correlated to 68.7% of the total variability. Figure 2.3 showed that Nutrients and soil pH contributed to the major variability during the monsoon season, while salinity and temperature were the major variables during the pre-monsoon season. Such a definite pattern for the post-monsoon season was not evident in most stations.

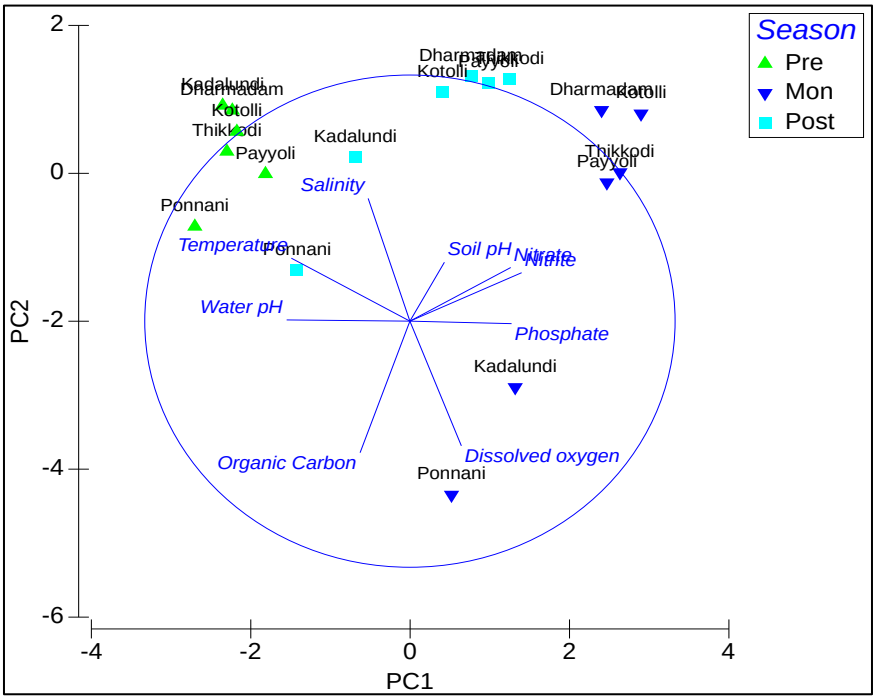


Figure 4.3 Principal Component Analysis (PCA) correlating seasonal variability to environmental parameters

Clear segregation of stations during different seasons was evident in PCA, indicating that all environmental variables influenced the actinomycetes assemblage during different seasons in the study area. Therefore, it is understood that environmental parameters prevailing in a region during a particular season greatly influence the species assemblage of actinomycetes in that region. Variability of actinomycetes is also related to their species diversity and for which a detailed analysis is further required. In order to ascertain the species diversity of actinomycetes, a series of diversity indices were worked out from the assemblage patterns.

Table 4.6 Diversity indices of Actinomycetes from the study area

		Sum of Squares	df	Mean Square	F	Sig.
No. of Species	Between Groups	385.611	5	77.122	7.755	.002
	Within Groups	119.333	12	9.944		
	Total	504.944	17			
Total Individual	Between Groups	1017.833	5	203.567	5.257	.009
	Within Groups	464.667	12	38.722		
	Total	1482.500	17			
Margalef's Species Richness	Between Groups	20.770	5	4.154	8.803	.001
	Within Groups	5.663	12	.472		
	Total	26.433	17			
Pielou's Evenness	Between Groups	.000	5	.000	2.084	.138
	Within Groups	.000	12	.000		
	Total	.000	17D			
Shannon	Between Groups	3.275	5	.655	10.043	.001
	Within Groups	.783	12	.065		
	Total	4.058	17			
Simpson	Between Groups	.007	5	.001	7.201	.002
	Within Groups	.002	12	.000		
	Total	.009	17			
Taxonomic Distinctness	Between Groups	73.824	5	14.765	6.967	.003
	Within Groups	25.430	12	2.119		
	Total	99.253	17			
Average Taxonomic Distinctness	Between Groups	66.147	5	13.229	5.415	.008
	Within Groups	29.315	12	2.443		
	Total	95.462	17			
Variance in Taxonomic Distinctness	Between Groups	3930.130	5	786.026	4.925	.011
	Within Groups	1915.291	12	159.608		
	Total	5845.422	17			

Table 4.7 Result of variation in the mean diversity indices of actinomycetes in the study area

		Sum of Squares	df	Mean Square	F	Sig.
No. of Species	Between Groups	385.611	5	77.122	7.755	.002
	Within Groups	119.333	12	9.944		
	Total	504.944	17			
Total Individual	Between Groups	1017.833	5	203.567	5.257	.009
	Within Groups	464.667	12	38.722		
	Total	1482.500	17			
Margalef's Species Richness	Between Groups	20.770	5	4.154	8.803	.001
	Within Groups	5.663	12	.472		
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	Within Groups	.000	12	.000		
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	Within Groups	.783	12	.065		
	Total	4.058	17			
Simpson	Between Groups	.007	5	.001	7.201	.002
	Within Groups	.002	12	.000		
	Total	.009	17			
Taxonomic Distinctness	Between Groups	73.824	5	14.765	6.967	.003
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Average Taxonomic Distinctness	Between Groups	66.147	5	13.229	5.415	.008
	Within Groups	29.315	12	2.443		
	Total	95.462	17			
Variance in Taxonomic Distinctness	Between Groups	3930.130	5	786.026	4.925	.011
	Within Groups	1915.291	12	159.608		
	Total	5845.422	17			

Among the diversity indices, except for Pielou's Evenness index, all other indices showed significant ($P<0.05$) variation among the different stations. Similarly, except for Variation in Taxonomic Distinctness, which measures the irregularities and divergences in the distribution of actinomycetes within a sample, no other diversity indices showed a significant difference in diversity actinomycetes within seasons.

Table 4.8 Results of Multiple Regression analysis of diversity indices of Actinomycetes in the study area

	Ponnani	**Kadalundi**	**Thikkodi**	**Payyoli**	**Dharmadam**	**Kotooli**
No. of Species	12.3333^{bc}	19.6667^{d}	7.6667^{ab}	14.3333^{cd}	5.3333^{a}	13.0000^{bc}
No. of individuals	19.6667^{bc}	30.3333^{c}	9.6667^{ab}	21.3333^{bc}	8.0000^{a}	20.0000^{bc}
Species Richness	3.7963^{bc}	5.4927^{d}	2.8983^{ab}	4.3657^{cd}	2.1007^{a}	4.0023^{bc}
Species Evenness	0.9858^{ab}	0.9901^{b}	0.9829^{a}	0.9868^{ab}	0.9882^{ab}	0.9906^{b}
Shannon diversity	2.4550^{b}	2.9260^{b}	1.9887^{a}	2.6127^{b}	1.6267^{a}	2.5120^{b}
Simpson diversity	0.9608^{b}	0.9795^{b}	0.9562^{b}	0.9715^{b}	0.9193^{a}	0.9658^{b}
Taxonomic Diversity	34.8433^{a}	40.6633^{b}	39.3533^{b}	39.1100^{b}	41.0833^{b}	39.3333^{b}
Average Taxonomic Distinctness	37.6433^{a}	41.0467^{b}	40.2167^{ab}	40.2067^{ab}	44.1667^{c}	41.0033^{b}
Variance in Taxonomic Distinctness	153.7000^{b}	143.1667^{b}	148.4667^{b}	146.8000^{b}	108.5700^{a}	142.9667^{b}

Results indicate a distinct assemblage pattern of actinomycetes in all the six stations with better abundance, diversity, and taxonomic distinctness in Kadalundi followed by Ponnani mangroves. A closer observation shows that the diversity varies based on the substratum, with the mangrove regions showing the highest diversity during the rocky nearshore regions with the least abundance. Similarly, the taxonomic diversity of species from Ponnani was distinct compared to other stations, indicating the chances for isolating a diverse group of actinomycetes from Ponnani compared to other stations. However, while analyzing actinomycetes' diversity and distribution pattern during a different season, no significant ($P>0.05$) variation could be seen, indicating that the variability is more location-specific rather than a season. Hence all the environmental parameters which have been assumed to have contributed to the distribution pattern of actinomycetes in Northern Kerala is related to the geographic distribution and significantly less to the seasonal changes.

BEST BIO-ENV analysis was carried out to ascertain the most influencing parameter that contributed to the distribution of actinomycetes in the region. Results showed a Rho value of 0.909, indicating that variables such as water temperature, salinity, nitrate, water pH and organic carbon were responsible for over 90% of the distribution of actinomycetes in the region. Hence a variability of these parameters in different stations was responsible for the actinomycetes assemblage structure at a particular time. However, the standout parameter determining the diversity and distribution of actinomycetes in these stations was organic carbon ($P<0.05$).

Table 4.9 Results of mean variation in the diversity indices of actinomycetes during different seasons in the study area

		Sum of Squares	df	Mean Square	F	Sig.
No. of Species	Between Groups	94.778	2	47.389	1.733	.210
	Within Groups	410.167	15	27.344		
	Total	504.944	17			
Total Individual	Between Groups	364.000	2	182.000	2.441	.121
	Within Groups	1118.500	15	74.567		
	Total	1482.500	17			
Margalef's Species Richness	Between Groups	4.687	2	2.344	1.617	.231
	Within Groups	21.745	15	1.450		
	Total	26.433	17			
Pielou's Evenness	Between Groups	.000	2	.000	.329	.724
	Within Groups	.000	15	.000		
	Total	.000	17			
Shannon	Between Groups	.711	2	.355	1.592	.236
	Within Groups	3.347	15	.223		
	Total	4.058	17			
Simpson	Between Groups	.001	2	.001	1.245	.316
	Within Groups	.008	15	.001		
	Total	.009	17			
Taxonomic Distinctness	Between Groups	10.247	2	5.124	.863	.442
	Within Groups	89.006	15	5.934		
	Total	99.253	17			
Average Taxonomic Distinctness	Between Groups	2.795	2	1.397	.226	.800
	Within Groups	92.667	15	6.178		
	Total	95.462	17			
Variance in Taxonomic Distinctnesss	Between Groups	38.712	2	19.356	.050	.951
	Within Groups	5806.709	15	387.114		
	Total	5845.422	17			

Table 4.10 Results of BIO ENV analysis of the environmental parameters against actinomycetes distribution in the region

Variable	SS(trace)	Pseudo-F	P	Prop.
Temperature	1524.4	0.61152	0.738	3.68E-02
Salinity	2343.9	0.96	0.428	5.66E-02
Dissolved oxygen	3981.9	1.7023	0.101	9.62E-02
Soil pH	881.17	0.34788	0.926	2.13E-02
Nitrite	3114	1.3011	0.256	7.52E-02
Nitrate	3717.4	1.578	0.136	8.98E-02
Phosphate	1526.9	0.61258	0.727	3.69E-02
Water pH	1588.4	0.63825	0.69	3.84E-02
Organic Carbon	6550.7	3.0068	0.01	0.1582

Results of Marginal tests to delineate the most significant factor contributing to the diversity and distribution of actinomycetes in the region reconfirmed the result of BEST BIO ENV, indicating that organic carbon ($P<0.01$) of soil alone was the sole factor responsible for inducing a change in the distribution pattern in all the stations and seasons.

Multidimensional Scaling (MDS)

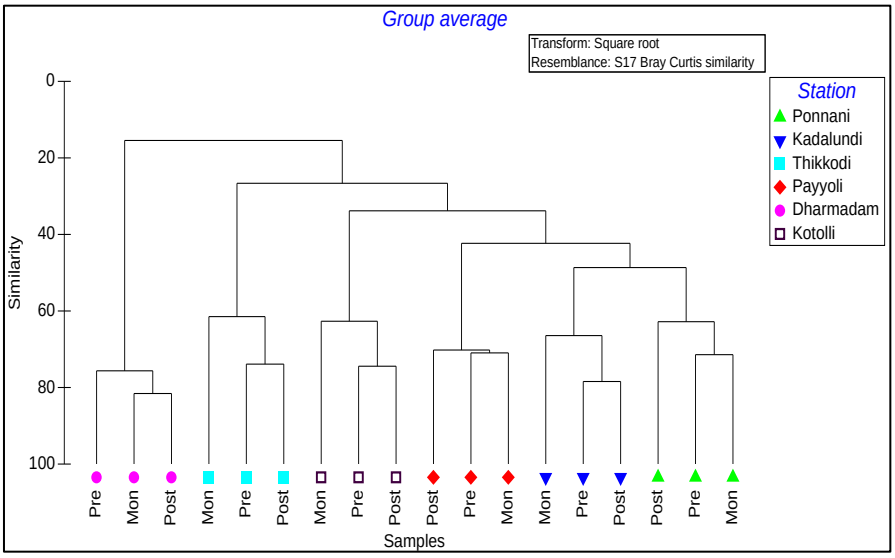


Figure 4.4 Result of Multidimensional Scaling (MDS) to group distribution of actinomycetes

Multidimensional Scaling (MDS), which is done to visualize the level of similarity of seasonal distribution of actinomycetes in the study region, showed a clear and specific pattern. The grouping of station indicated that the diversity of actinomycetes in all the six-station were significantly diverse, thereby grouping separately. While a distinct grouping for species in Dharmadam, Thikkodi and Kootoli indicates, they share a typical assemblage. The species from Ponnani, Kadalundi and Payyoli, seemed similar, which could be due to the similarity in the sediment structure of the region. However, from the analysis, it could be seen that the community in Dharmadam is very diverse from the rest of the station in the study area.

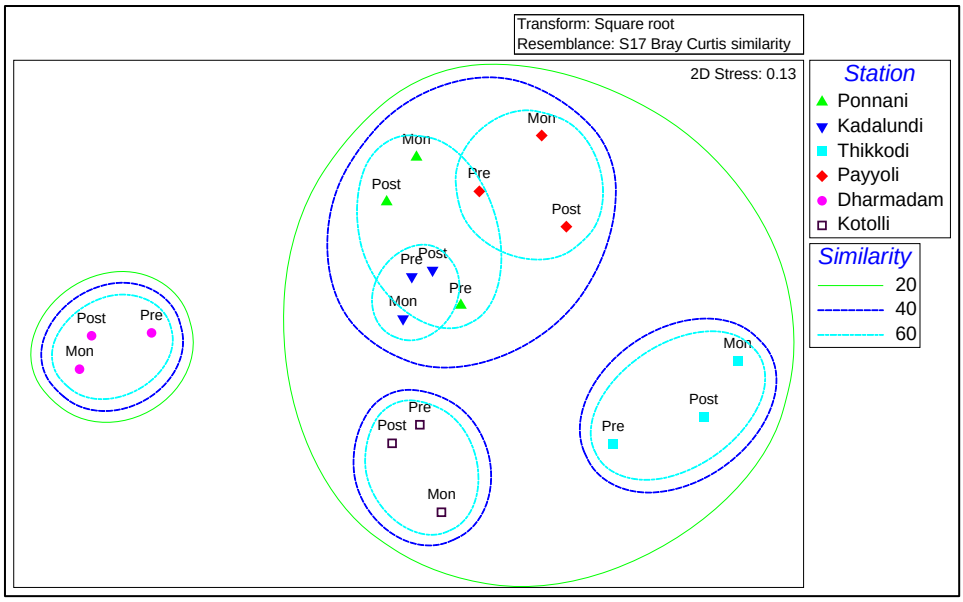


Figure 4.5 Result of cluster analysis indicating similarity in the species assemblage

Results of Cluster analysis based on 40% and 60% similarity in the population of Actinomycetes supported the result of MDS, showing a distinct grouping for species encountered in Dharmadam, Thikkodi and Kootoli. Actinomycetes assemblage did not vary with the season in any of the six stations studied. Although all other stations were closely grouped, there existed a clear demarcation in their grouping, too, at 60%

resemblance indicated in the MDS. This could be due to the difference in the organic carbon of sediment, which was attributed to the BIO ENV results.

Table 4.11 Results of PERMANOVA on Biological Diversity of actinomycetes in the study area

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique Perms
Station	5	35524	7104.8	16.638	0.001	999
Season	2	1614.2	807.09	1.89	0.123	998
Residual	10	4270.3	427.03			
Total	17	41409				

Permutational multivariate analysis of variance (PERMANOVA) is a non-parametric multivariate statistical test done to compare groups and their dispersion in space and time in relation to all groups. An interesting result that emerged following PERMANOVA analysis was that although the diversity of actinomycetes within the six stations were significantly ($P<0.01$) different, there existed very little variation ($P>0.05$) while comparing their distribution season-wise. This indicates that the assemblage structure of actinomycetes in Northern Kerala is determined more by the geographical location, the nature of the substrate and certain environmental parameters, which are not influenced by the changes in the seasonal pattern of environmental variability. Therefore it can reasonably be confirmed that the region, type of substratum, sediment nature, organic carbon load of soil etc., are the major contributing factors influencing the diversity of actinomycetes in the region.

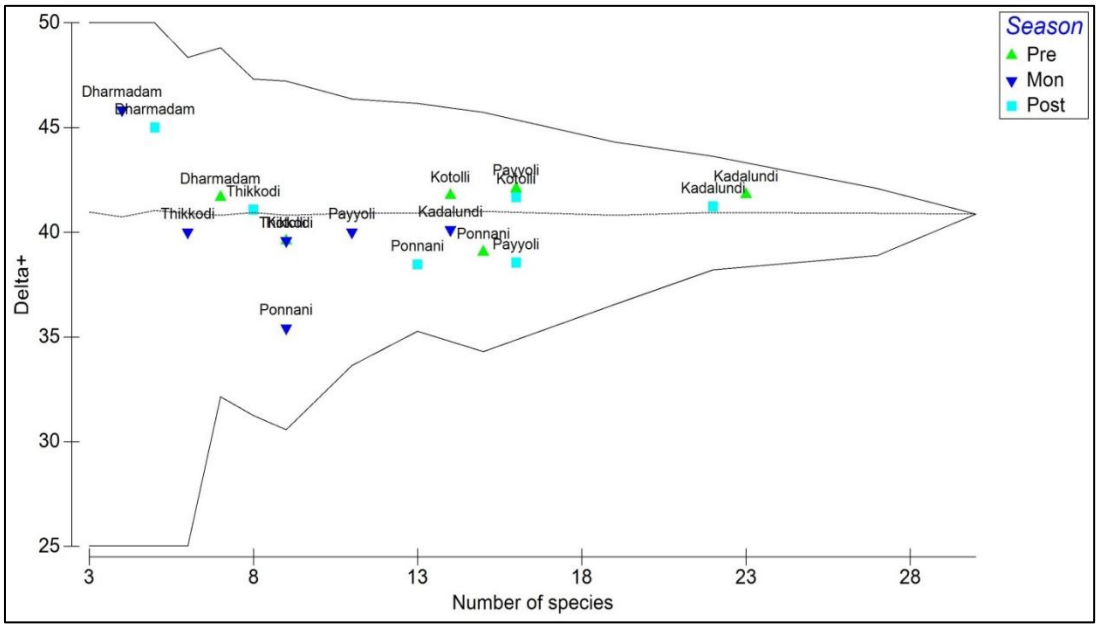


Figure 4.6 Results of funnel plot showing the Average taxonomic distinctness (AvTD)

Average taxonomic distinctness (AvTD) showed that in all the stations, the taxonomic distribution of actinomycetes was relatively uniform within 95% of the global mean. Hence the species distinction of actinomycetes was somewhat similar. However, the influence of habitat type and species diversity could not be ruled out, and this may be why certain stations such as Ponnani and Dharmadam in the monsoon season showed lower and higher values of AvTD than the group mean.

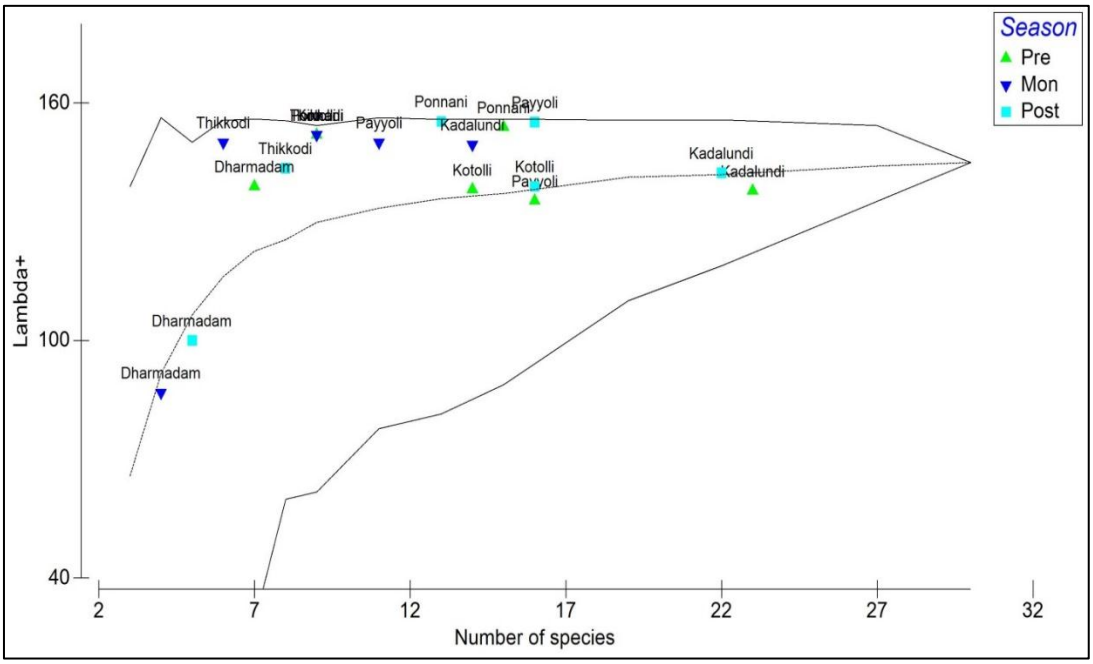


Figure 4.7 Results of funnel plot showing variations in Average taxonomic distinctness (VarTD)

An analysis of the seasonal influence on the variation in the taxonomic distinctness of actinomycetes in the study region indicated that though stations such as Kadalundi had higher species number compared to other stations during pre and post-monsoon seasons, the VarTD values were comparatively less compared to Payyoli and Ponnani during the same season. This indicates that a broader taxonomic representation was discernible even in certain stations that did not have the highest species richness. Hence from the study of AvTD and VarTD, it is clear that the taxonomic diversity of actinomycetes in the study area was a reciprocal impact of various associated factors as discussed earlier, including environmental variations.

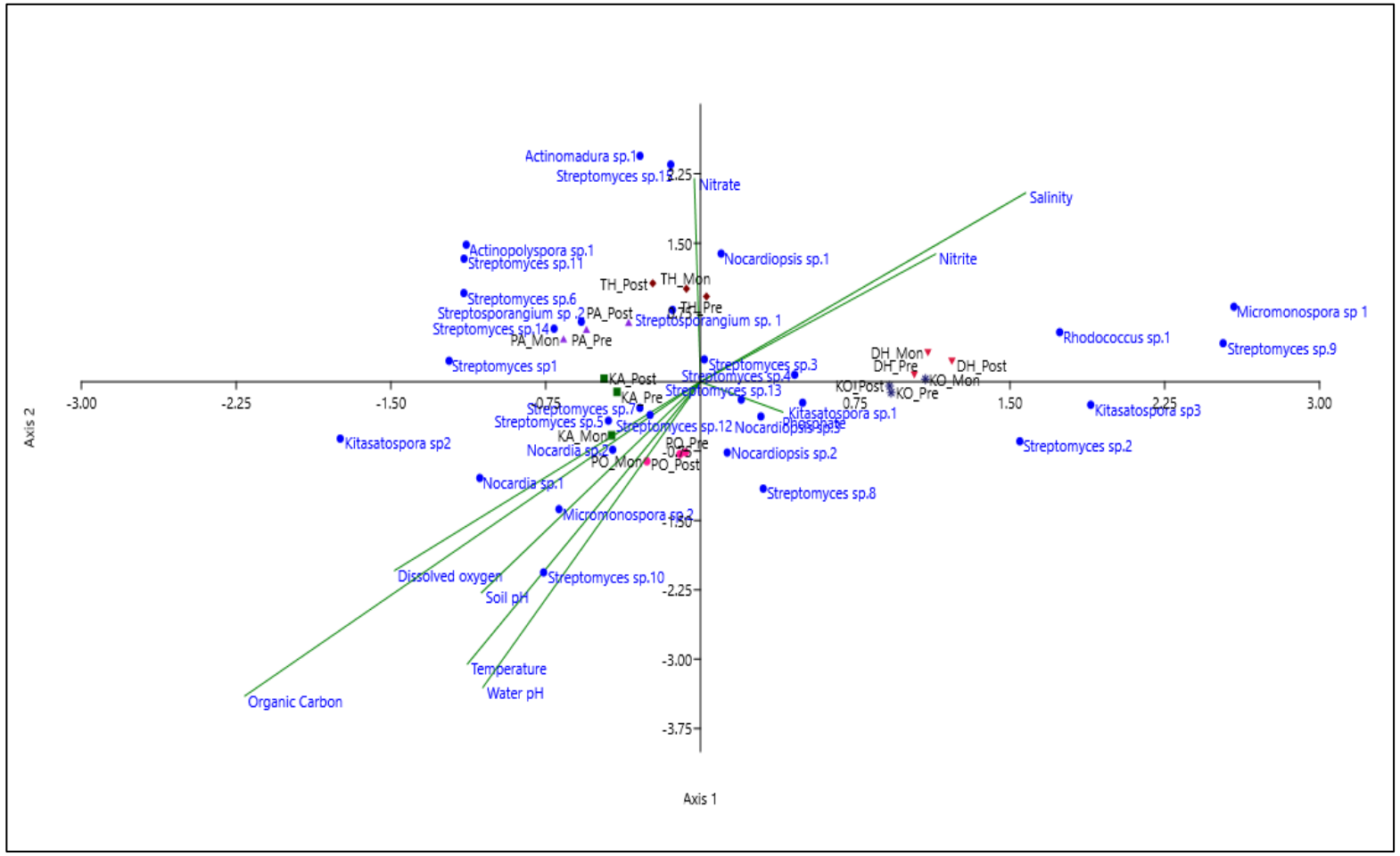


Figure 4.8 Results of Canonical Correspondence Analysis

As a final step for ascertaining the environmental influences on the distribution of actinomycetes in the Northern Kerala, Canonical Correspondence Analysis (CCA) was employed. The result indicated a clear pattern of distribution of specific actinomycetes based on the environment;l; profile. Among them, the most noted ones were those depicted by *Rhodococcus* sp. and *Micromonospora* sp., which showed a positive correlation with salinity and nitrite. In contrast, most *Streptomyces* sp. were influenced by the availability of organic carbon, temperature and pH. Hence the study demarcates the groups that were impacted by changing environmental variables. Although most actinomycetes were affected by the changing physico-chemical parameters, their degree of influence varied considerably. However, certain groups, such as *Nocardiopsis,* were not influenced by changing environmental variabilities and remained viable throughout the year. Hence, it is understood from the study that environmental variabilities and habitat characteristics power the occurrence and distribution of actinomycetes in Northern Kerala.

4.4 Discussion

The present study discusses the pattern of an assemblage of actinomycetes from the selected regions of Northern Kerala to understand their temporal and seasonal diversity and distribution. A total of 529 isolates of actinomycetes were isolated from the region, coming under nine genera and seven families. The majority of the isolates belonged to the genera *Streptomyces*. This genus is one of the most widely distributed actinomycetes groups in the nearshore regions of the tropical and sub-tropical marine realm. Having originated from a terrestrial environment, these groups represent a wide variety of species that are also good sources of novel bioactive compounds (Madrid *et al*., 2001). From this study, it was clear that there exists a significant ($P<0.01$)

difference in the diversity and distribution of actinomycetes based on the sediment characteristic of the regions, i.e., mangrove sediments > nearshore sediments > seaweeds. It is also confirmed that actinomycetes diversity in Northern Kerala was quite diverse related to the sediment characteristics of the region. Mangrove sediments showed higher abundance compare to nearshore sediments. Sediments of mangrove are of marine alluvial origin, transported as sediments and deposited by rivers and sea. These sediments are comprised of different percentage of sand, silt and clay (Hossain and Nuruddin, 2016). Further, a mixture of silt and clay forms mud, which is rich in organic matter and harbours a rich fauna of actinomycetes. The significant fluctuation of salinity and tidal gradients makes the mangrove forests unique environments and, therefore, good resources of actinomycetes that can withstand wide variations in environmental conditions (Hong *et al*., 2009). Results similar to the present study have been reported in earlier studies (Gong *et al*., 2018), which underlines that genomic analysis of these groups can also bring out more species diversity. In the present study, too, most of the actinomycetes encountered in the nearshore coastal regions of Northern Kerala were found to be from a single genus, *Streptomyces*. Although a number of other genera of Actinomycetes were also observed in the present study, they could not outnumber *Streptomyces* in the region. Similar results were observed in the study of Karthikeyan *et al*. (2013) from the Southeast coast of India. In consonance with present results, they reported a wide variety of actinomycetes coming under the genera *Actinobispora*, *Actinomadura*, *Actinosynnema*, *Catellospora*, *Jonesia*, *Microtetraspora*, *Nocardia*, *Pseudonocardia*, *Rhodococcus*, *Saccharopolyspora*, *Saccharothrix*, *Streptomyces*, *Streptoverticillium* and *Thermoactinomycetes* from mangrove soil of Mamallapuram, Tamil Nadu, India. Other studies have also confirmed the dominance of

Streptomyces in the actinomycetes population from coastal sediments (Alexander, 1971) and have accounted for over 80% of the actinomycetes population in some habitats (Jensen *et al*., 2005a). It is also reported that *Streptomyces* and *Micromonospora* species are best adapted to coastal and bathyal sediments (Takizawa *et al*., 1993; Mexson and Bull, 1999). A similar assemblage was reported by Leena and Raju (2019) from the coast of the Bay of Bengal. In the present study, 78 actinomycetes isolates were collected from the seaweeds samples of Thikkodi and Dharmadam rocky shores. Macroalgae have shown to harbour a rich diversity of actinomycetes as several of them lead an epiphytic or endophytic life in association with these seaweeds (Egan *et al*., 2013). The quest for exploring these microorganisms as a source for the production of novel bioactive compounds, as seen in endophytes of terrestrial plants, have lead to the emergence of a new branch of marine biotechnology (Strobel, 2003; Zhao *et al*., 2011). The results emerging from the present study are comparable with similar studies from other parts of the world (Girao *et al*., 2019). In the present study, the macroalgae screened came under the family Phaeophyceae, Chlorophyceae, and Rhodophyceae. It is reported that the region has a rich source of seaweeds both attached to the rock substratum as well as in the intermittent marine sediments. It is also observed that the permanent contact of the algae with marine sediments and coastal rocks enable actinobacteria to predominate in these unique habitats (Maldonado *et al*., 2005a; Abdelmohsen *et al*., 2014). Several Streptomyces strains have been isolated from algae, especially those belonging to the family Phaeophyceae (Wiese *et al*., 2009; Brana *et al*., 2015; Ismail *et al*., 2018). In the present study, however, most of the actinomycetes were recovered mainly from red seaweeds (Rhodophyceae) predominantly, *Gracilaria corticata* and *Gratiloupia filicina.*

In this study, the result of multidimensional scaling indicated that a distinct grouping for species was evident in Dharmadam, Thikkodi, and Payyoli, and the community in the Dharmadam was very diverse. This indicates that the substrate plays a vital role in the distribution and diversity of actinomycetes in these regions. Multiple regression analysis showed a distinct assemblage of actinomycetes in all six stations with better abundance, diversity, and taxonomic distinctness in Kadalundi followed by Ponnani. This could be attributed to the conducive environments of the mangrove regions that include less salinity, better productivity, high organic load, suitable sediment texture and favourable temperature regimes. Seasonal-wise variation in the abundance was also noticed with the maximum diversity seen during Premonsoon and least during Monsoon seasons. Studies of Varghese *et al*. (2014) have shown that the actinomycetes abundance in the soils of myristica swamps of Kerala varied with space and time, with a higher abundance in the pre-monsoon season. Similar to the present study, they also witnessed rich species diversity of actinomycetes comprising mainly six genera such as *Streptomyces, Nocardia, Micromonospora, Pseudonocardia Streptosporangium, and Nocardiopsis. The load of the actinomycetes* population was lesser in the monsoon season, possibly due to the reduction in nutrients and salinity. The result showed that Actinomycetes were mostly vigorous through the pre-monsoon season due to enhanced soil temperature, which might favour microbial activity. Temperature is considered one of the major environmental factors that control the activities and survival of microbes (Kumar and Sharma, 2020, Kumar *et al*., 2013; Takacs-Vesbach *et al*., 2008; Abou-Shanab, 2007).

There are several reports about the relationship between physico-chemical parameters and the distribution of actinomycetes in marine soils (Nadimuthu,1998;

Chandhuri *et al*., 2009; Karthikeyan *et al*., 2013). The distribution of soil microbial population is determined by a number of environmental factors like pH, moisture content and soil organic matter etc. (Kennedy *et al*., 2005). In the present study, however, the most contributing factor was found to be organic carbon which varied significantly between the stations. Similar results were observed by earlier research such as Miller *et al*.(1998), who reported that organic carbon showed to vary with depth and concluded that there was a strong relationship between topographic position and soil physicochemical properties. Soil temperature was found to positively correlate with actinomycetes distribution (Cho *et al*., 2008; Varghese *et al*., 2012). Total organic carbon that varied with the texture of the sediment in the range of 2.3–5.9 mg/g dry weight and the type of sediments, especially clayey soils, also had a good assemblage of marine actinomycetes (Das *et al*., 2008). An interesting observation on the analysis of environmental variable with nutrient suggests that physico-chemical parameters such as pH and electrical conductivity of soil and the concentration of sodium and potassium alter the total number of colonies in the coastal realm (Karthikeyan *et al*., 2013). In this study, out of the six sampling stations selected, except for Ponnani and Kadalundi, all other stations showed a uniform trend in the variation in environmental parameters for all the season and environmental variables such as nutrients and salinity was the major factor that varied during the year. In the Ponnani mangrove region, organic carbon, water pH and dissolved oxygen turned out to be the major variables. In Kadalundi, it was the temperature and soil pH that determined the species assemblage. Various analysis to correlate the significance of environmental parameters on the distribution of actinomycetes showed that though the species diversity was attributed to various factors, the majority of change in the community structure was contributed by three

variables: temperature and water nutrients and organic carbon in the sediments. Li *et al*. (2019b), through redundancy analysis, reported that moisture content, water-soluble carbon (WSC) and pH ($p < 0.05$) determined the temporal variations of actinomycetes community composition, accounting for 86.6% of the variation in the community structure.

In conclusion, this chapter describes the relationship between the physico-chemical parameters and the distribution and diversity of actinomycetes from the northern coast of Kerala. It is summarised that most of the environmental parameters studied influenced the spatial and temporal distribution of actinomycetes in the Northern Kerala coast. The diversity of actinomycetes in the marine habitat has also been related to the substrate where they are found. Seaweeds and coastal mangrove patches are a haven for a wide variety of actinomycetes. The mangrove environment, in particular, functions as a source of microorganisms, including actinomycetes capable of producing primary and secondary metabolites, and the actinobacteria here have rich diversity and various biological activities. Seaweeds (macroalgae) support a unique association with the marine actinomycetes, usually which is symbiotic in nature. Generally, the symbiosis of marine bacteria and marine organisms has a greater probability of producing chemical compounds than marine bacteria, which live freely (Mearns-Spragg *et al*., 1998). *Streptomyces* was the single largest genus encountered, and it has an enormous biosynthetic potential that remains unchallenged without a potential competitor among other microbial groups. Variables such as water temperature, salinity, nitrate, water pH and organic carbon were responsible for over 90% of the distribution of actinomycetes in the region. Seasonal-wise variation in the abundance was also noticed, with the maximum diversity seen during pre-monsoon and

least during monsoon seasons. Seasonal influence on the distribution of actinomycetes showed that wide fluctuations in their species assemblage were recorded during the monsoon season. Hence, the present study provides the first comprehensive description of the diversity of actinomycetes in the Northern Kerala coast, emphasizing its distribution in terms of habitat and season. Results indicate that the region to have a good population of actinomycetes which have sustained survival in diverse ecological niches. Since most of these groups produce secondary metabolites to adapt to wide variations in environmental conditions, it is, therefore, worth investigating species that produce these compounds extensively to survive under different climatic environments.

Chapter 5

ISOLATION AND IDENTIFICATION OF BIOFOULING BACTERIA FROM PONNANI COAST

5.1 Introduction

The marine realm, which covers around 70% of the earth's surface, has an enormous microbial diversity spread in various major and minor habitats. Several workers have reported that though more than half of the earth's biomass is microbial, they are not conspicuous in the natural environment (Perez *et al*., 2006; Ventosa*et al*., 1998; Lakshmi and Akondi, 2013). Microbial adaptability to varying habitats has been attributed to their diverse morphology, physiology and metabolism that they display to survive even in extreme environments (Lakshmi and Akondi, 2015). One among them is the halophiles that are adapted to harsh, hypersaline conditions. Being halotolerant microorganisms, they grow over a wide range of salt concentrations inhabiting natural hypersaline brine in arid, coastal and deep-sea waters as well as in artificial salt pans used to mine salt from the sea (Azam*et al*., 1983; Button, 2004). It is a known fact that these halophiles use various mechanisms to withstand the extreme environmental changes through the production of secondary metabolites that enable them to survive and distribute well in some of the most incompatible situations.

In the marine environment, the competition for living space is intense, and all surfaces, living or dead, are susceptible to fouling. The colonization of living or non-living surfaces by sessile organisms, plants or animals is a universal phenomenon in the aquatic environment. Generally, biofouling is referred to as the attachment and growth

of aquatic organisms on any artificial solid surfaces. Although such assemblages may sound harmless, the problems associated with biofouling are ever so alarming, which has paved the wait for substantial research the world over (Nandakumar and Yano, 2003). The biofouling growth, species composition, accumulation rate, community dynamics, and problems caused are various as the types of material used and the place and season of the year. Thus, the variability in biofouling is governed mainly by the geographical location, the reproductive cycle of the organisms, depth of the water column, and the season (Kawahara, 1969; Keough and Raimondi 1996; McDougall, 1943; Menon*et al*., 1977; Nandakumar*et al*., 1996). Biofouling in freshwater systems is less pronounced than seawater, which has high salt content and forms a complicated solution containing most known elements.

Marine biofouling is the colonization of submerged surface by unwanted marine organisms such as microorganisms, barnacles and seaweeds. Marine biofouling is a global problem for maritime industries creating both economic and environmental issues. It has harmful effects on the shipping and fishing industry, heat exchangers, oceanographic sensors, and aquaculture systems.Fouling on ships is significantly detrimental and results in increased drag, surface corrosion, decreased fuel efficiency, loss of speed, and increased pollution. Submerged structures become corroded, followed by rusting due to the intense actions of the fouling organisms. The prevalence of marine biofouling is naturally higher in the shallower water along the coast. The development of new aquaculture facilities has led to an increase in submerged structures such as floats, ropes, cages and nets that inadvertently provide favourable substrates for fouling organisms (Durr and Watson, 2010). These biofuels significantly interfere with culture operations, cause significant economical impacts on marine aquaculture and are widely

recognized as one of the main problems faced by any aquaculture facility. It is also estimated that the annual economic direct cost of controlling biofouling on aquaculture is around 5-10% industry value (Lane and Willemson, 2004).

The process of biofouling generally begins with the formation of a film on which bacteria and other organisms colonize. The macroforms include various eukaryotic organisms like marine invertebrates and algae (Lakshmi and Akondi, 2015). Once underwater, the surface of the substrate immediately starts to adsorb a molecular 'conditioning' film primarily consisting of dissolved organic material (Jain and Bhosle, 2009). The conditioned surface is later colonized by microorganisms, including bacteria, algae (especially diatoms), fungi and protozoa. The attachment, colonization and growth of microorganisms on the surface result in the formation of a slimy layer called 'biofilm'. The biofilm layer can aid (or deter) the subsequent succession of 'macrofouling' species, such as barnacles, by facilitating adhesion or through the production of bioactive molecules (D'Sousa *et al.*, 2010). Thus, it has been assumed that the type of biofilm formation determines the nature of fouling to succeed. However, it is now proven that successional colonization of a surface necessarily implies only a causal relationship between one stage to the next. Thus, by mere controlling or blocking initial stages of colonization, such as biofilm formation, it may necessarily not reduce or eliminate macrofouling. On the other hand, it is undoubtedly clear that attachment of spores and larvae can be influenced by other organisms, notably by bacterial biofilms, and positive, negative and neutral effects have been detected in controlled laboratory experiments when biofilms of specific bacteria have been tested against algal spores and larvae of invertebrates (Callow and Callow, 2006; Dobretsov*etal*., 2006; Hugget*et al*., 2006; Dobretsov*et al*., 2009; Huang and Hadfield, 2003).

Microbial biofilms are biological structures composed of surface-attached microbial communities embedded in an extracellular polymeric matrix. Natural biofilms are composed of several microbial species and their extracellular polymeric substances (EPS) coatings. The EPS coating is both an adhesive and protective layer that modulates the diffusion of molecules in the biofilm. Consequently, cells in biofilms are more resistant to antibiotics and antibacterial agents (Costerton*et al*., 1987). In aquatic environments, the microbial colonization of submerged surfaces is a complex process involving several factors related to environmental conditions and the physical-chemical nature of the substrates (Caruso, 2020). Biofilm formation on substrate surfaces is the first step in biofouling formation (Dang and Lovell, 2016; Dobrestov*et al*., 2013; Flemmingand Wuertz, 2019). According to Donlan (2002), microbial biofilms consist of several microorganisms, both prokaryotic (bacteria and archaea) and eukaryotic (algae and fungi), strictly adhering to a substrate surface. Within microbial biofilms,microcolonies of bacterial cells are wrapped into an exopolysaccharide matrix consisting of "extracellular polymers of biological origin which participate in the formation of microbial aggregates" (Geesey*et al*., 1996). Therefore, each microbial biofilm is composed of a complex microbial community, which creates a microenvironment where over 4000 species can co-exist in a complex structure (Nielsen and Molin, 2000; Jefferson, 2004). Several factors, both environmental and those related to the nature of the substrate, may influence microbial colonization at sea (Fletcher, 1998; Characklis*et al*., 1990). It has become evident that several physico-chemical variables, such as temperature, salinity, pH, and nutrient concentration, together with the geographic location, seasonal periodicity, light availability, water depth, presence of tides, competition among biofilm components, can affect the

formation and composition of biofilm communities in marine environments (Dang and Lovell, 2016; Salta *et al*., 2013). The success in controlling fouling depends upon the generation of basic information on bacterial assemblage in the biofilm, species composition, growth, biomass build-up, diversity, seasonality, succession, and distribution of the biofouling bacterial community. The most effective method to curb the growth of biofuels is by using antifouling paints that contain biocides, such as tributyltin (TBT) and tributyltin oxide (TBTO) which were proven to be harmful to non-target organisms and the environment (Sonak*et al*., 2009). Moreover, being broad-spectrum agents, they also deleteriously affect the useful bacterial growth that has nutritional and bioremedial properties. Hence it is pertinent to first assess the bacteria that facilitate the biofilm formation for successive biofouling processes. In order to understand this, a baseline study on the biofilm-forming bacteria on different surfaces that are wholly or partially exposed underwater need to be studied. An understanding of the bacterial diversity would be beneficial in isolating the most potent species that form biofilm readily on a wide variety of solid structures. Therefore, the strains of actinomycetes that were isolated and characterized in Chapter 3 could be tested on these locally available bacterial strains for their antifouling activities. Hence against the above pretext, a study was conducted to screen different strains of biofilm-forming bacterial groups from the harbour and coastal regions of Ponnani to understand their diversity and potential for producing EPS and successive biofouling in the region.

5.2 Materials and Methods

5.2.1 Study area

Figure 5.1 Map showing the sampling area Ponnani, Malappuram district, Kerala, India.

The present study was carried out from coastal areas of Ponnani, mainly from Ponnani fishing harbour (10^0 47' 12" N, and 75^0 54' 59" E), and sampling was done periodically during 2017-2018.

5.2.2 Sample collection

Biofouling (Biofilm) samples were scrapped from immersed surfaces of boats, ropes, oysters and other hard materials from the Ponnani fishing harbour using a sterilized spatula. The collected samples were placed in a sterile zip lock cover and placed in an icebox. The samples were then aseptically brought to the laboratory for further analysis.

5.2.3 Isolation of biofouling bacteria

Biofouling bacteria were isolated by standard spread plate method using Zobell's marine Agar (Peptone 5.000 g/L Yeast extract 1.000 g/L Ferric citrate 0.100 g/L Sodium chloride 19.450 g/L Magnesium chloride 8.800 g/L Sodium sulphate 3.240 g/L Calcium chloride 1.800 g/L Potassium chloride 0.550 g/L Sodium bicarbonate 0.160 g/L Potassium bromide 0.080 g/L Strontium chloride 0.034 g/L Boric acid 0.022 g/L Sodium silicate 0.004 g/L Sodium fluorate 0.0024 g/L Ammonium nitrate 0.0016 g/L Disodium phosphate 0.008 g/L Agar 15.000 g/L;pH (at 25°C) was 7.6 ± 0.2 (Dhanasekaran*et al*., 2009). About 10 gram of fouling sample was mixed in 90 ml buffer and serially diluted up to 10^{-5} using sterile distilled water blanks. After serial dilution, 1 ml of an aliquot from each dilution were transferred into the prepared agar plates and spread using a sterile 'L' rod. Plating was done in duplicate, and all the plates were incubated at 28^0C for 3-6 days. After enumeration, morphologically different bacterial colonies were selected, purified and sub-cultured on nutrient agar slants supplemented with 2% NaCl (Bavya*et al*., 2011).

5.2.4 Biofilm formation study on isolated bacteria

5.2.4.1 Plate method

The mucoid nature of the bacterial colonies was studied by the cultivation of all the strains on Congo Red Agar (CRA) plates (Mariana *et al*., 2009). Eighteen hours old bacterial cultures were taken and spotted on the CRA plates. Plates were incubated at 28°C for 24-48 hours and observed for the characteristic colony morphology.

5.2.4.2 Tube method

The biofilm formation capability by the bacterial isolates was noticed by way of adherence to the walls of culture tubes (Mathur*et al*., 2006). The inoculum was prepared using 2 ml of nutrient broth. After 24 hours of incubation at 28^0C, turbidity was adjusted to 0.5 McFarland standards. For the biofilm experiment, 100µl of inoculum was transferred into 3ml of nutrient broth in 10 ml test tubes. All the test tubes werekept in a shaker at 95 "rpm" speed for 24-48 hours. After incubation, culture broth which contains the free cells, if any, were discarded. The tubes were washed with 3ml of 1X phosphate buffer saline (PBS). About 3ml of 2 % crystal violet solution was added and allowed to act for 5 minutes. All the tubes were washed with sterile water after discarding the crystal violet solution and allowed to dry. All the tubes were visually observed for the presence of biofilms on the inner walls of the test tubes. All the tubes were added with 1.5 ml of 33 % glacial acetic acid and mixed gently. The optical density (OD) value was measured in a colorimeter at 570 nm. The OD values of the test samples were compared with the PBS present in the control tube.

5.2.5 Characterization and identification of biofouling bacteria

5.2.5.1 Morphological characterization

Gram's staining

The isolates that showed strong biofilm formation were subjected to Gram' staining according to the procedure (Cappuccino and Sherman, 2005). The isolate was smeared in the slide and heat-fixed. The crystal violet dye was added, kept for 1 minute and washed in running water. Gram's iodine was added, kept for 1 minute and washed in running water, then the smear was decolorized with ethanol, and finally, the counterstain safranin was added. After a minute, it was washed in running water. It was observed under the Oil immersion microscope (Olympus CX21i, Japan). Gram-positive cells were purple in colours, and Gram-negative cells were red in colours.

Spore staining

All the selected isolates were smeared in the slide and heat-fixed. The malachite green dye was added, and it was heat fixed, kept for 1 minute and washed in running water. It was decolorized with tap water, and counterstain safranin was added after a minute, washed in running water. The isolate was observed under the binocular microscope (Olympus CX21i, Japan). Spore formers were green in colours, and non-spore formers were red in colour.

Hanging drop method

The motility test was carried out by the hanging drop technique (Gunasekaran, 2000). Coverslip was coated with Vaseline on its edges. The isolate was transferred into the centre of the coverslip. The cavity slide was placedover the coverslip and turned over to prepare hanging drop; the slide was viewed under the microscope. The motility was determined from the swarming movement of the microorganism.

5.2.5.2 Biochemical characterization

Indole production test

The indole production test was used to test whether the organism can oxidize tryptophan resulting in the formation of indole, pyruvic acid and ammonia. Tryptone broth was inoculated with the isolate, and one tube was kept as an uninoculated comparative control incubate tubes for 24-48 hrs at 37ºC. The indole production during the reaction was detected by adding Kovac's reagent (dimethylaminobenzaldehyde), which produces a deep-red layer in the top of the test tube, was indicates positive. There was no development of deep red colour in the top layer of the tube indicates a negative reaction.

Methyl red test

The methyl red test was employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of Acid and acid products. MR-VP broth tubes were inoculated with the isolate. One tube was kept as an uninoculated comparative control. Incubate tubes for 24-48 hrs at 37ºC. After 48 hrs of incubation, five drops of methyl red indicator was added, the colour of methyl red turned to red indicates a positive test. The colour of methyl red turned to yellow indicates a negative test.

Voges-Proskauer test

The Voges-Proskauer test was used to differentiate the microorganisms to produce non-acidic or neutral end products such as acetyl methyl carbinol and 2, 3 butanediol. The isolate was inoculated with test tubes containing MR-VP broth. One tube was kept as an uninoculated comparative control. Incubate tubes for 24 hrs at 37ºC. Development of deep red colour in the inoculated tubes 15 minutes after addition of Barrit's reagent indicated positive the absence of red colouration indicates negative.

Citrate utilization test

Citrate test was used to differentiate bacteria based on their ability to utilize citrate as the sole carbon source. The utilization of citrate depends on the presence of enzyme citrate produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. Simmon's Citrate agar slants were inoculated with the selected isolate. An uninoculatedSimmon's Citrate agar slant was kept as control. Then these tubes were incubated at 37ºC for 24-48 hrs. Development of green to blue colour and this constitutes a positive test. No change in the colour of the medium indicates negative.

Catalase tests

Trypticase soy agar slants were inoculated with the selected isolate. An uninoculatedtrypticase soy agar slant was kept as control. Then these tubes were incubated at 37ºC for 24-48 hrs. While holding the inoculated tube at an angle, allow 3-4 drops of hydrogen peroxide to flow over the growth of each slant culture. Catalase positive microorganisms were indicated by the production of bubbles of oxygen within one minute after the addition of H_2O_2. Catalase negative microorganisms were indicated by no bubble of oxygen production.

Oxidase test

Oxidase test to check the presence of the electron transport chain that is the final phase of aerobic respiration of microorganisms, was carried out. Usually, oxygen is the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N,N,N',N'-tetramethylphenylenediaminedihydrochloride) was used in the place of oxygen. This acceptor changes colour to a dark blue or purple when it takes the electron from the last element, cytochrome oxidase, in the electron transport

chain. With a sterile swab, a small amount of isolated culture from an agar plate was obtained. One drop of oxidase test reagent was placed onto the culture on the swab. Positive reactions turned the bacteria culture violet to purple immediately or within 10 to 30 seconds. Delayed reactions are ignored.

5.2.6 Isolation of exopolysaccharides (EPS)

Exopolysaccharides are high molecular-weight polymers containing sugar residues secreted by the bacteria into the surrounding environment before biofilm formation. EPS formation is necessary for the successive bacterial strains to colonize the substrate and therefore acts as the primary step in biofouling. EPS isolation was done following Van Geel-Schutten*et al*. (1998) from the culture medium. EPS pellets so formed were resuspended in hot distilled water, and the total sugars from final solutions were determined by the Anthrone method (Morris, 1948) by measuring absorbance at 630 nm using glucose solutions, and a standard curve was drawn. The obtained O.D value (Y) was substituted in the standard curve equation to get the value for X which gave concentration of EPS in 0.5 ml of the sample and calculated as, EPS (mg/L) = {(X / 0.5 * 10) * 1.1} * 100.

5.2.7 Molecular characterization of selected strains of biofouling bacteria

The 16S rRNA gene was amplified from genomic DNA obtained from biofouling bacterial cultures with forward primer- F243 (5'-GGATGAGCCCGCGGCCTA-3') and reverse primer R513GC (5'-CGGCCGCGGCTGCTGGCACGTA-3') in PCR (QiagenRotorGene Q). The reaction mixture contained 50 ng of DNA, Ex Taq PCR buffer, 1.5 mM MgCl2, 10 mMdeoxynucleoside triphosphate mixture, 50 pmol of each primer and 0.5 U of Ex Taq polymerase chain reaction (PCR). Conditions consisted of an initial denaturation at

94°C for 5 min; 30 cycles at 94°C for 1 min, annealing at 63°C for 1 min and 72°C for 1 min; and final 5 min extension at 72°C. Amplification products were examined by agarose gel electrophoresis purified using a QI quick PCR clean up kit (Fredimoses, 2010). The complete 16S rRNA gene was sequenced by using an ABI 377 automated DNA sequencer. A Maximum- Likelihood (ML) tree was constructed using MEGA V. 7 (Kumar *et al*., 2016) by including the 16S rDNA sequence of *Bacillus* strainscollected from the Ponnani region, together with those retrieved from NCBI GenBank with *Nocardiopsis sp.* used as an outgroup.

5.3 Results

5.3.1 Isolation of biofouling bacteria

Only isolates present in appreciable quantities (based on the number of CFU) were screened for biofilm studies. As a result, in the present study, 28 biofouling bacterial isolates were recovered from various fouling samples of Ponnani harbour during2017-18.

5.3.2 Confirmation for biofilm formation

All the 28 bacterial isolates were subjected to biofilm formation study by plate method and tube method. Out of this, only 13 isolates showed positive results for biofilm formation, of which seven isolates showed good biofilm production.

5.3.3 Identification of selected biofouling bacteria.

The isolates that showed strong biofilm-forming capacity under laboratory conditions were subjected to cultural and biochemical characterization, and the results are depicted in Table 5.1. The seven isolates identified belonged to four genera viz., *Bacillus*, *Aeromonas*, *Staphylococcus*, and *Serratia*.

Table 5.1 Cultural and biochemical characteristics of selected biofilm-forming isolates

Characteristics	Bacterial isolates			
	Bacillus	Aeromonas	Staphylococcus	Serratia
Gram staining	G +ve rods	G-ve rods	G+vecocci	G +ve rods
Endospore staining	+ve	-ve	-ve	G+ve rods
Motility	Motile	Variable	Non-motile	Motile
MacConkey agar	Colourless	Brilliant Red	Orange	Pink
Cetrimide agar	No growth	_	No Growth	_
TCBS agar	No growth	_	_	_
MSA agar	No growth	_	_	_
Oxidase	-ve	+ve	-ve	+ ve
Catalase	+ve	+ve	+ve	+ ve
Indole	-ve	+ve	-ve	-ve
Methyl red	-ve	+ve	+ve	-ve
VogesPrauskaeur	+ve	+ve	+ve	+ve
Simmon citrate	+ ve	+ve	+ve	+ve
NaClTolerence				
0%	+	+	+	+
1%	+	++	++	+
2.5%	++	++	+++	++
5%	++	++	+++	++
7.5%	+	+	++	+
10%	-	+	+	+

+ ; Positive -; Negative ++; Good +++; High

5.3.4 Exopolysaccharides production

Since biofilm formation and subsequent biofouling are related to the secretion rate of exopolysaccharides (EPS), a by-product in the process, a quantitative analysis of the same was carried out. It was seen that in most instances, the rate of production of

EPS as a secondary metabolite was directly related to the bacterial biomass besides other factors. Therefore, the amount of EPS produced per unit biomass was determined, and the results are shown in Fig 5.2. It is evident from the study that two strains, namely PPL7 (175.27 mg/L)and PPV2 (149.53 mg/L), produced maximum EPS per unit biomass, while all other strains had significantly less EPS production,

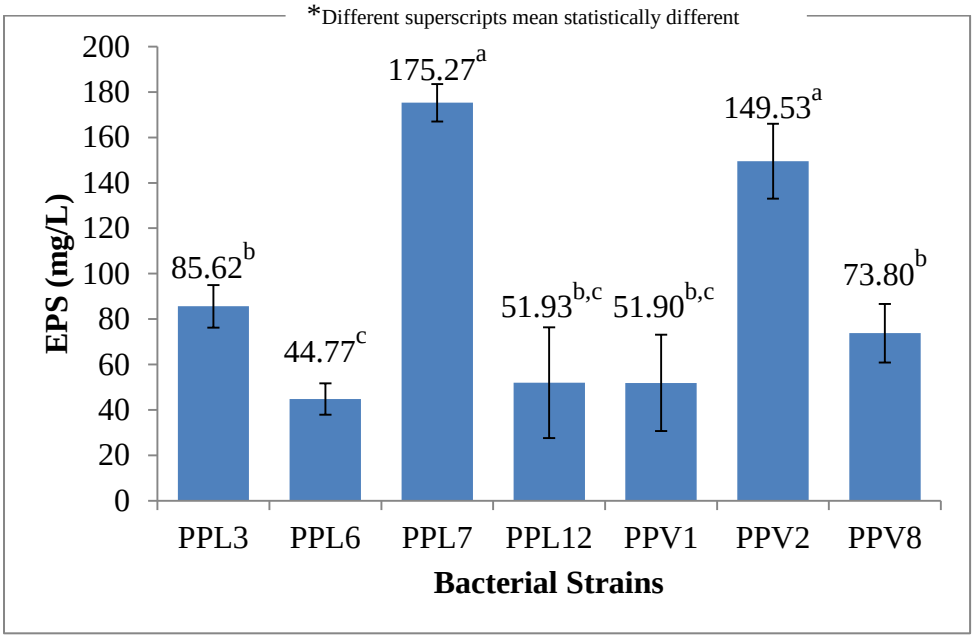


Figure 5.2 EPS production by seven selected biofouling bacterial strain

5.4 Molecular characterization of selected strains of biofouling bacteria

The two isolates PPL7 and PPV2 of biofouling bacteria that showed better EPS production were subjected to molecular taxonomy. With better EPS production, these two strains indicated high biofilm production. The PCR products of 16S rRNA were sequenced, and nucleotide sequences of the two bacterial strains PPL7 and PPV2 were submitted to NCBI Genbank with accession numbers MT348573 and MT348574, respectively. The results of phylogenetic analysis based on 16S rRNA gene sequence showed that PPL7 is closely related to *Bacillus subtilis* while PPV2 is closely related to *Bacillus licheniformis* (Fig. 5.3).

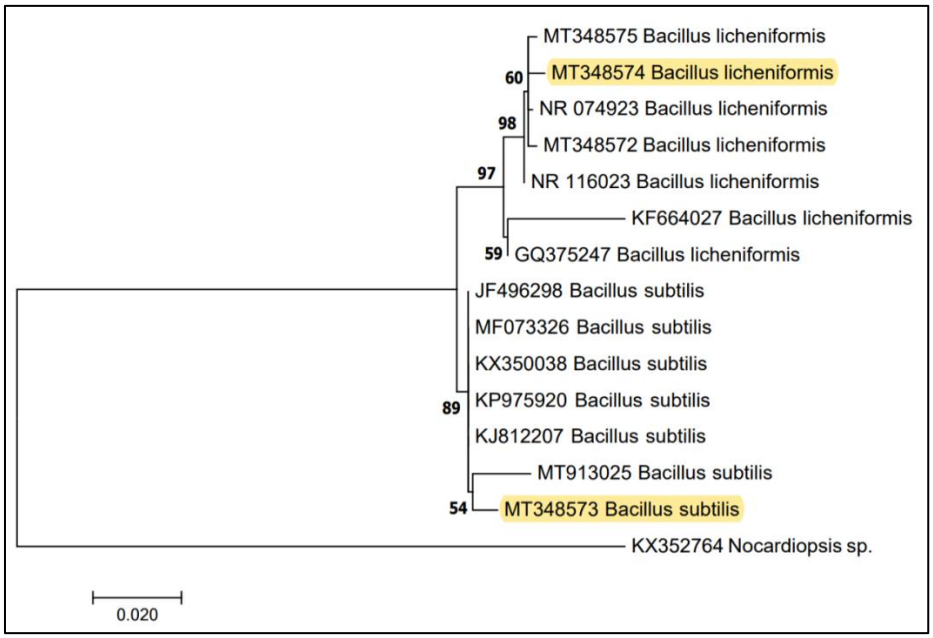


Figure 5.3 Phylogenetic tree of two strains of *Bacillus spp.* reconstructed applying maximum likelihood method

5.4 Discussion

In the present study, the bacterial strains involved in biofilm production in immersed surfaces of various hard surfaces from Ponnani harbour were isolated and characterized. Most of these primary settlers on these substrates are bacteria, and as a result, their isolation was done on Zobell's marine agar. Similar media were used to successfully isolate biofouling bacteria from similar surfaces by several workers earlier (Dhanasekaran*et al*., 2009; Bavya*et al*., 2011; Gopikrishnan*et al*., 2013). In the present study, 28 biofouling isolates were isolated, which may be less because representative sampling was done only based on the season and the limitation in the types of immersed structures. In the present study, the main onus was to identify the different strains of biofilm-forming bacteria and not on their distribution pattern. Hence, a quantitative assessment of their distribution in the different substrates or their seasonal diversity was not assessed Ponnani fishing harbour. Out of 28 isolates obtained, 13 isolates showed positive results for biofilm formation, of which seven isolates showed strong biofilm

formation. The biofouling bacteria have already been reported from different habitats (Lee *et al*., 2014). However, their potential for producing biofilms vary based on various biotic and abiotic factors, with nutrition being the most important one (Ryu*et al*., 2004). Although there have been various studies carried out in other parts of the world that have characterized the availability of biofilm-forming bacteria such as *Pseudomonas fluorescence* by Sillankorva *et al.* (2008), *Vibrio alginolyticus* by Kreig and Hoit (1984) and Sonak and Bhosle (1995), *Escherichia coli* by Nidal *et al.* (2004), *Aerobacteraerogens* by Chaudhary *et al.* (1996), very few studies have tried to correlate the biofilm formation and diversity of bacterial population from Northern Kerala coast. In India, such a study Gopikrishnan*et al*. (2013) have shown that a particular genus of bacteria can produce strong biofilm, including the genera *Staphylococcus, Micrococcus, Vibrio and Alcaligenes.* In the present study, however, the diversity of genus *Bacillus* as a major biofilm-forming bacterial group in the Ponnani harbour is worth mentioning. Other works that fall in line with the present study are of Saravanan*et al*. (2006), reporting *Bacillus sp* and *Staphylococcus sp*in cooling water system and their role in developing complex multi-species biofilm community.However, the one work that has a close resemblance to the present study in the region is byBavya *et al*. (2011), who identified and characterized three biofouling bacterial isolates as *Bacillus*sp, *Serratia*sp, and *Alteromonas* sp. from the Kerala coast. In the present study, seven bacterial isolates showed strong biofilm formation, and on further biochemical and enzymatic characterization, they were identified to belong to the genus *Bacillus, Aeromonas, Staphylococcus and Serratia.* Bacillus is known to colonize diverse environments linked to their spore-forming properties to its high number of operon copies per genome, enabling them to withstand adverse environmental conditions (Klappenbach*et al*.,

2000).Thus, it is evident that *Bacillus* spp. has a better adaptability and broader distribution in diverse habitats which vary widely in space and time and also diverse environmental conditions as witnessed in the present sampling.Having a better chance to be the pioneers in biofilm formation, *Bacillus* sp. dominated the biofilm-forming bacterial community in Ponnani harbour. In the present study, having a greater salt tolerance similar to *Staphylococcus, Bacillus* sp. in the present study was more widely distributed groups and seen in all the substrates studied.

Microbial exopolysaccharides (EPS) are a wide group of secreted polymers that can be highly attached to the cell surface or released as extracellular slime in the surroundings of the cell (Knoshaug*et al*., 2000). Since the characteristics of biofilm formation are attributed to the community of attached microorganisms connected by an extracellular polysaccharide (EPS) coating, their quantification gives ready information on the susceptibility of the bacterial group to prepare biofilms (Magin*et al*., 2010). Stewart and Franklin (2008) have reported that bacteria undergo multiple developmental stages from planktonic to attached cells. This transformation from the planktonic to the attached state induces a phenotypic change that facilitates increased secretion of an EPS coating. Biofilm formation in bacteria is closely linked with the production of EPS, which acts as a glue, aiding cell surface adhesion to the submerged structures. In the present study, based on the concentration of EPS production per unit volume, it is found that two strains of biofouling bacterial isolates that showed high biofilm production are suitable candidates for the formation of biofilms prior to fouling. In a similar study, Saravanan*et al*. (2008) have informed that the quantitative variations in EPS production and biofilm-forming ability among bacteria are directly proportional. EPS are also responsible for the viscoelastic properties of the biofilms that strongly affect the

formation of microbial flocs and biofouling layer by subsequent invaders of the floc. Eventually, EPS are recognized as the most direct and significant factor affecting biofouling (Sweity*et al*., 2011). Hence, the present study was an attempt to understand the primordial biofilm formation communities present in different hard structures of Ponnaiharbour. Although there have been other studies related to the production and characterization of Exopolysaccharides (EPS) formed by different strains of bacteria during biofilm formation, especially in marine bacterium by Majumdar*et al*. (1999), Saravanan*et al*. (2008) and Kumar *et al*. (2011) from the Indian subcontinent, their studies were more related to the physical features of these EPS and subsequent biofilm. However, in the present study, emphasis was given to the molecular taxonomy of the most active biofilm-forming bacterial strains.

In the present study, the molecular taxonomic study of the two biofouling bacterial isolates PPL7 and PPV2 with high Exopolysaccharide (EPS) production through 16S rRNA gene sequencing showed that both belonged to the genus *Bacillus*, with PPL7 being *Bacillus subtilis* and PPV2 as *Bacillus licheniformis*. The production of EPS from both these strains was much higher than any isolated strains, indicating that they had better adapted to the habitat from where they were isolated. Recent studies by Newman *et al*. (2013) indicate that the ability to form biofilms, especially in *B. subtilis*, is through a regulatory pathway that involved a protein SinR, which acts as a master regulator of biofilm formation. Hence this strain is among the major biofilm-forming groups that have impacted ocean-going vessels, formation of dental plaque, infections of the urinary tract, and contamination of surgical instruments. Similarly, the thermophilic bacterium *Bacillus licheniformis* is also a strong biofilm producer, which is known to produce three different types of biofilms (Sadiq*et al*., 2016). Hence the two

strains isolated in the present study are classic examples of biofilm-forming bacteria that are tolerant to a wide range of environmental variations and hence are suitable for studied pertaining to antifouling by actinomycetes.

The present investigation has revealed the presence of biofouling bacteria in the marine biofouling samples collected from the submerged surfaces of fouling materials in the Ponnani harbour, Kerala, India. Morphological, biochemical and molecular characterization studies showed the diversity of different strains of *Bacillus sp, Aeromonassp, Staphylococcussp* and *Serratia sp.,* with *Bacillus sp.* notablybeing a predominant genus.The rate of biofilm production was different in each strain. The present study provides a clear understanding of the diversity of biofilm-forming bacteria in Ponnani harbour and the variation in their potential for forming a biofilm. Among the different genus, although the quantity of EPS produced by *Bacillus* was highly *Staphylococcus* strain was found to be more saline tolerating.Since the rate at which different bacterial biofilms contribute to the acceleration in biofouling by different organisms was not part of the study, such an estimate could not be done. However, this preliminary study provides some valuable inputs on the species diversity of the microbial community that have good biofilm-forming potential in the region.In order to minimize the economic loss, fuel consumption and metal corrosion due to the formation of biofilms and subsequent biofouling, there is an urgent need for identifying suitable natural antifouling agents to control the biofouling, which is abundant in the marine environments. Therefore, it is important to note that the present study results have great potential in screening available biofilm-forming bacteria of Ponnani harbour, which could be suitably used for further antifouling studies using isolated actinomycetes**.**

PLATE 5.1

Sampling area

Selected strains of biofouling bacteria

Bacillus sp *Aeromonas sp* *Staphylococcus sp* *Serratia sp*

Chapter 6

SCREENING ACTINOMYCETES FOR ANTIFOULING ACTIVITY AGAINST BIOFOULING BACTERIA

6.1 Introduction

Actinomycetes, the heterogeneous spore-forming, filamentous gram-positive bacteria, are primarily saprophytic microorganisms of the soil. Being one of the archaic microbial groups, they have significantly contributed to the soil fertility, nutrient replenishment, and structural breakdown of complex biopolymers, such as lignocellulose, hemicellulose pectin, etc. to simpler compounds that higher forms could utilize. Seeing the importance of these groups, especially its diversity, has lead them to be classified as a separate class, namely, Actinobacteria (Stackebrandt*et al*., 1997) under the Order: Actinomycetales. Their members have an unparalleled ability to produce diverse secondary metabolites (Mincer *et al*., 2002). Having explored their potential from the terrestrial environment in a diverse field of science such as agriculture, forestry, mining, solid waste management, bioremediation, pharmacology etc., they are now widely isolated from aquatic environments, predominantly coastal marine habitats. Due to the rich diversity, extreme environmental conditions, and vastness, marine ecosystems are considered goldmines for these microbial communities with immense potential in present-day bioprospecting. Actinomycetes represent the most prominent group of microorganisms, which produce bioactive compounds (Krzesniak*et al*., 2018). A traditional approach in obtaining novel bioactive agents, especially with unique chemical structures and biological significance, relies on distinct microorganisms isolated from different, often secluded, environments.

Actinobacterial strains are commonly derived from soil (Guo*et al*., 2015), but they are also abundantly present in seas and oceans (Hassan *et al*., 2015; Xu*et al*., 2017). Moreover, extreme habitats such as caves, deserts or Antarctic ecosystems are recognized as valuable sources of actinomycetes producing novel metabolites of pharmacological importance (Lee *et al*., 2012; Jiang *et al*., 2015; Goodfellow*et al*., 2017; Singh *et al*., 2018). As the frequency of novel bioactive compounds discovered from the terrestrial *Actinomycetes* decreases with time, much attention has been focused on screening *Actinomycetes* from diverse environments for their ability to produce new secondary metabolites. Studies have shown that *Actinomycetes* isolated from the marine environment are metabolically active and have adapted to life in the sea. *Streptomyces* are incredibly prolific and can produce many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites (Thenmozhi and Krishnan, 2011). Approximately 23,000 bioactive secondary metabolites produced by microorganisms have been reported, and over 10,000 of these compounds are produced by *Actinomycetes*, representing 45% of all bioactive microbial metabolites discovered (Vimal*et al*., 2009). The immense diversity of marine habitat and its exploitation is the fundamental reason for attracting researchers towards Actinomycetes to discover novel metabolite producers (Ward and Bora, 2006).

Marine actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities, including antibacterial, antifungal, anticancer, insecticidal and enzyme inhibition. As marine environmental conditions are extensively different from those of terrestrial ones, the marine actinomycetes are other than terrestrial strains in producing various bioactive compounds (Williams *et al*., 2007). *Streptomyces* alone contributes to the presently known bioactive molecules (Challis and

Hapwood, 2003). It has an enormous biosynthetic potential that remains unchallenged without a potential competitor among other microbial groups. In the marine environment, biofouling is a serious problem, which results in the accumulation of living organisms on an artificial surface by adhesion, growth and reproduction. The organisms involved in marine fouling are primarily attached, or sessile forms commonly occur in shallow waters along the coastline. The biofouling process occurs in the natural environment through a series of sequential steps. Within minutes of immersion, a pristine surface becomes 'conditioned' through the adsorption of organic layers of macromolecules. The conditioned surface is later colonized by microorganisms, including bacteria, algae (mainly diatoms), fungi and protozoa. The attachment, colonization and growth of microorganisms on the surface result in the formation of a slimy layer called 'biofilm'. The biofilm layer can aid (or deter) the subsequent succession of 'macrofouling' species, such as barnacles, by facilitating adhesion or through the production of bioactive molecules (D'Sousa *et al.*, 2010).

Biofouling is an ongoing problem for water-immersed artificial structures such as ship's hulls, oceanographic instrumentation, pipelines, membranes, heat exchangers and aquaculture equipment, resulting in severe economic consequences (Schultz, 2007). Biofouling is especially economically significant on ship hulls, where high fouling levels can reduce the vessel's performance and increase its fuel requirements. Fouling causes huge material and economic costs in the maintenance of mariculture, shipping industries, naval vessels, and seawater pipelines. Biofouling can also occur in groundwater wells where build-up can limit recovery flow rates and in the exterior and interior of the ocean-laying pipes. The latter case has been shown to retard the seawater flow through the pipe and remove it with the tube cleaning process. Antifouling refers

to all systems that prevent the attachment of biofouling organisms on immersed surfaces. To avoid marine biofouling, broad-spectrum metal biocides, such as tributyltin (TBT) and copper, have been traditionally used as antifouling compounds (Albert *et al.*, 1992; Thomas *et al.*, 2001). These biocides were very effective but highly toxic to non-target organisms (Alzieu, 2000; Konstantinou and Albanis, 2004). Due to this adverse effect, International Maritime Organization (IMO) and Marine Environmental Protection Committee (MEPC) banned the usage of TBT or other substances containing tin as biocides in antifouling paints from January 2008 (Xu*et al.*, 2010). Several physical, mechanical, chemical, and biological methods for preventing marine biofouling have been tested in the last 40 years (Abarzua *et al.*, 1999). Current antifouling technology is based on the application of toxic substances that can be harmful to the natural environment (Dobrestove*et al.*, 2013).Alternate methods to curb the fouling have been cumbersome and highly uneconomical. Due to increasingly restrictive regulations on the polluting antifouling compounds, there is a growing need for other methods to prevent marine biofouling (Gademann, 2007). This has necessitated the development of alternative antifoulants that are environmentally friendly and economically viable for maritime domains. For this reason, there is a need for the development of ‘‘environmentally-friendly’’ antifoulants. Actinomycetes are a promising group of bacteria in terms of biodiversity and bioactive metabolite production. In recent years, marine-derived actinomycetes are under exploration for novel bioactive metabolites, including antifouling compounds (Lam, 2006).

Despite success in discovering antibiotics and anticancer agents, marine actinomycetes have not been paid much attention to discovering antifouling agents. Actinomycetes in general and marine actinomycetes, in particular, have shown to have

the potential for producing antifouling bioactive compounds. Among the several stages for isolating a potent antifouling compound from the actinomycetes, the first step involves screening a wide variety of such microbes from a diverse environment. Later these are cultivated in laboratory conditions. This is followed by characterization of these groups for accurate identification of their taxonomic status, quality and quantity of extracellular metabolites/enzymes that they produce to counter biofilm formation. Then the most prolific actinomycetes are cultured in different media and under different environmental conditions to check the variation in the production of antifouling compounds. Finally, the most prolific group is subjected to biochemical tests to extract the compound responsible for the antifouling. This biogenic compound can either be produced in mass through *in vitro* biosynthesizers or the compound's chemical structure to be synthetically manufactured and used in different forms to retard the growth of bacteria causing primary biofilm formation retard biofouling.

Exopolysaccharides (EPSs) are high-molecular-weight carbohydrate polymers produced by marine bacteria, dispersed onto the surface as slime (Sutherland, 1982). EPS are produced by these microorganisms as a strategy for growth, adhering to solid surfaces, and surviving adverse conditions. Not only this, EPS production and subsequent biofilm production by different bacteria have also been effectively used for controlling metal corrosion in marine structures (Finkenstadt and Willett, 2011). Hence the present understanding of microbial biotechnology relies on discovering and developing new microbial EPSs that possess novel industrial significance (Nicolaus*et al*., 2010). Despite these advantages, many biofouling bacteria also produce EPS as a precursor for their establishment on solid surfaces. Therefore, it is necessary to identify strains of actinomycetes that can produce antibacterial compounds and are effective

EPS produces since a combination of this would enable the actinomycetes to adhere to the surfaces that already have biofilm formation. Being enzymatic in nature, EPS from the actinomycetes would easily attach to the surface, and the secondary metabolites produced by them would destroy the prevailing biofilm to creates a conducive surface for the actinomycetes to grow. It is also essential to ascertain that the EPS produced by these actinomycetes work in a wide range of environmental conditions, especially the pH of the surface on which they adhere. In India, research on marine actinobacteria with particular reference to antifouling compounds is still in its infancy. Since a vast region of our coastal habitats has not been explored for any novel molecules that have the potential for producing antifouling compounds, especially from the lesser-known habitats of Northern Kerala, a study was conducted to screen isolated actinomycetes from the region. Owing to the diversity in the species assemblages, as seen in an earlier chapter, actinomycetes associated with mangroves, nearshore sediments and seaweeds from the area were screened to isolate specific strains to determine their antifouling potential.

6.2 Materials and Methods

6.2.1 Screening of actinomycetes for enzyme production

All the selected actinomycetes isolates (as mentioned in Chapter 3) were screened qualitatively for the production of six important enzymes such as Catalase, Amylase, Gelatinase, Caseinase,

Catalase

Catalase test is performed by adding 3% hydrogen peroxide solution (H_2O_2) to a heavy suspension of isolates on a microscopic glass slide. Within one minute after the

addition of H_2O_2, Catalase positive cultures produce bubbles of oxygen. The release of free oxygen gas bubbles is considered a positive catalase test.

Amylase

Amylase activity was tested using starch (1%) supplemented Nutrient Agar medium (Agar 2 g; Beef extract 0.3 g; Peptone 0.5 g; sea water 100 ml; pH 7.0). Spot inoculation of the culture plates was followed for testing the activity of enzymes. The inoculated culture plates were incubated for seven days. Then, 2% potassium iodide solution was flooded on the culture plate. A clear zone around the culture spot was reported as a positive result for amylase activity.

Gelatinase

Nutrient Agar medium (peptone 0.5 g; beef extract 0.3 g; agar 2 g; sea water 100 ml; pH 7.0) supplemented with gelatin (2%) were prepared for gelatinase assay.Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 3 to5 days. Clearing around the growth after flooding with Frazier's reagent was recorded as positive results for gelatinase production.

Caseinase

Screening of enzyme Caseinase was done using skim milk agar (Skim milk powder 10 g; Agar 2 g; Distilled seawater 100 ml; distilled water 100 ml). Autoclaved 10 g of skim milk in 100 ml distilled water. Then two grams of agar was added to 100 ml of seawater and was autoclaved separately. Sterile skim milk and agar were mixed thoroughly and poured into Petri plates. Isolates were spot inoculated, and the plates were incubated at 36 ± 1.0ºC for 5-7 days. Decomposition of Casein could be observed due to the clearing of skim milk agar around the colony and was recorded as positive.

Lipase

Lipase activity was tested using Nutrient Agar medium (peptone 0.5 g; beef extract 0.3 g; agar 2 g; sea water 100 ml; pH 7.0) supplemented with tributyrin (1%). Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 3 to 5 days, and observations were made. A clear zone around the colonies on tributyrin agar for the production of lipase was noticed.

Urease

Christensen's Urea Agar medium (HiMedia; composition; Peptone 1g; Dextrose (Glucose) 1g; Sodium chloride 5g; Disodium hydrogen phosphate 1.20g; Potassium dihydrogen phosphate 0.8g; Phenol red 0.012g; Agar 15g; Distilled water 1L; Final pH (at 25°C) 6.8±0.2) was used to test the production of urease. The above ingredients except urea were dissolved in 950 ml of distilled seawater and autoclaved at 15 lbs for 15 minutes. Aseptically prepared 50 ml of 40% sterile urea solution was added to the basal medium and prepared agar slants (3 ml each). Cultures were inoculated, and tubes were incubated at 36 ± 1.0ºC for 18-24 hours. A change of colour in the medium from yellow to pink was noted as positive for urea hydrolysis.

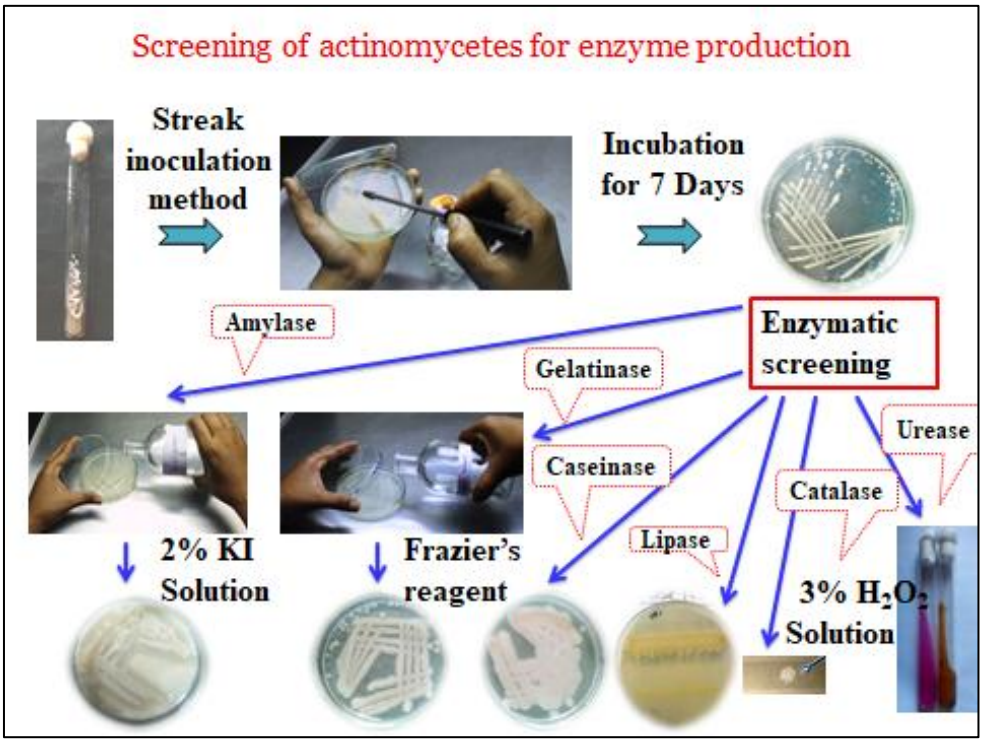


Figure 6.1Screening of actinomycetes for enzyme production

6.2.2 Screening of actinomycetes for antifouling activity

Isolates that showed high enzymatic activity for most of the six enzymes were subsequently tested for their antifouling potential. Likewise, a total of eighteen crude ethyl acetate extracts were tested against seven bacterial isolates that showed substantial adherence property (as mentioned in Chapter 5) for further analysis. The ethyl acetate extracts were prepared from cell-free supernatant of soybean meal medium cultured with actinomycetes. Antifouling activity of actinomycetes extracts was tested against biofouling bacteria by disc diffusion method. About 0.25 mg of crude extract were impregnated on sterile disc and placed on Nutrient Agar plates inoculated with selected biofouling bacteria. All the plates were incubated at 28^{o} C for 24 hours and observed for the zone of inhibition.

6.2.3 Isolation and estimation of Exopolysaccharides (EPS) production

All the eighteen isolates of actinomycetes that gave positive enzymatic results were subjected for isolation of EPS. Exopolysaccharide contents from each strain of selected actinomycetes were isolated and quantified from the culture medium according to the method described by Quesada *et al*. (1994). Initially, the actinomycetes strains were cultured in Starch-Casein Agar. Growth of actinomycetes and EPS production was monitored in batch cultures in 500-mL Erlenmeyer flasks containing 100 mL medium (three replicate flasks per experiment). The culture after a definite period of growth was centrifuged at 8,000×g for 20 min at 4ºC., three volumes of chilled ethanol were added to the supernatant and kept overnight at 4°C for the precipitation of the EPS. The precipitate was then collected by centrifugation at 12000×g for 30 min. The cell-bound EPS was extracted from the cell mass residue by treating with normal hot saline for 10 min under vigorous shaking. It was re-centrifuged at 10,000×g for 15 min to separate the cell mass. These steps resulted in the isolation of crude precipitate of EPS from the cell mass. The soluble and cell-bound EPS were pooled, dissolved in a known volume of deionized water and quantified for total carbohydrate. The total carbohydrate content of EPS was quantified following the Dubois method (Dubois *etal*., 1956). In 1 mL of EPS solution, 0.5 mL of 5% phenol and 3.5 mL of concentrated sulfuric acid were added and incubated at 50°C for 20 min. Absorbance was recorded at 490 nm, and the concentration was determined from the calibration curve prepared in the same method using glucose as standard (Kodali*et al*., 2009). Mean values of EPS produced (mg EPS/100 ml) under different conditions were statistically analyzed through one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) using SPSS 20.0 software of IBM to determine the significance in the quantity of EPS produced (Gomez and Gomez, 1984).

6.2.4 Optimization of media for EPS production by selected Actinomycetes strains

To understand the most optimum conditions for maximizing EPS production, a series of experiments were conducted. Initially, under normal conditions, the EPS production from the 18 selected isolates was monitored every 24 hr for six days to standardize the incubation time (1–6 days). Based on the results, the strains that showed better EPS production, both in terms of quantity and extent of the duration of production, were identified, and further standardization was done only on those strains. The standardization included the incubation temperature (22, 32 and 42 °C), salt concentration (1, 2, 5, 10% (w/v)), carbon source (glucose, sucrose, mannose, galactose), glucose concentration (0, 1, 2, 5, 7 and 10% (w/v)) and pH of the media (5, 6, 7, 8 and 9).

6.2.5 Antibacterial activity of selected actinomycetes against biofouling bacteria

The actinomycetes cultures with comparatively better antibacterial activity were further chosen for crude extract preparation during secondary screening. These actinomycetes strains also produced relatively higher EPS under optimum conditions and therefore were tested against potent biofilm-forming bacterial strains of *Bacillus subtilis* and *Bacillus licheniformis* (as shown in Chapter 5). The effectiveness of these extract on selected bacteria was estimated following the disc diffusion method described by Kirby-Bauer with modification (Bauer *et al*., 1966). The selected antagonistic actinomycetes were inoculated into 100 ml of actinomycete isolation broth (composition: glycerol: 5.0 g, sodium propionate: 4.0 g, sodium caseinate: 2.0 g, K_2HPO_4: 0.5 g, asparagine: 0.1 g, $MgSO_4 \cdot 7H_2O$: 0.1 g, $FeSO_4 \cdot 7H_2O$: 1.0 mg, water: 1000 ml and pH: 8.0±0.1) and incubated in an orbital shaker at 28 °C and 190 rpm for

seven days. On the 7th day, the fermented broth was centrifuged in a 50 ml centrifuge tube at 10000 rpm for 5 min. The supernatant was collected and filtered to avoid any cell debris. The fermentation broth was subjected to sequential extraction using ethyl acetate in a 1000 ml separating funnel. The organic phase of the supernatant was dried under reduced pressure using a rotary vacuum evaporator, and concentrated extracts were collected, dried and used for bioassay studies (Valli*et al*., 2012). The supernatant was transferred aseptically into a screw-capped bottle and stored at 4°C for further assay.

About 1 mg of the crude extract was dissolved in 100µl sterile DMSO, and 400µl of sterile double distilled de-ionized water was added and mixed thoroughly. From this, 30µl of the treatment solution was impregnated onto sterile discs (6 mm; Himedia) in a Laminar air chamber. The discs were allowed for complete drying in the oven at 45°C. Sterile Nutrient agar plates were prepared, and the two strains of biofilm-forming bacteria (PPL7 and PPV2) were swabbed onto the surface of the agar plates under aseptic conditions. The discs impregnated with the extracts were placed on the plates and incubated at 37°C for 24 hr. The zone of inhibition was recorded for all the extracts against these biofouling bacteria. DMSO was used as solvent control, and Streptomycin(10µg/µl) was used as a reference control.

6.2.6 Molecular characterization of selected actinomycetes strains

Based on the results of bioinhibition studies, the most active actinomycetes isolates against these biofouling bacteria were then subjected to molecular characterization to identify the strain to species level. The molecular identification of bacteria was carried out by the 16S rDNA sequence-based method. Here, genomic DNA from purified bacteria was isolated using a commercially available HiPurA™ Fungal

DNA Purification Kit (HIMEDIA). Total genomic DNA from these selected actinomycetes strain was isolated as per standard protocols (Sambrook*et al*., 1989). The selected isolates were inoculated into 50 ml of Luria Bertani's broth in a 250 ml conical flask and incubated at 28°C for four days at 100 pm. Then the cells were collected by centrifugation, and the pellet (approximately 500 mg) was used for DNA isolation as per the manufacturer's protocol. The isolated DNA was further analyzed by electrophoresis using 0.8% agarose gel. DNA sequencing of 16S rDNA of selected isolates was carried out according to Kumar *et al.* (2010). The 16S rDNA was amplified using the following universal primers 8f (5'-AGAGTTTAGATC TGGCTCAG -3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The sequence of the primers was selected based on the previous reports (Chun and Goodfellow 1995). The polymerase chain reaction (PCR) was processed as 50 µL working volumes, which included 50 ng of genomic DNA, 20 pmol of each primer, 200 µM of each dNTPs, 5 µl of 10X PCR buffer and 1.25U of Taq DNA polymerase. The PCR procedure was initiated by a 2 min denaturation at 94°C, followed by 30 cycles of 30 seconds denaturation at 94°C, 1 min annealing at 45°C, and a final extension for 7 minutes at 72°C. PCR was carried out using a 96 well Thermal Cycler (applied Biosystems). The size and quality of PCR products were visualized in 1% agarose gel. The products were purified using the Wizard SV gel and PCR Cleanup System (Promega). Cycling sequencing for the PCR products was performed using ABI PRISM BigDye Terminators V 3.1Kit (Applied Biosystems), and the sequences were analyzed by capillary electrophoresis using an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems) at SciGenom Cochin, India Ltd. The gene sequence obtained was compared by aligning the result with the sequence in GenBank using the BLAST search program at the NCBI. A Maximum-likelihood

(ML) tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) V. 7 (Kumar *et al*., 2016).

6.3 Results

6.3.1 Enzymatic characterization of the isolated actinomycetes

Of all the actinomycetes screened for enzyme production, 18 isolates showed good enzymatic activity. Table 6.1 depicts the results of enzymatic activity of these selected strains, which showed positive activity for at least three enzymes. On further analysis, it was observed that twelve (12) strains belonged to the genus *Streptomyces*, three (3) to *Kitasatospora*and one each to *Micromonospora, Actinomadura*and *Rhodococcus.* A noteworthy observation was that among these 18 strains, only three (3) strains recorded positive results for all six enzymes.

Table 6.1 Enzymatic activity of selected actinomycetes strains

No	Isolates	Catalase	Amylase	Caseinase	Gelatinase	Lipase	Urease
1	*Streptomyces sp1.*	_	+	+	_	+	+
2	*Streptomyces sp2.*	+	+	+	_	_	+
3	*Streptomyces sp3.*	+	_	+	+	_	+
4	*Streptomyces sp4.*	+	_	_	+	+	_
5	*Streptomyces sp5.*	+	_	_	+	+	_
6	*Streptomyces sp6.*	_	+	+	_	+	+
7	*Streptomyces sp7.*	+	_	+	+	+	_
8	*Streptomyces sp8.*	_	_	+	+	+	_
9	*Streptomyces sp9.*	+	+	+	+	+	+
10	*Streptomyces sp10.*	+	+	+	_	+	_
11	*Streptomyces sp11.*	+	_	_	+	+	+
12	*Streptomyces sp12.*	+	+	_	+	+	+
13	*Kitasatospora sp1.*	_	+	+	_	+	_
14	*Kitasatospora sp2.*	+	+	+	_	_	_
15	*Kitasatospora sp3.*	+	+	+	+	+	+
16	*Micromonospora sp.*	+	+	+	+	+	+
17	*Actinomadura sp.*	_	+	+	+	+	_
18	*Rhodococcus sp.*	+	_	+	+	+	+

6.3.2Estimation of Exopolysaccharides (EPS) from the selected actinomycetes

Results of quantitative estimation of Exopolysaccharide production among all the eighteen (18) actinomycetes isolates indicated that the production rate in all these cultures was different, indicating that each actinomycete has a specific rate at which it produces the EPS under normal conditions (Table 6.2). Except for four isolates, all the

other actinomycetes strains ceased to produce EPS beyond 72 hrs (3 days) of the incubation period, and the quantity of EPS in these cultures decreased thereafter. However, for the four strains that showed continued EPS production, their total carbohydrate content reduced only after 96 hrs (5 days). A noteworthy observation was that all the four strains that had extended EPS production belonged to the Genus *Streptomyces.* Based on the results of quantity and extended period of EPS production, seven strains of actinomycetes (*Streptomyces sp.* strain KSA9; *Streptomyces sp.* strain APP59; *Streptomyces sp.* strain K2M4; *Streptomyces sp.* strain K2M6; *Streptomyces sp.* strain KML4; *Kitasatospora sp.* strain KSA7 and *Micromonospora sp.* strain APP56) were identified for further optimization of culture media. Among them, the strain that produced significantly high EPS (1039.5 mg EPS/100 ml) was *Streptomyces sp.* strain KML4.

Table 6.2.Exopolysaccharide production in selected actinomycetes under different incubation time

No	Isolates	Incubation time (days)					
		1	2	3	4	5	6
1	*Streptomyces sp1.*	21.4±5.5^{a}	142.4±32.6^{b}	256.4±63.2^{c}	182.4±35.2^{b}	114.1±49.4^{b}	93.5±33.2^{b}
2	*Streptomyces sp2.*	16.5±2.7^{a}	146.8±40.6^{c}	306.8±75.3^{d}	261.0±76.8^{d}	98.8±39.96^{c}	42.8±20.4^{b}
3	*Streptomyces sp3.*	45.4±7.4^{a}	256.4±67.4^{b}	582.7±174.2^{c}	722.7±266.3^{d}	782.1±248.7^{d}	432.6±134.0^{c}
4	*Streptomyces sp4.*	10.5±2.6^{a}	87.1±28.8^{b}	157.2±45.1^{b}	111.5±39.4^{b}	54.6±38.5^{b}	34.9±11.4^{a}
5	*Streptomyces sp5.*	11.5±7.3^{a}	130.3±73.1^{b}	165.1±112.4^{b}	156.4±77.4^{b}	69.7±49.2^{a}	18.3±9.9^{a}
6	*Streptomyces sp6.*	8.5±4.1^{a}	92.8±57.1^{b}	202.5±88.3^{c}	123.5±45.1^{b}	86.4±42.8^{b}	15.5±9.2^{a}
7	*Streptomyces sp7.*	72.5±9.3^{a}	368.7±107.5^{c}	548.4±153.1^{d}	333.4±113.1^{c}	251.2±62.3^{b}	135.2±83.5^{b}
8	*Streptomyces sp8.*	7.4+1.3^{a}	70.4+15.2^{b}	127.7+51.9^{b}	101.1+57.0^{b}	74.9+51.7^{b}	25.7+21.2^{b}
9	*Streptomyces sp9.*	96.1±11.4^{a}	376.2±120.3^{b}	563.6±250.9^{c}	692.4±272.0^{d}	808.2±264.8^{e}	573.2±211.4^{c}
10	*Streptomyces sp10.*	88.6±10.5^{a}	469.1±92.0^{b}	702.7±244.3^{c}	919.8±292.1^{d}	777.6±264.0^{c}	395.1±99.3^{b}
11	*Streptomyces sp11.*	12.7±3.4^{a}	145.5±19.5^{b}	255.5±81.7^{c}	83.9±33.2^{b}	32.7±19.5^{a}	28.3±13.2^{a}
12	*Streptomyces sp12.*	79.6±6.2^{a}	454.6±88.5^{b}	747.5±128.1^{c}	958.3±276.9^{d}	1039.5±214.2^{e}	866.9±173.6^{d}
13	*Kitasatospora sp1.*	11.6±4.3^{a}	89.3±20.6^{b}	262.7±115.5^{c}	162.5±55.1^{c}	111.1±32.5^{b}	21.5±5.8^{a}
14	*Kitasatospora sp2.*	9.3±3.2^{a}	96.3±19.2^{b}	172.4±102.6^{c}	96.8±34.4^{b}	52.5±18.2^{b}	12.4±6.8^{a}
15	*Kitasatospora sp3.*	61.1±10.2^{a}	374.9±114.4^{c}	546.8±145.3^{d}	488.6±181.1^{d}	423.5±97.0^{d}	255.4±63.4^{b}
16	*Micromonospora sp.*	95.3±15.5^{a}	445.3±154.5^{b}	756.4±262.8^{c}	246.8±40.6^{c}	112.0±37.8^{a}	81.5±22.7^{a}
17	*Actinomadura sp.*	9.5±1.3^{a}	97.5±20.3^{a}	187.1±44.8^{b}	256.4±67.4^{b}	133.6±44.1^{b}	72.3±21.4^{a}
18	*Rhodococcus sp.*	13.5±5.1^{a}	111.5±25.1^{b}	150.3±53.3^{b}	87.1±28.8^{b}	32.4±17.4^{a}	14.2±4.5^{a}

*Means with same superscript are homogenous

6.3.3. Optimization of culture media for enhanced EPS production

Enhancing EPS production in selected actinomycetes through modification of culture environment was attempted and optimized. Temperature is a major variable for the effective growth of actinomycetes was assessed under three different conditions. Being a spore-forming bacteria, a clear assessment of the optimal range for their growth was required for maximizing their production. Results of the EPS production under the three temperatures, as shown in Figure 6.2, indicate that the values varied considerably below and above 32°C. In all the seven strains, the temperature that gave maximum EPS production was 32°C. However, a noteworthy observation was except for two strain (K2M6 and KML4), all other strains produced comparatively higher EPS in lower temperatures (22°C) than the higher temperatures (42°C).

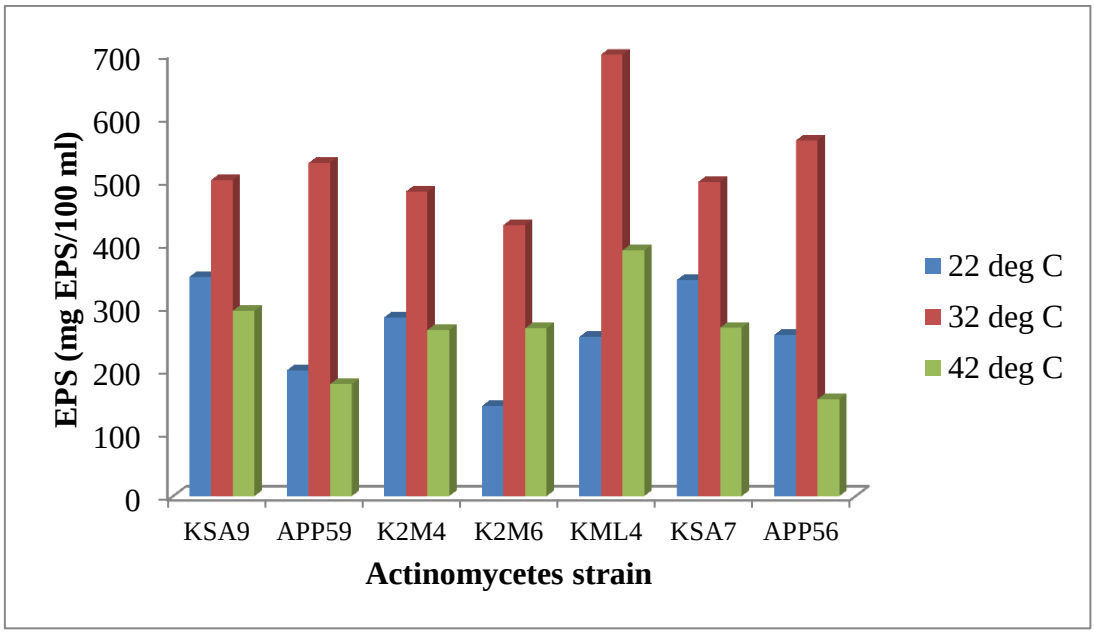


Figure 6.2. EPS production by actinomycetes under different incubation temperature

The salt concentration is yet another factor that directly determines the EPS production in marine actinomycetes. Since bacterial metabolism can be directly related

to the salt content in the culture environment, a range of salt concentrations was used to delineate the most appropriate percentage (w/v) to maximize EPS production. Figure 6.3 depicts the variation in EPS production among the seven actinomycetes strains used to monitor the impact of salt concentration. Results indicate that in most strains (except APP59 and KSA7), the EPS production declines beyond 2% (w/v) of sea-salt concentration. However, in the other two strains, though the EPS concentration (mg/100ml) decreased beyond 5%, the difference in EPS value between 2% and 5% was less than 15%. This indicates that 2% (w/v) can be considered a standard salt concentration to enhance EPS production.

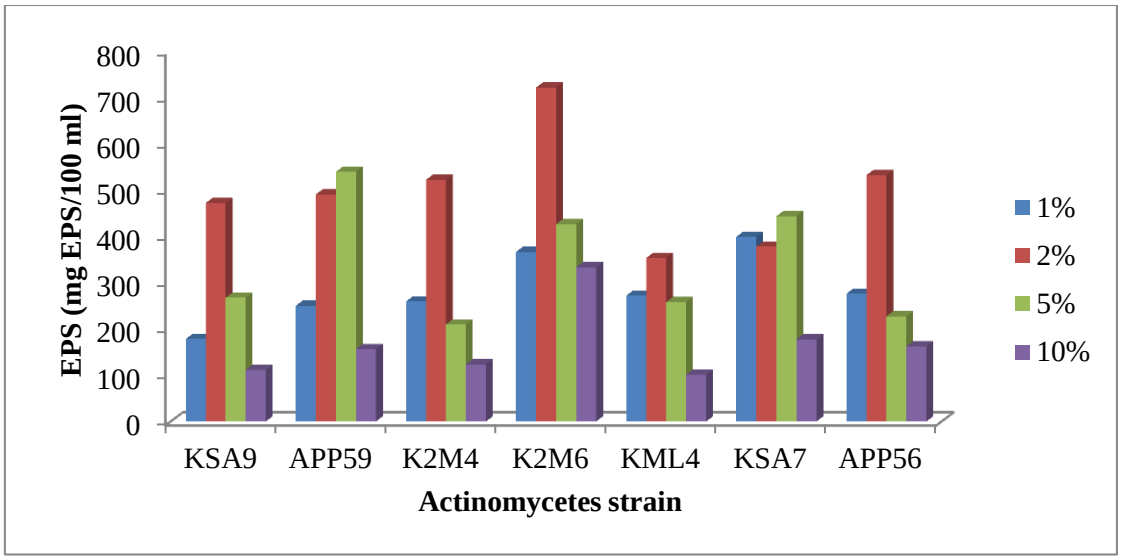


Figure 6.3. EPS production by actinomycetes under different salt concentration (% w/v)

Actinomycetes utilize a wide range of carbon sources during their growth. The EPS production can be improved by carefully modifying this carbon source. In this regard, the results of four commonly used carbon sources on the growth and EPS production of selected strains of actinomycetes indicated that all the four sources contributed to the formation of EPS (Figure 6.4). Among the different carbon sources,

high EPS production for all the strains was seen in cultures incorporating glucose. On the contrary, sucrose utilization resulted in low EPS production. Although the utilization of carbon sources varied between strains, a definite pattern for its utilization could not be seen. However, the two strains (K2M4 and KML4) that produced maximum EPS relied on glucose compared to other carbon sources.

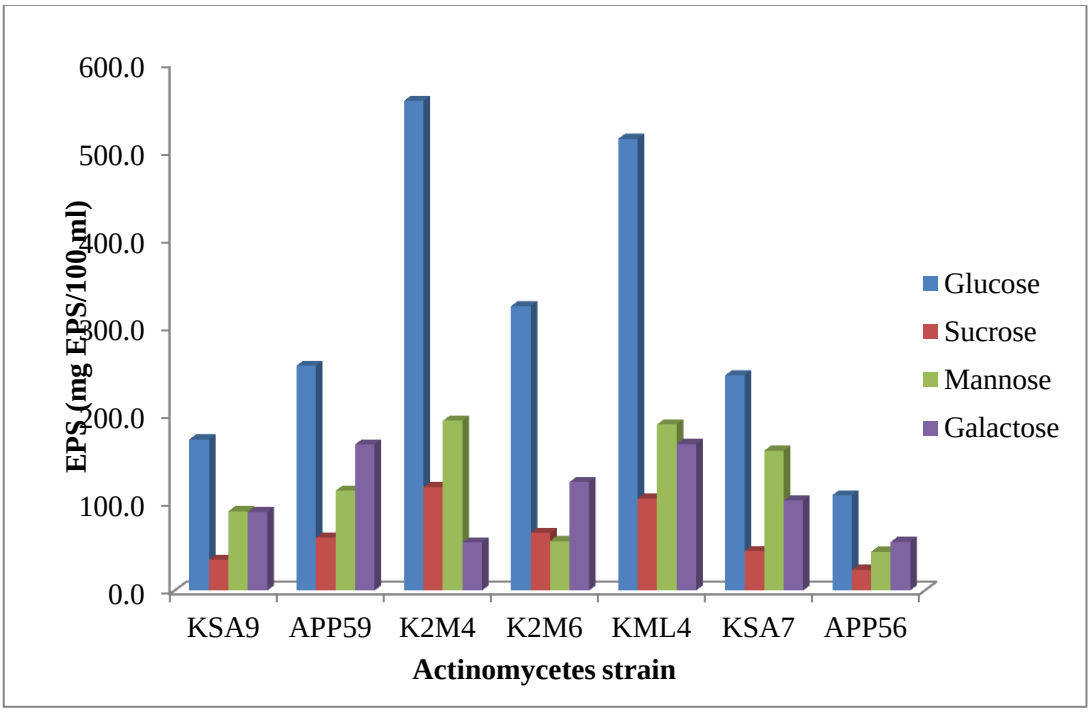


Figure 6.4. EPS production by actinomycetes under different carbon sources

Since glucose was the most influential carbon source responsible for maximizing EPS production in the selected strains of actinomycetes, further optimization of their concentration in the culture media was carried out. Based on a series of concentration (%w/v), it is envisaged that standardization of concentration of this carbon source could aid in the commercial production of EPS. Results indicated that except for strain K2M6, all other strains produced the highest EPS at a concentration of 1%, beyond which their production decreased (Figure 6.5). However, for K2M6, the maximum output was noticed at the 2% level. Hence, it is clear that a concentration of

1% of glucose in the medium is optimal for maximizing EPS production in these strains. A noteworthy observation of the present study was that though the concentration of EPS decreased beyond 2%, the growth of actinomycetes was unimpeded even at 10% glucose concentration.

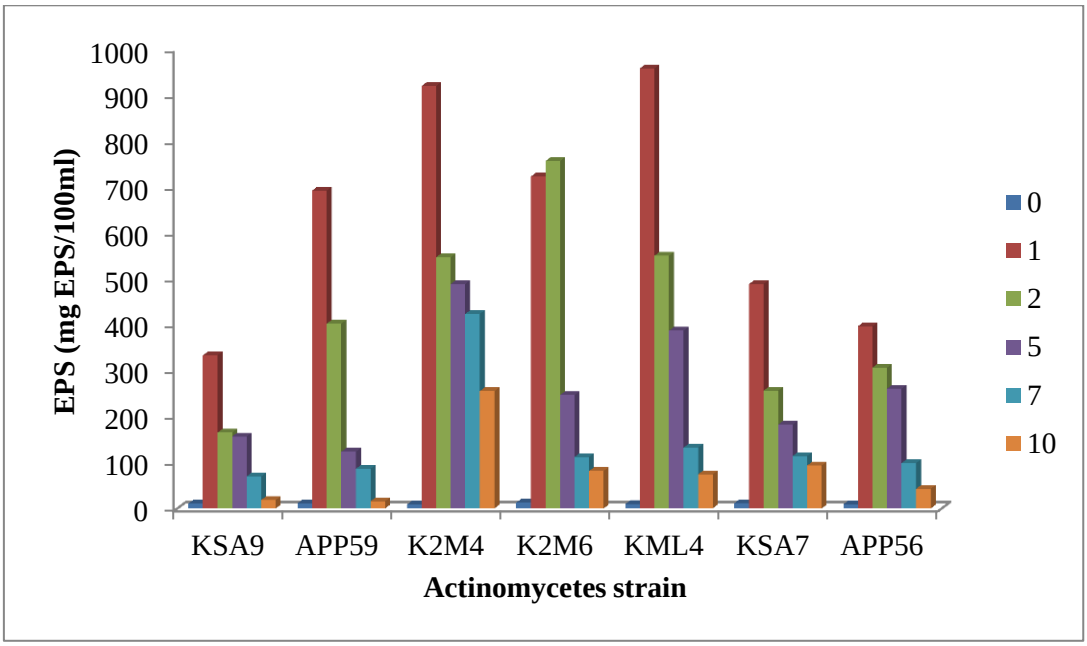


Figure 6.5. EPS production by actinomycetes under different glucose concentrations (% w/v)

Surface pH is considered a primary component that determines the fate of biofilm production by many biofouling bacteria. Taking a cue from this, it was imperative to understand the role of pH in EPS production. Likewise, the selected strains of actinomycetes were cultivated in media with different pH levels. Results indicated a diverse EPS production pattern under different pH levels (Figure 6.6). Most of the strains efficiently produced EPS in pH ranges of 6-9, with maximum production in a slightly alkaline pH of 8. Four strains of Actinomycetes viz., K2M6, KML4, APP56 and APP59 had better EPS production in a wide range of pH.The results aligned with

the effective range at which biofilm formation have been recorded, indicating that these strains also have a similar affinity for biofilm formation as other biofouling bacteria.

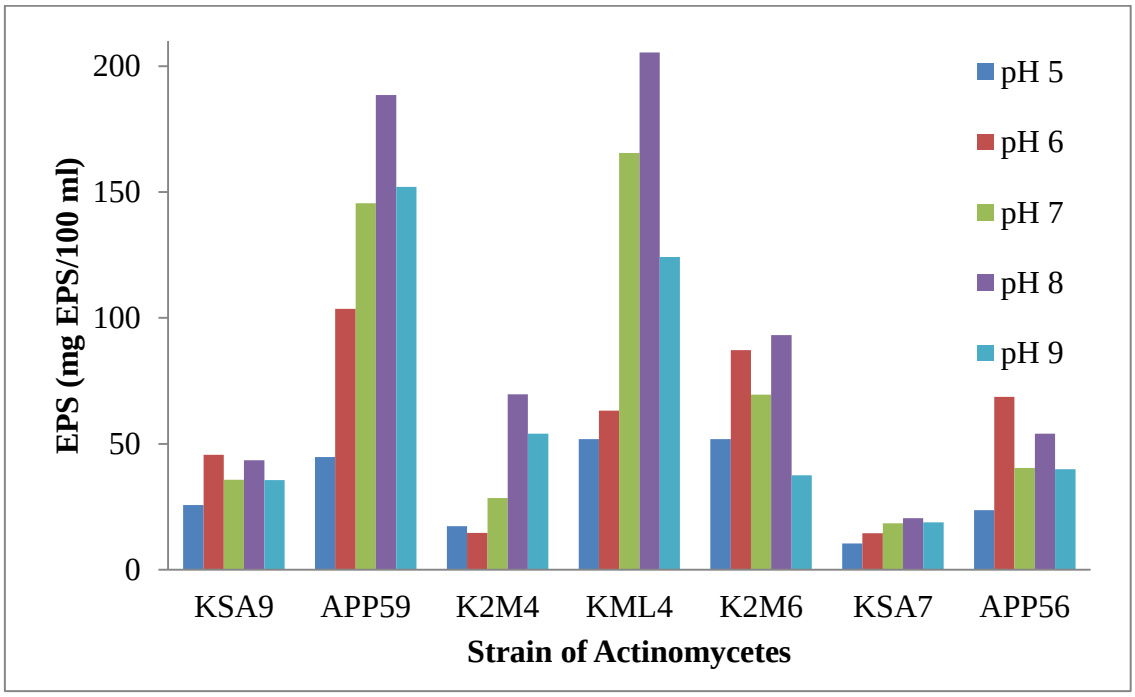


Figure 6.6.EPS production by actinomycetes under different pH

6.3.4Screening of actinomycetes for antifouling activity.

The seven actinomycete isolates that showed good enzymatic activity and exopolysaccharide (EPS) production were tested for their antagonistic activity over biofouling bacteria. Two biofilm-forming bacteria (*Bacillus subtilis and B. licheniformis*) identified in Chapter 5 were used. These fouling bacteria were evenly grown on Nutrient Agar after swabbing pure culture on the culture plates. After 24 hrs of incubation, the antimicrobial activity of the selected actinomycetes strains was carried out following the disc diffusion method. Results showed that although all the seven isolates showed antimicrobial activity against the two *Bacillus* strains, four isolates (K2M6, KML4, APP56 and APP59) gave a better zone of clearance within the first 24 hrs (Table 6.3; Plate 6.1).

Table 6.3 Antifouling activity of selected actinomycetes strains against *Bacillus subtilis* and*Bacillus licheniformis*

Actinomycetes strain	**Biofouling bacteria strain** (Zone of inhibition in millimetre)	
	***Bacillus subtilis* PPL7**	***Bacillus licheniformis*PPV2**
Streptomyces sp. K2M6	26, 28, 28	30, 28, 33
Streptomyces sp. KML4	32, 34, 34	36, 32, 34
Streptomyces sp. APP56	26, 24, 27	10, 11, 10
Streptomyces sp. APP59	23, 26, 22	18, 16, 10
Streptomycine	30, 34, 32	10, 12, 11

A closer observation of the zone of inhibition indicated that among these four strains of actinomycetes, the one that showed the highest inhibition on both *Bacillus subtilis*PPL7and *Bacillus licheniformis*PPV2was *Streptomyces sp.* KML4 (Plate 6.2). Hence it was imperative to taxonomically identify this strain along with the other three for further characterization studies.

6.3.5 Molecular characterization of the four *Actinomycetes* strains.

The 16S rDNA of four actinomycetes strains which showed good antibacterial activity against biofouling bacteria were sequenced, and the product included sequences of 680 to 820 bp, which were submitted to the GenBank database under accession numbers MT317112, MT317114, MT913026 and MT913027. These partial sequences were aligned and compared with all the available 16S rDNA sequences in the GenBank database using the BLAST comparison tool. The phylogenetic tree constructed using MEGA software Version 7 using the Maximum-Likelihood method grouped the four species systematically along with other similar sequences of NCBI Genbank (Figure 6.7). The results depicted that the four strains K2M6, KML4, APP56 and APP59 to be

Streptomyces *variabilis*(MT913027),*Streptomyces* *griseorubens*(MT913026),*Streptomyces* *rubrogriseus*(MT317112) and *Streptomycesspiroverticillatus*,respectively.

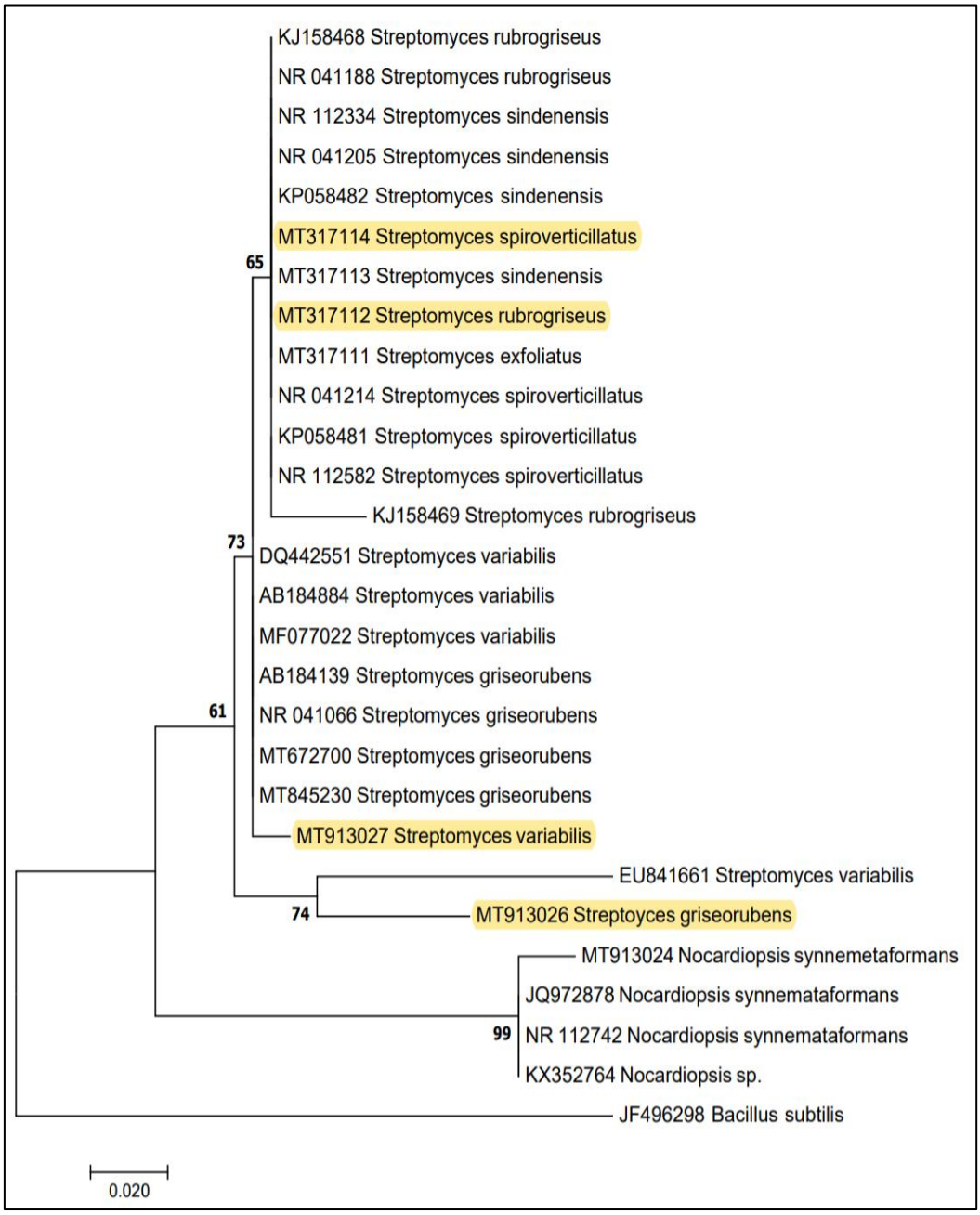


Figure 6.7. Maximum-Likelihood tree constructed based on partial 16S rDNA gene sequence of the selected actinomycetes strains

6.4 Discussion

Marine actinomycetes are a rich source of natural products exhibiting many diverse biological properties such as cytotoxic, antifungal, and antibiotic activities (Fenical, 1993; Jensen *et al*., 2005a). From a natural products perspective, marine microbes are better resources for novel antifoulants to control biofouling in marine structures. Having isolated from similar habitats, these microorganisms are exposed to the same degree of environmental perturbations as other fouling organisms. This gives them an added edge in combating the climatic vagaries as their bacterial counterpart responsible for producing the preliminary biofilm for the biofuels to attach to the substrate. Actinomycetes have proved to adapt to a wide array of habitats. They are also diverse not only in their taxonomic level but also in their genetic diversity. This genetic diversity enables them, a species of actinomycetes, to adapt to different environments. They are also known to produce various extracellular metabolites, which not only aid them to adjust to these changing climatic conditions and establish them as pioneer settlers in ecological succession. Hence, from an ecological perspective, a better understanding of these secondary bioactive compounds produced by these actinomycetes has immense importance. However, studies aiming to explore these compounds have limited the discovery of antimicrobial molecules required for drug discovery and exploring antifoulants from marine actinomycetes are sparse. Though there have been reports of the potential of marine actinomycetes, especially *Streptomyces* to produce antifouling metabolites that are active against micro and macro fouling organisms (Gopikrihnan*et al*., 2016; Prakash*et al*., 2015), most of the studies have focused on other groups such as Bacteria (Morales *et al*., 2008), Algae (Iyyaparaj*et al*., 2012), Fungi (Kwong*et al*., 2006) and Sponges (Henrikson*et al*., 1995) as sources

for isolating bioactive compounds with antifouling activities. Except for very few compounds like Diketopiperazines, Lobocompactol and Napyradiomycins (Li *et al*., 2006; Cho *et al*., 2012; Perier*aet al.,* 2020), very few products have commercially been exploited as antifouling agents from marine actinobacteria. It is even more alarming to see no antifouling compound reported from marine actinobacteria in India, though there have been studies on different antifouling activities of marine actinomycetes (Kumaran*et al*., 2011; Bavya*et al*., 2011).

Biofouling in the marine environment includes primary colonization of the substrate by microorganisms, including diatoms and macroalgal zoospores (Silva-Aciares and Requelme, 2008). Microbial cells produce extracellular polymers and biofilms (Deccho, 2000). These biofilms are utilized by marine invertebrate as a settlement indicator (Wieczoreck and Todd, 1998). Inhibiting this primary colonization may be an effective way to eliminate fouling organisms. Therefore, this study has focused on screening mangrove derived and seaweed associated actinomycetes from the northern coast of Kerala for antifouling activity against biofouling bacteria. Care was taken to isolate actinomycetes from diverse environments and habitats to derive a cross-section of their representation in the screening process. After a series of assessments that included ease of culturing them in artificial media, resistance to wide environmental conditions, potential to produce exopolysaccharides and extracellular enzymes, natural pigmentation, etc., eighteen isolates primarily associated with the mangrove and seaweeds were identified to have potential antifouling activity. The antibacterial activity of these strains was tested against the two potent strains of biofouling bacteria isolated from biofilm samples of various fouling objects of the Ponnani coastal region and fishing harbour, as described in Chapter 5. Further characterization of these strains

based on the production of exopolysaccharides (EPS) provided a better understanding of the nature of their activity. It was therefore found pertinent to utilize this quality of actinomycetes as a foundation for their commercial exploitation. Based on these studies, the search was further siphoned down to seven strains with all the characteristics mentioned above for being utilized as antifouling agents.

A close perusal of earlier studies in the application such antifungal agents have reported the limitation in the molecules to have specificity in their inhibitory effect, difficulty in their usage, and other emulsions, low resistance to wide temperature and pH changes (Satheesh*et al*., 2016). This may be the reason, though, in spite of discovering a wide array of compounds belonging to terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides and lactones extracted from the marine organisms, very few end up reaching a commercial level as antifouling agents (Feng*et al.,* 2009). Hence, the present study was designed to produce potent antifungal molecules and check for their commercial viability based on their smaller molecular size, better retention in emulsion bases, and natural colour. In this study, all the eighteen isolates showed an inhibitory effect against one or more biofouling bacteria, but seven isolates specifically were active against both the tested biofouling bacterial strains. Gopikrishnan*et al.,* 2013 reported that two strains of *Streptomyces* PM33 and PE7 showed promising activity against a maximum number of biofouling bacteria tested. He further reported *that Streptomyces sampsonii* associated with mangrove yielded a maximum amount of antifouling compounds (Gopikrishnan*et al*., 2019). In the present study, too, a similar result was observed. Among the seven strains of actinomycetes that were finally selected for the test, five belonged to the genus *Streptomyces.* Five among the seven strains were isolated from mangrove habitats, while the remaining two were

from seaweeds. Bavya*et al*. (2011), in their study, concluded that marine actinomycetes strain *Streptomyces filamentosus* as a potential source for the development of eco-friendly antifouling compounds and six out of twenty actinomycetes isolates showed maximum inhibition against all the biofouling bacteria tested. In a similar study, Manikandan and Vijayakumar (2016) isolated 55 actinomycetes isolates, and twenty isolates were found to have antifouling producers against six biofouling bacteria isolated from poultry fouling samples. The present study indicated that actinomycetes strains showed greater inhibition against both *Bacillus subtilis* and *Bacillus licheniformis*.

It is a known fact that the underlying mechanism of retarding biofilm formation by actinomycetes strains includes both antibiotic as well as anti-adhesion property, and this is greatly influenced by the extracellular EPS production, which is essential for biofilm formation (Satheesh*et al*., 2012). In the present study, media optimization results for enhancing EPS production among the seven isolates showed that all the parameters assess viz., incubation temperature, salt concentration, carbon source, percentage of glucose in the medium, and the media's pH had a direct influence. These assessments are essential, especially for understating whether a bioactive compound can be used successfully as antifouling agents by incorporating in suitable emulsions. However, the preparation of an antifouling coat using such biomolecules is a major challenge as these compounds are susceptible to rapid breakdown in aquatic environments. Hence, to ensure better durability of the compound, it is necessary to delineate their structural integrity and the quality of their bioactivity when applied as antifouling coatings in diverse substrates and environments. Results of the present optimization studies identified the strains that remained consistent in EPS production

under different conditions. Likewise, four strains (K2M6, KML4, APP56 and APP59), which incidentally were all species under genus *Streptomyces*, were shortlisted as ones having excellent potential for antifouling. In the present study and earlier studies, it has been reported that Bacillus species were frequently isolated and reported from many biofouling samples than other biofouling bacteria. The antibacterial studies conducted for these actinobacteria produced a reasonable zone of inhibition for the two strains of *Bacillus* viz., *B. subtilis*PPL7 and *B. licheniformis*PPV2. However, a noteworthy observation of this study was that one strain (KML4) among these actinomycetes showed the highest inhibition against both *Bacillus subtilis* and *Bacillus licheniformis*. Since this strain recorded a good antifouling effect on both Bacillus species, it was further required to ascertain their exact taxonomic status and further analyze the potent compound this strain was producing, which enabled it to be the best actinomycetes strain among the isolate having potential for antifouling Cho *et al*. (2012) in a similar study found that actinomycetes associated with seaweed *Ulvapertusa* and diatom *Naviculaannexa* showed highest antifouling activity against. Through molecular studies of 16s rDNAsequences, they identified the strain as *Streptomyces praecox.* In this study also, the selected isolates of actinomycetes with better EPS production and antibacterial properties were molecular characterized and identified up to species level as *Streptomycesvariabilis* K2M6(MT913027), *Streptomyces griseorubens* KML4 (MT913026), *Streptomyces rubrogriseus*APP56 (MT317112) and *S. spiroverticillatus* APP59 (MT317114). Hence the strain with the highest zone of inhibition was identified as *Streptomyces griseorubens*. This is a species known to produce compounds with antimicrobial and antibiofilm potential (Al-Askar*et al*., 2014; Baygar and Ugur, 2017). Another significance of this species is that it is an extremophilicactinomycete with great

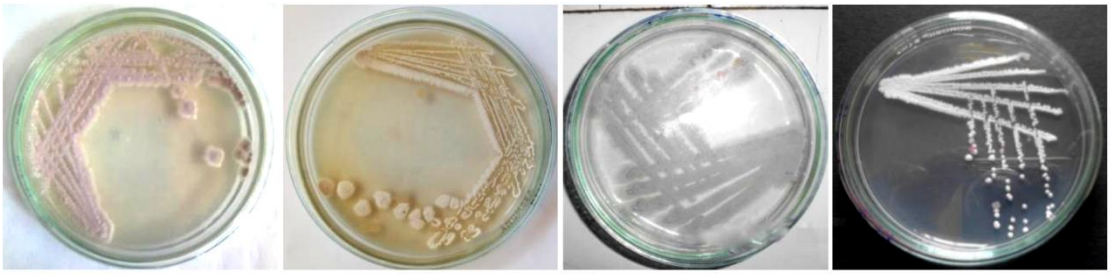
diversity in the production of a wide array of novel compounds presently being used as antimicrobial agents such as Oxaphenalene, Anthracycline, Mersaquinone derivatives etc. (Hamed*et al*., 2017, Paderog*et al*., 2020; Kim *et al*., 2020).

The fouling process, which includes both macro and micro biofouling, is facilitated through a series of stages, starting from biofilm formation by bacteria. To date, most of the biological attempts to curtail the process of biofouling has been through retarding the growth of these bacterial settlers on the substrate through competitive expulsion technique either with the help of other microbial strains that do not allow settling of larger foulers or through the use of natural antifouling compounds. The majority of these compounds were isolated from the marine organisms that were epiphytic or symbiotic in nature, including those associated with mangroves, seaweeds and other macroinvertebrates. These microbes synthesize a complex mixture of bioactive compounds in small quantities to enable them to survive in those environments. However, due to their lower quantity, the extraction and purification of compounds and natural harnessing from marine habitats would mean sacrificing their host organisms, notably sponges, corals, and rare species, which has been a cause of concern from the biodiversity conservation point of view. Contrary to this, it was seen that if such microbes could be cultured in lab environments and their production of bioactive compounds standardized, they could be produced on an industrial basis. It is also possible that if the genes responsible for this compound could be identified and through transgenesis incorporated into other bacterial strains, which are much easier to produce in bioreactors, a large quantity of these antifouling compounds could be made. Alternately, these biomolecules could be synthesized artificially to counter any disadvantages they pose related to their stability and potency. In this regard, the present

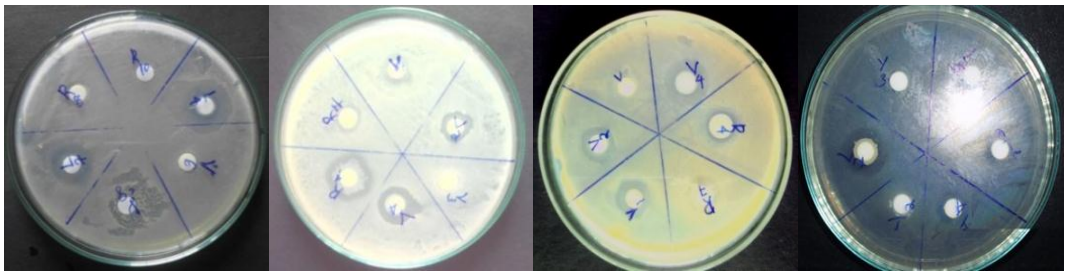
study provides valuable information on the antifouling potential of selected actinomycetes from the coastal regions of Northern Kerala. Though the findings are preliminary and require thorough assessment for identifying the potent biomolecule, the present chapter discusses the probable sources and species of actinomycetes that could be used for such studies. Results also indicate that the region is less studied, and with the diversity available, there are strong chances that more species of actinomycetes could be available that have potential in bioprospecting.

PLATE 6.1

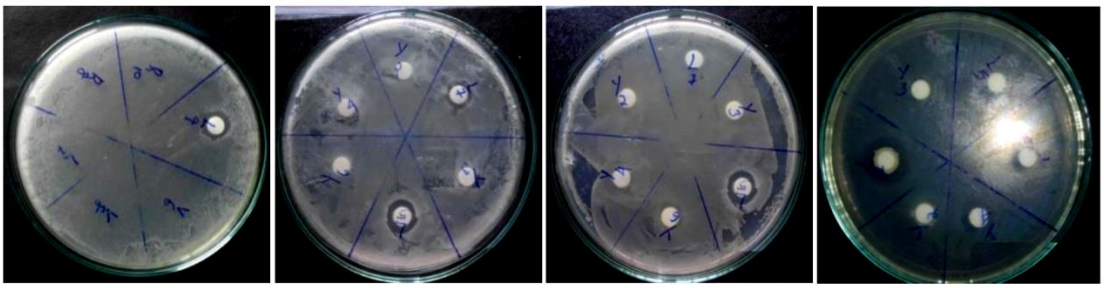
Pure isolates of four selected Actinomycetes strains



Streptomyces K2M6 *Streptomyces* APP56 *Streptomyces* KML4 *Streptomyces* APP59

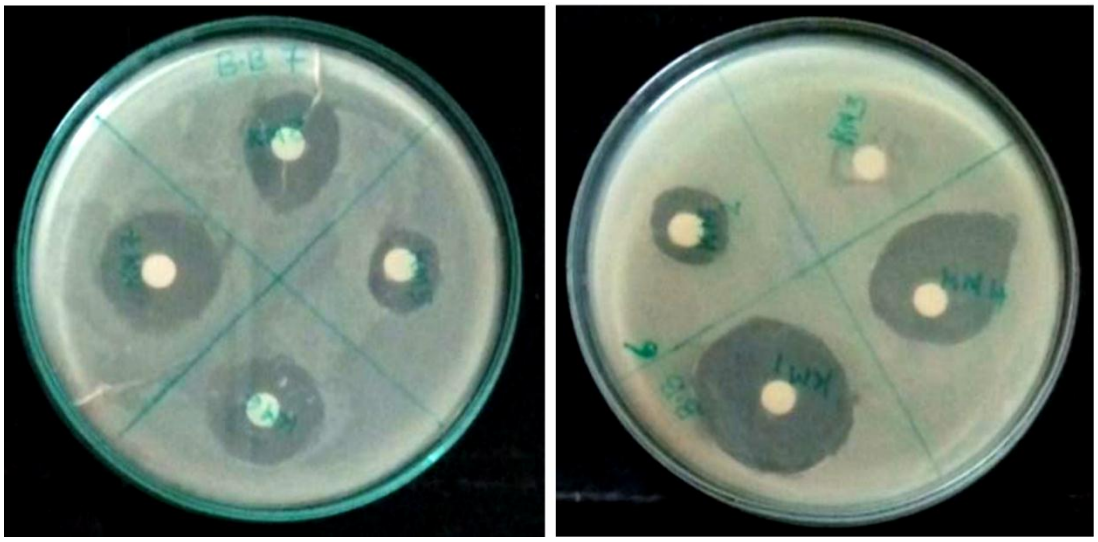


Antifouling activity of four selected strains against *Bacillus subtilis* PPL7 bacteria



Antifouling activity of four selected strains against *Bacillus licheniformis* PPV2 bacteria

PLATE 6.2



Zone of inhibition by the four strains against *Bacillus subtilis*

Zone of inhibition by the four strains against *Bacillus licheniformis*

Antifouling activity of four selected actinomycetes strains against *Bacillus subtilis* and *Bacillus licheniformis*

Chapter 7

ISOLATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF BIOMOLECULES ISOLATED FROM *STREPTOMYCES GRISEORUBENS* KML4

7.1 Introduction

Actinomycetes, which prevail all over the globe, fall only next to bacteria in abundance and play an essential role in producing a diverse range of secondary metabolites that have multiples roles that of antibiotics, anticancer and antitumor agents. Marine actinomycetes, among various other microbes, have played an important role in being the greatest source of novel compounds (Silambarasan *et al*., 2012; Deepa *et al*., 2013). They synthesize approximately two-thirds of all naturally derived antibiotics currently used in medicine, veterinary, and agriculture. The majority of these molecules originate from the genus *Streptomyces* (Barka *et al*., 2016; Chater, 2016). Bioactive metabolites are similar to those obtained from other organisms like plants, animals, fungi, bacteria etc., demonstrate biological activity and have diverse and unusual chemical structures with low molecular mass (Donadio *et al*., 2002; Demain and Sanchez, 2009). Out of 200,000 – 250,000 bioactive metabolites presently known, more than 22,500 are produced by microorganisms. Among them, about 45% (10,100) are products of actinomycetes fermentation, 38% (8600) are of fungal origin, and 17% (3800) are metabolites of unicellular bacteria, especially *Bacillus spp.* and *Pseudomonas spp*. (Demain and Sanchez, 2009; Berdy, 2005). Among filamentous actinomycetes, about 75% (7600) of metabolites are produced by species of a single genus viz., *Streptomyces* (Lam, 2007). Many and varied types of structurally unique and

biologically active secondary metabolites were recently discovered from marine *Streptomyces*. Secondary metabolites obtained from Actinomycetales are being rampantly used as a potential source of many novel compounds with antibacterial, antitumour, antifungal, antiviral, antiparasitic and other properties (Solecka *et al*., 2012).

Environmental conditions of the sea are highly different from that of the terrestrial (Carte 1996; Kijjoa and Sawangwong 2004), and this has led to a significant divergence of actinomycetes and their potential to produce novel bioactive compounds and new antibiotics (Ellaiah and Reddy 1987; Ramesh and Mathivanan, 2009). The discovery of several new marine actinomycete taxa and their ability to form stable populations in different habitats and produce novel compounds with various biological activities hence has been one of the most researched subjects in microbial ecology (Magarvey *et al*., 2004; Jensen *et al*., 2005b, 2007; Lam 2006; Fenical and Jensen, 2006; Prudhomme *et al.*, 2008; Olano *et al*., 2009; Asolkar *et al.*, 2010; Rahman *et al.*, 2010). Similarly, marine-derived *Streptomyces*, one of the prolific groups in marine environments, has focused on the research for novel secondary metabolites (Nandhini *et al*., 2015). Their extremophilic nature, unique characteristics, notably their complex fungal-like life cycle and earthy odour; they occur widely and show a higher diversity in colour formation than most other bacteria. This genus is defined by both chemotaxonomic and phenotypic characters, notably the presence of LL-DAP, an isomer of diaminopimelic acid, which is present in the cell wall peptidoglycan; this feature and the characteristic substrate and aerial mycelium are diagnostic for *Streptomyces* (Shinwari *et al*., 2013). However, the importance for this group increased after discovering many vital bioactive compounds with high commercial values from

them and their ability to produce a wide variety of antibiotics and extracellular enzymes (Narendhran *et al*., 2014). Hence this genus is considered a goldmine for new metabolites, and greater impetus is laid on unravelling their chemical composition using various techniques from different habitats (Zotchev, 2011, Kim *et al*., 2011). The biosynthesis of secondary metabolites depends on the growth conditions of each strain. For years researchers have been applying different nutrients and physicochemical factors during fermentation processes to optimize the production of bioactive compounds (Rajnisz *et al*., 2016). Currently, the statistical optimization approach performs modelling and analysis of fermentation processes, e.g. response surface methodology, which enables enhanced production of antibiotics, enzymes, and probiotics (Latha *et al*., 2017). With the advent of new technologies, the rate of discovering new potential drug in marine bioprospecting has increased manifold.

Marine actinomycetes have the potential to produce non-toxic antifouling compounds as they can produce substances that inhibit not only the attachment and/or growth of microorganisms but also the settlement of invertebrate larvae and macroalgal spores. Research on antifouling metabolites from natural resources has been taken a cue from this phenomena to derive new compounds in recent time. Notably, marine organisms were found to be among good resources for the production of non-toxic antifoulants. Previous studies have reported several natural product antifoulants from marine organisms, mainly invertebrates (Limnamol *et al*., 2009; Qian *et al*., 2010; Qian *et al*., 2013). Apart from them, marine microbes have gained much attention and are being explored as potential sources for producing environmentally friendly antifouling metabolites. In comparison to marine invertebrates, marine microbes are the authentic sources for natural products, including antifoulants (Jin *et al*., 2016). Once a strain of

actinomycetes is found effective against fouling bacteria, the normal process involves the mass culture of this microbe in laboratory environments. For this, the media optimization is done to enhance the production of their EPS, from which the bioactive compounds could be extracted. The extracellular metabolites are then subjected to a series of analytical techniques for purification, identification and isolation. Different fractionation techniques have been used to isolate components from secondary metabolites, which are then characterized to identify the bioactive molecules responsible for the antifouling property. Although Indian coastal ecosystems have been identified as having a plethora of biodiversity and primary source of organisms with the potential to produce a bioactive compound, very few such compounds have so far been commercially exploited (Sivakumar *et al*., 2007; Radhakrishnan *et al*., 2016). In the above background, the present study was envisaged to unravel the actinobacterial diversity in lesser-explored Northern coastal regions of Kerala to isolate, characterize, and partial purification of biomolecules that have antifouling potential. In continuation to Chapter 6, the strain of actinomycetes *Streptomyces griseorubens* (KML4) derived from mangrove ecosystems in the region. It showed better antifouling properties than the other selected *Streptomycetes* was further analyzed to derive the bioactive molecule responsible for the antifouling activity.

7.2 Materials and Methods

7.2.1 Secondary metabolite preparation

Mass culture of *Streptomyces griseorubens*, KML4 isolates was attempted by inoculating purified culture in 2L conical flasks containing Starch Casein media and incubating with a shaker at 29°C for two days**.** At the end of 48 hrs, the volume of the medium was scaled up to 8L by inoculating 2% of pre-inoculation media into the 8L of

production media and incubated at 29°C with periodical shaking for 8 to 12 days. The culture was then extracted using ethyl acetate, agitated overnight and separated using a separating funnel. The crude extract was then concentrated in a vacuum rotatory evaporator.

7.2.2 Analysis of secondary metabolite by Gas Chromatography-Mass Spectroscopy

The crude ethyl acetate extract of the pure culture of the isolates of KML4 was subjected to gas chromatography-mass spectrometry (GC-MS) analysis using a Thermo Scientific TSQ 8000 Triple Quadrupole MS and TG-5MS fused silica capillary column (30m, 0.25mm, 0.1mm film thickness). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used, and helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperature were set at 280°C. The oven temperature was programmed (50°C for 1 min, then 50–280°C at a rate of 5°C/min) and subsequently held isothermally for 20 min (Boussaada *et al.,* 2008). The peaks of the obtained compounds in the gas chromatography were subjected to mass spectral analysis. The spectra were then analyzed from the library data, NIST-MS search (version 2.0) (Al-Tameme *et al*., 2015).

7.2.3 Partial purification of the active crude compound by HPLC

High-Performance Liquid Chromatography (HPLC) was used to separate and quantify different compounds based on their elution time to identify and isolate bioactive compounds from *Streptomyces griseorubens* strain KML4. Only the fractions that had a higher probability ratio (>60%) and greater retention time (which indicate a higher quantity of the compound) alone were isolated. To separate the compounds, a

reversed-phase high-performance liquid chromatography (HPLC) (ThermoFisher Scientific, Dionex UltiMate 3000) was used, having a C18 column (2.1 mm×150 mm, 3.0 µm, 120A) kept at a constant temperature of 40 °C. The flow rate was maintained at the rate of 1 mL/min, and the eluate was detected using a UV detector. The partially purified components so separated were concentrated in a vacuum rotatory evaporator and used for further analysis.

7.2.4 Estimation of Minimum Inhibitory Concentrations

Minimum inhibitory concentration (MIC) of partially purified antibacterial metabolite was accomplished using the conventional agar dilution method against the two test biofouling bacteria, as mentioned in Chapter 5. Both *Bacillus subtilis* PPL7 and *B. licheniformis* PPV2 were inoculated onto the Mueller-Hinton medium containing different active metabolite concentrations (10-100 µg/mL). After an incubation period of 24 hours at 37˚C, the plates were examined for growth. The lowest antibiotic concentration that inhibited the growth of one or both organism was noted. The Mueller-Hinton media without test biofilm-forming bacteria and active compounds were used as a control. MIC of broad-spectrum antibiotics streptomycin was also carried out as a standard for comparison.

7.2.5 Antibacterial (antifouling) activity test

The antifouling test was performed against two Gram-positive biofouling bacterial species, *Bacillus subtilis* PPL7 and *Bacillus licheniformis* PPV2. The compounds were collected separately and concentrated. A known volume of the most active component (based on the MIC) was tested to ascertain the antifouling potential against biofouling bacterial species of *Bacillus subtilis* PPL7 and *Bacillus licheniformis* PPV2 strains by disc diffusion method. Each test organism was inoculated on the

surface of Zobell marine agar plates. The MIC concentration of each extract was impregnated on a sterile disc and placed over each of the Petri plate inoculated with the test organism. The plates were then incubated at 37°C for 24 h, after which the resultant zone of inhibition was measured.

Thermo Scientific TSQ 8000 Triple Quadrupole GC-MS Analyzer

ThermoFisher Scientific, Dionex UltiMate 3000 HPLC

7.3 Results

7.3.1 Gas chromatography-mass spectroscopy (GC-MS) analysis of the extract

The crude extracts of *Streptomyces griseorubens* KML4 were analyzed using the GC-MS technique. Many fractionated compounds (122) were detected from the crude endocellular and extracellular extracts of this species. However, based on the quantity of the fraction (depending on the retention time), increased probability (greater than 60%) of compound identification (based on the reference library) and basic information on their antimicrobial activity, twenty-seven (27) compounds were identified for further analysis. All these fractions were partially purified using reverse-phase high-performance liquid chromatography (HPLC).

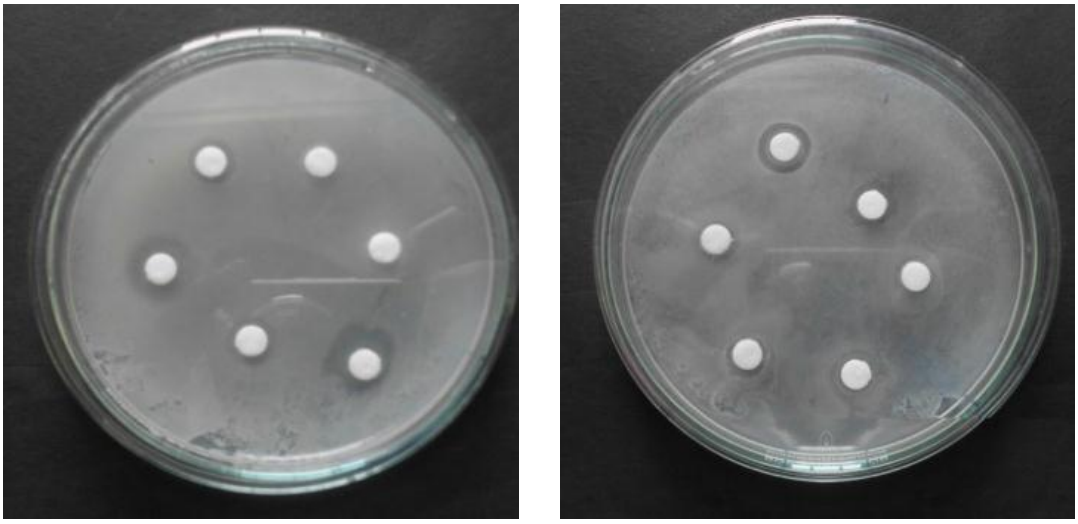
Table 7.1 Characteristics of six major compounds eluted in GC-MS analysis

Chemical Compound	Retention Time	Probability	Molecular weight	Minimum Inhibitory Concentrations (µg/mL)	
				***Bacillus subtilis* PPL7**	***B. licheniformis* PPV2**
Benzene,1-isocyano-3-methyl-3,6-Di-m-tolyl-1,2,4,5-tetrazine **(**C_8H_7N**)**	18.60-16.68	74.06	116	70	90
Pyrrolo[1,2a]-pyrazine-1,4-dione,hexahydro-3(2-methylpropyl) **(**$C_{11}H_{18}N_2O_2$**)**	33.42-33.65	94.62	210	65	30
Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl), (5'à, 10à) **(**$C_{33}H_{35}N_5O_5$**)**	40.60-40.72	94.98	583	30	45
1-H-Indole-3-carboxylic acid, methyl ester **(**$C_{10}H_9NO_2$**)**	31.78-3.92	72.66	175	55	70
2,3-Diphenyl-cyclopropyl-methyl phenyl sulfoxide **(**$C_{22}H_{20}OS$**)**	34.75-34.82	67.73	332	80	80
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester **(**$C_{19}H_{38}O_4$**)**	43.20-43.30	82.44	330	40	50

These isolated fractions were dried in a rotary vacuum evaporator and tested for their antibacterial potential against the two *Bacillus* strains PPL7 and PPV2. Of the twenty-seven fractions, only six (6) compounds showed any antagonistic activity against any of the two biofilm-forming bacterial strains. On further analysis, an assessment of their Minimum Inhibitory Concentration (MIC) was recorded to be between 30-95 µg/mL. Results of the MIC of these compounds on the two *Bacillus* strains, their chemical composition (molecular formula), retention time, percentage probability and molecular weight are shown in Table 7.1. Results indicate that two among these compounds viz., *Pyrrolo[1,2a]-pyrazine-1,4-dione,hexahydro-3(2-methyl propyl)* ($C_{11}H_{18}N_2O_2$) and *Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl), (5'à, 10à)* ($C_{33}H_{35}N_5O_5$) showed comparatively better MIC values against *Bacillus licheniformis* PPV2 and *Bacillus subtilis* PPL7 respectively.

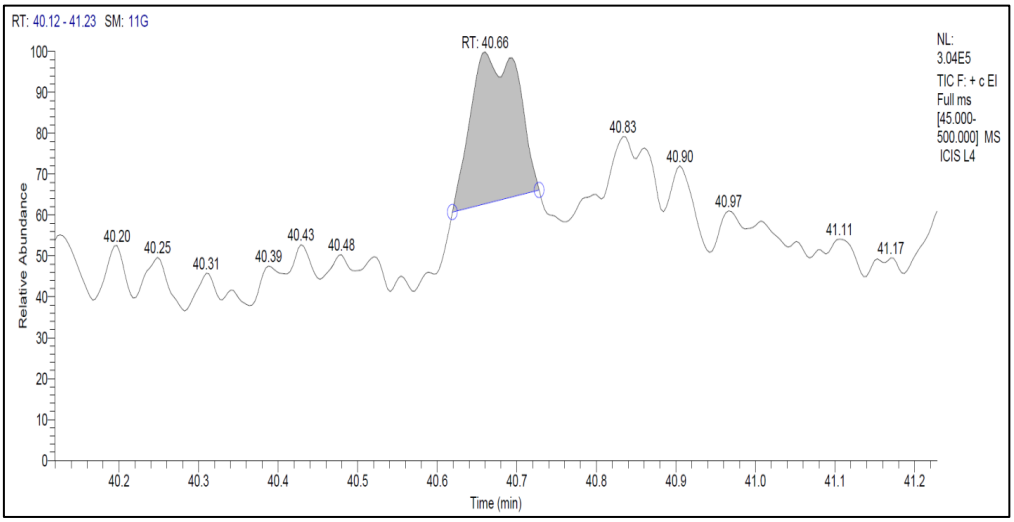
7.3.2 Antifouling assay

Based on MIC results, the zone of inhibition was recorded through the disc diffusion method incorporating the two partially purified compounds at 30 µL/mL concentration against the two bacterial strains of *Bacillus subtilis* PPL7 and *Bacillus licheniformis* PPV2. Figure 7.1 shows the antifouling activity of the two compounds against the bacterial strains. Results indicated that Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl) ($C_{33}H_{35}N_5O_5$) was effective in retarding the growth of *Bacillus subtilis* PPL7, while Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methyl propyl) ($C_{11}H_{18}N_2O_2$) was specifically effective against *Bacillus licheniformis* PPV2. The elution peak (retention time) and molecular structure of these two compounds retrieved from the GC-MS profile and NIST library are depicted in Figures 7.2 and 7.3, respectively.



$C_{33}H_{35}N_5O_5$ $C_{11}H_{18}N_2O_2$

Figure 7.1 Zone of clearance for the two partially purified compounds against *Bacillus subtilis* PPL7 and *Bacillus licheniformes* PPV2



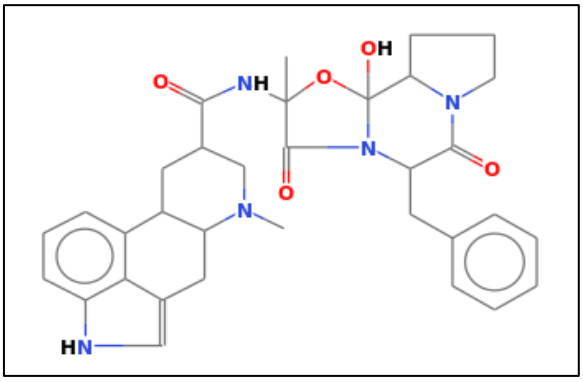


Figure 7.2 Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)

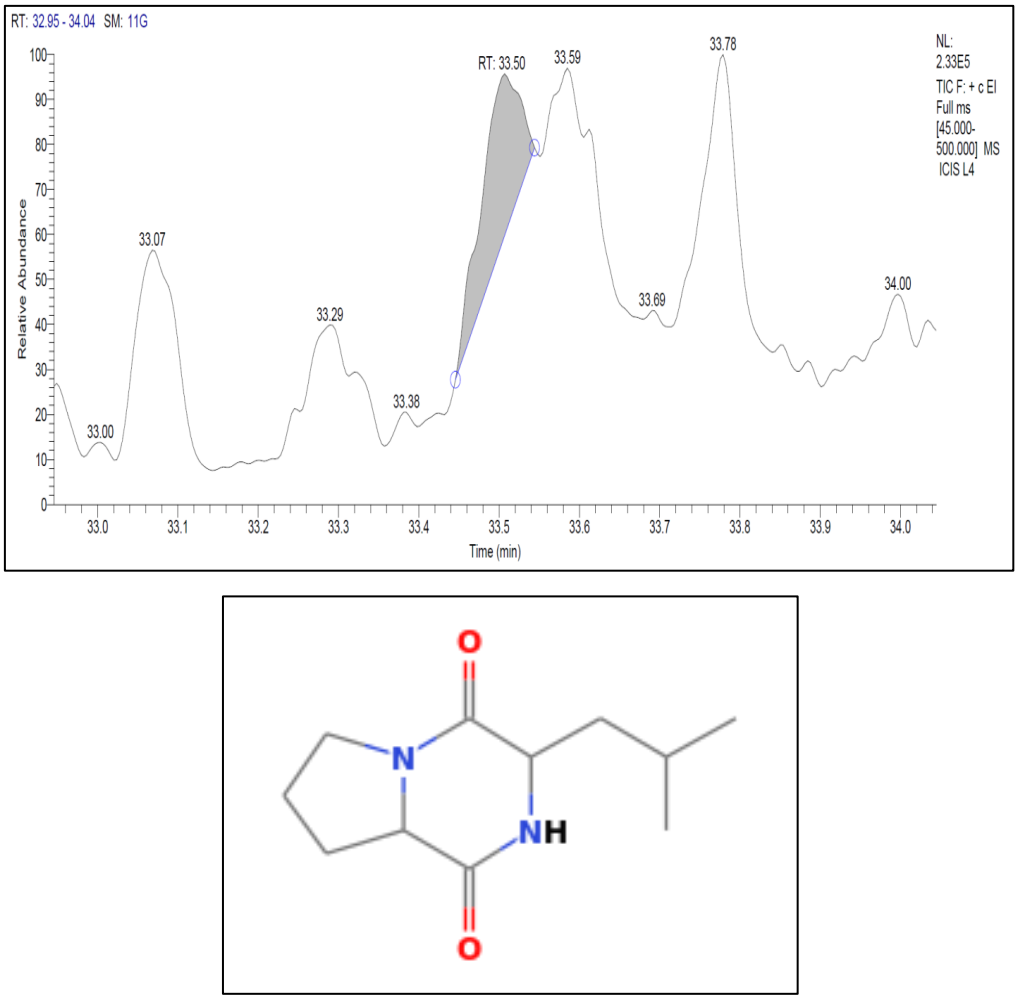


Figure 7.3 Pyrrolo[1,2a]-pyrazine-1,4-dione,hexahydro-3 (2-methylpropyl

7.4 Discussion

This chapter discusses the screening and identification of novel antifouling bioactive compounds from marine Actinomycetes *Streptomyces griseorubens* strain KML4. Mangrove actinomycetes occupy a special place in the microbial community based on their potential to produce a wide array of bioactive compounds not previously known to humanity. Hence, screening secondary metabolites have always resulted in discovering new compounds (Mahajan and Balachandran, 2012). Earlier studies on solvent-based extraction of crude extracts from actinomycetes have shown ethyl acetate to be the most effective solvent capable of eluting maximum number of compounds (Kumar and Jadeja, 2018). Hence, ethyl acetate was used to extract the different

fractions in the crude extract in the present study. Results indicated that a large number of compounds, most of which were derivatives of esters, alcohol, ketones, phenol, organic acids and lipids. In the present study, 122 types of secondary metabolites were isolated from this actinomycetes strain, of which 27 compounds were known antibacterial compounds. The results are in consonance with earlier reports of Xu *et al.* (2014) and Kumari *et al.* (2019), who have reported similar levels of secondary metabolites from mangrove associated *Streptomyces* strains. Members of the genus *Streptomyces* are common and widely distributed in soils, and their ability to produce a wide range of secondary metabolites and extracellular enzymes is of use in pharmacy, agriculture and industrial development (Suzuki *et al*., 2000). *Streptomyces griseorubens* KML4 is aerobic, mesophilic, halotolerant, Gram-positive actinomycete that forms an extensively branched substrate mycelium and aerial hyphae. They are characterized by the frequent formation of open hooked spore chains with ellipsoidal, smooth-surfaced spores. On Starch Nitrate agar medium, they produce a creamy substrate mycelium and a greyish aerial spore mass. Although this species does not produce diffusible pigments, they produce melanin pigments on peptone/yeast extract/iron or tyrosine agars. Owing to the capacity to synthesize numerous compounds that exhibit extreme chemical diversity, *Streptomyces* strains have always been a topic of research due to their ability to be a suitable candidate for the industrial production of new bioactive molecules (Wezel *et al*., 2006).

Various compounds have been isolated from actinomycetes with antibacterial, antifungal, antiviral, antitumour, antiprotozoal and hypocholesterolemic properties (Solecka *et al*., 2012). It is understood that out of the 14,000 known compounds so far isolated from microbes, the majority have antibacterial properties. A closer analysis

shows 66% of them have antagonistic activity against gram-positive bacteria, 30% against gram-negative bacteria, and 5% against mycobacteria. Likewise, against other microbes, 34% of compounds exhibited antifungal activity. 21% acted against yeasts, 11% against phytopathogenic fungi, and 24% against other fungi (Bredy, 2005). Among microbial products, *Streptomyces* fermentation products are the richest source of antibiotics and other industrially important compounds (Watve, 2001; Ruiz *et al*., 2010). Although different forms of compounds were segregated in the present study, most of them had not been previously analyzed for antibacterial effects. Hence, it was imperative to explore all these compounds produced in sufficient quantity to ascertain their biological role. Therefore, twenty-seven (27) compounds were delineated based on their novelty, amount, and prior bioactivity information for preliminary screening. Among them, only six compounds showed effective resistance against the known biofilm-forming *Bacillus* strains PPL7 and PPV2. Results of the present study are in confirmation with various other studies that have identified the antimicrobial potential of *Streptomyces griseorubens.* Al-Askar *et al*. (2014) have reported that this species has suitable antifungal and antibacterial activities and holds the potential to be used in the biological control of fungal and bacterial diseases of essential crops. Similar results have been recently reported by Sengupta *et al.* (2015) and Nguyen *et al*. (2019).

Among the six compounds finally isolated for assessing the Minimum Inhibitory Concentration (MIC), three viz., Pyrrolo[1,2a]-pyrazine-1,4-dione,hexahydro-3(2-methyl propyl); 1-H-Indole-3-carboxylic acid, methyl ester and Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester had previously been recorded for their antimicrobial activities (Kiran *et al*., 2018; Choppara *et al*., 2019; Kumari *et al*., 2019). While three compounds viz., Benzene, 1-isocyano-3-methyl-3, 6-Di-m-tolyl-1,2, 4,5-

tetrazine; Ergotaman-3',6', 18-trione, 9,10-dihydro -12'-hydroxy -2'-methyl-5'-(phenylmethyl), and 2,3-Diphenyl-cyclopropyl-methyl phenyl sulfoxide had no prior reports of being antimicrobial though these compound have shown to have anticancer and antioxidant properties. Results of MIC indicated that though Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester was equally effective against both PPL7 and PPV2 strains of *Bacillus*, its MIC concentration was higher than that of Pyrrolo[1,2a]-pyrazine-1,4-dione,hexahydro-3(2-methyl propyl) and Ergotaman-3',6',18-trione,9,10-dihydro-12'- hydroxy-2'-methyl-5'-(phenylmethyl), (5'à, 10à), the latter being a first record for showing antibacterial property. In the past, Gas Chromatography-Mass spectrometry (GC-MS) has been successfully used fingerprint analysis of secondary metabolites from various sources (Kell, 2005). In the present study, based on the antibacterial property and better MIC values, the two compounds were found effective against fouling bacteria. While Pyrrolo[1,2a]-pyrazine-1,4-dione,hexahydro-3(2-methyl propyl) was found effective against *Bacillus licheniformis* PPV2, Ergotaman- 3',6',18-trione, 9,10- dihydro- 12'- hydroxyl-2'- methyl-5'- (phenylmethyl), (5'à,10à) showed promising results against *Bacillus subtilis* PPL7 strains. Results emerging from the study are in tune with those reported by Cetinkaya *et al*. (2020), indicating a similar presence of pyrrole compound, Pyrrolo[1,2-a] pyrazine-1,4-dione,hexahydro-3-2-methyl propyl, from another strain of *Streptomyces griseorubens*. Pyrrole compounds are produced as the characteristic secondary metabolite by the species of *Streptomyces*, *S. lateritius* and *S. mangrovisoli sp*. (Awla *et al*., 2016; Ser *et al*., 2015). In yet another bioactivity study on this compound extracted from *Streptomyces* species, VITPK9 isolated from the salt spring habitat of Manipur, Sanjenbam and Kannabiran (2016) reported the rich potential to be used as a bioactive compound. However unlike the

Pyrrole counterpart Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)- ,(5'α,10α) have not shown much antibacterial effects. Ergotamine receives its nomenclature from an alkaloid peptide ergot, traditionally used in medicine as an analgesic, a vasoconstrictor agent, a serotonergic agonist, and an alpha-adrenergic agonist and a mycotoxin (Awla *et al*., 2016). However, recently their use as the potential antifungal compound has gained this compound much acclaim. Though they are profoundly isolated from epiphytic and endophytic bacteria extracted from the foliage of various plants (Nxumalo *et al.,* 2020), they have also been reported from certain species of *Streptomyces*. However, they have not been assessed for their antimicrobial properties (Awla *et al*., 2016).

A common drawback of compounds extracted from epiphytic bacteria is the higher volatility of the extracted compounds (Kavitha and Savithri, 2017). In the present study, however, the antimicrobial property of both the selected compounds prevailed even in lower concentration and for a longer period of time. This indicates that the compounds were stable and could be used further for commercial applications. Certain compounds, especially those with the pyrrole ring, are among the most explored heterocycles in drug discovery programs. In this regard, isolation of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-2-methyl propyl is a good indicator for testing their efficacy against various bacterial and fungal microbes. The mycotoxin effect of Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl), (5'α, 10α) enables it to be considered for antibacterial drug discovery (Lyagin and Efremenko, 2019). For both the compounds, the MIC was found to be 30 µl/mL. However, both these compounds were specific in their effectiveness against the tested bacterial strains. While Ergotaman-3',6', 18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-

5'-(phenylmethyl) effectively retarded the growth of *Bacillus subtilis* PPL7, Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methyl propyl) retarded the growth of *Bacillus licheniformis* PPV2. A similar pattern of inhibitory effect against *Bacillus subtilis* by extracts of *Streptomycetes sp.* was reported by Shinwari *et al*. (2013). Though there have been other reports which identify an array of compounds from actinomycetes that have an inhibitory role against biofilm-forming bacteria or biofouling organisms, most of these studies have focused only on compounds such as 2,3-Butanediol, Cyclobutanol, Octadecanal etc. or chemical constituents such as (6S,3S)-6-benzyl-3-methyl-2,5-diketopiperazine (bmDKP) and (6S,3S)-6-Isobutyle-3-methyl-2,5-diketopiperazine (imDKP) extracted from selected actinomycetes strains such as *Streptomyces praecox* (Cho *et al*., 2012). Similarly, Quercetin and Taxifolin, two antifouling metabolites isolated from mangrove derived *Streptomyces fradiae* PE7, and *Streptomyces sampsonii* PM33 was also reported to be a promising candidate for the development of eco-friendly antifouling preparation (Gopikrishnan *et al*., 2016; 2019).

The results of the present study suggest that actinomycetes, like other microbes, have immense potential to produce novel antifouling compounds. Though many attempts in the past identified different compounds from microbes and invertebrates that could effectively be used against antifouling bacteria, very few compounds could be synthetically produced (Yang *et al.*, 2007). A major hindrance to this was the complex structure of the identified molecule which rendered them chemically unstable in natural conditions. The isolation of microbes from other invertebrates, such as those found in symbiotic association with bacteria, is a bottleneck with great concerns from a conservation perspective. In this regard, the advantages are more when microorganisms

associated with marine organisms are used for bioprospecting of antifouling compounds. The standardization of culture conditions and the optimization of media are some limitations; once their production is optimized in laboratory conditions, their commercial applications could be attempted. It was also seen that better recovery of the extracellular components in microbes could be enhanced with standardization (Jensen *et al*., 1996). Compared to normal actinomycetes, the strains with good fermentation potential produce an excellent quantity of metabolites for bioassays (Penesyan *et al.*, 2010). In the present study, *Streptomyces griseorubens* KML4 strain showed good fermentation and subsequent production of bioactive compounds in their crude extracts. The antibacterial activity of most of the extracted fractions indicated that this actinobacterium has various biochemical mechanisms to survive in the natural environment. The higher EPS production is also a testimony for their acclimatization to adverse environmental parameters. It was also noted that through standardization of the culture medium and optimization of the culture media, the production of EPS and bioactive compounds could be enhanced. Most importantly, the bioactive compounds that showed good antibacterial properties could be easily separated, and partial purification could be achieved for testing their efficacy. Results of the present study point out two compounds that have not been known to retard biofilm-forming bacteria and therefore biofouling. Another advantage of the two compounds isolated was that being relatively small; they could be tried to produce synthetically. Derivatives of both these compounds have shown their capacity to be commercially manufactured viz., Ergotamine and Pyrroles. Hence the present study introduces two compounds that have novelty in their application against antifouling bacteria. In the past, a major limitation of marine bioprospecting is the inability of the compounds to perform in-field assays, as

seen in laboratory trials. Therefore the most significant challenge lies not in developing natural antifouling coatings but in maintaining their antifouling characteristics for a more extended period of time in the field. In the present study, such an attempt was not attempted. However, based on the inherent qualities of the compound, it is envisaged that it has the potential to prevent the formation of biofilms. This includes antibiofilm activities through the antibacterial and anti-adhesion property and production of extracellular polymers production (EPS), essential for biofilm formation.

From this study, it could reasonably be concluded that *Streptomyces griseorubens* KML4 has immense potential as an actinomycete in bioprospecting. The ability of this strain of *Streptomyces griseorubens* to curb the growth of two biofouling bacteria viz., *Bacillus subtilis* PPL7 and *Bacillus licheniformis* PPV2 makes it a remarkable candidate for screening other bioactive compounds. Since this species was isolated from mangrove associated habitats, it would be interesting to see the role that these extracellular metabolites play in the ecological functioning of the region. The two compounds that were isolated and partially purified from this species, i.e., Ergotaman-3',6', 18-trione,9,10 -dihydro-12' -hydroxy -2'-methy l-5'- (phenylmethyl) and Pyrrolo [1,2-a] pyrazine -1,4-dione, hexahydro-3-(2-methyl propyl) before synthesizing it on a commercial level require thorough assessment for ascertaining their bioactive potentials. It is also pertinent to explore whether these compound have a synergistic effect since both these compounds were either antimicrobial or antifungal, which could be the reason for its effectivity against both these biofilm-forming bacterial strains.

Chapter 8

SUMMARY AND CONCLUSION

The ocean is one of earth's most valuable natural resourcesand yet to be explored for natural resources. Life in these amazingly diverse systems includes the smallest microscopic bacteria and viruses to the largest animal ever lived on earth. Various offensive and defensive mechanisms have evolved to allow organisms to gain selective advantage and cope with competitors.Marine microorganisms form highly specific and symbiotic relationships with filter-feeding organisms like sponges, ascidians and marine plants.The host organism synthesizes compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment. To thrive in the competitive niche, these marine organisms produce bioactive metabolites. Almost every class of marine organisms, from the smallest microbes onwards, has various bioactive compounds with unique characteristics that may be entirely distinct from those of the terrestrial environment. These products are the current interest for multiple applications in the pharmaceutical, agricultural and food industries for new drugs and chemicals. Of all the marine microbes, actinobacteria have special consideration given the proven biosynthetic capabilities of numerous isolates from the soil and various marine environments. They are significant producers of bioactive secondary metabolites and extracellular enzymes with different biological activities, especially genus *streptomyces*. They are valued as producers of therapeutically essential compounds. Most natural products are derived from these marine microbes, and these valuable prokaryotes also show a universal distribution.

In this scenario, the present study was carried out to unravel the diversity and distribution of Actinomycetes from marine sediments and seaweeds of the less explored northern coast of Kerala for screening potential antifouling bioactive compounds. From the present study, potential bioactive molecules isolated from the selected strains of actinomycetes and tested their efficacy as antifouling agents. All the isolated actinomycetes were characterized up to a generic level based on biochemical characteristics and microscopic observation. All the selected isolates were screened for antifouling activity against biofouling bacteria.

The study area includes three districts of Northern Kerala (Malappuram, Kozhikode and Kannur). Monthly sampling from six ecologically diverse sites viz., Ponnani (Malappuram district), Kadalundi, Kotooli, Thikkodi and Payyoli (Kozhikode district) and Dharmadam (Kannur district) were conducted during 2016-17. These stations represented regions that were rich in mangrove and seaweed patch. Mangrove sediment samples were aseptically collected in sterile polyethene bags from Ponnani and Kadalundi mangrove areas, especially from the rhizosphere region of the soil using sterile PVC pipe at a depth of 5-15 cm. Likewise, typical coastal sediment samples were also aseptically collected from a depth of 10-20 cm, mainly from the nearshore regions in sterile polyethene bags from coastal areas of Kottoli and Payyoli stations. Seaweed samples were collected from the rocky intertidal shores of Thikkodi and Dharmadam during low tide periods. The seaweed samples were separately collected using a sterile spatula in sterile ziplock bags and marked accordingly. Sampling was conducted thrice in a year (representing the three-season) during pre-monsoon season (February to May), monsoon season (June to September) and post-monsoon season (October to January). For each sampling site, sub-samples of sediments were collected, pooled together and

homogenized to obtain a representative sample. All the collected samples were placed in an icebox and transferred aseptically to the laboratory. Water samples were also collected using clean plastic bottles for estimating water quality parameters.

During the present study, various physico-chemical parameters were analyzed at the stations and laboratory after fixing the samples. The isolation and characterization of these microbes were difficult since they grow slowly, relatively low numbers in relation to common unicellular bacteria. The collected sediment samples were subjected to pre-treatment by air drying at room temperature for a week, followed by oven drying at 55^0 C for 10 minutes to retard the growth of slime forming bacteria. Very few studies have been reported on the isolation and identification of Actinomycetes from mangrove sediment and seaweeds of the Northern coast of Kerala. In the present study, based on the results of colony morphology, biochemical and enzymatic characterization, the 529 isolates studied were grouped under 30 species falling under nine genera and seven families. On further analysis, it was seen that the majority of the actinomycetes belonged to the genera Streptomyces (53.9%) followed by *Kitasatospora* (9.1%), *Nocardiopsis*(8.7%), *Streptosporangium*(8.3%), *Micromonospora*(5.3%), *Nocardia*(4.9%), *Rhodococcus*(4.0%), *Actinopolyspora*(3.0%) and*Actinomadura*(2.8%). A total of 30 species were identified from the sediment and seaweed samples, and the species diversity was high for genus Streptomyces (15 species), followed by *Nocardiopsis*and *Kitasatospora* (3 each). From the present study, the results of enzymatic activity on isolates of actinomycetes showed better production of various hydrolytic enzymes viz., Amylase, Gelatinase, Caseinase, Lipase, Oxidase, Catalase, Urease. The Carbohydrate fermentation ability of the selected Actinomycete strains indicated that the isolates showed variation in their carbon source assimilation pattern.

A total of 529 Actinomycetes isolates of various genera were isolated during the study. The spatial distribution of actinomycetes showed that the Kadalundi Mangrove region showed the highest abundance represented by 150 isolates while Dharmadam recorded the least isolates (36). In all the station's Streptomyceteswas the dominant genera. The distribution pattern of actinomycetes among the different seaweed communities showed that a higher number of isolates (47) were collected from the red algae, followed by green algae (19) and brown algae (13). A genus-wise distribution of actinomycetes on the seaweeds showed a good association of these isolates on *Grateloupiafilicina.* It is clear from the study that the occurrence of actinomycetes in the sediment and seaweeds on the northern coast of Kerala greatly influenced by the season. Pre-monsoon had the highest actinomycetes abundance, while the monsoon had the least representation. Various physico-chemical parameters of the soil and water were collected to correlate the diversity and distribution of actinomycetes in the diverse systems of Northern Kerala. Soil pH was determined using a soil pH meter. Organic carbon in sediment was determined following Elwakeel and Riley's method. From each station, *in situ* measurements of temperature, pH, and salinity were recorded. For hydrological analysis, one litre of water was collected directly from the surface level. Dissolved oxygen (Winkler's method), Nitrate (Brucine method), and Phosphate were analyzed following standard protocol. Diversity indices, viz., Shannon-Weiner index ($H^{'}$), Margalef's index (d), Pielou's evenness index (J') and Simpson's diversity index (1- λ) were estimated from numerical abundance data using Plymouth Routines in Multivariate Ecological Research (PRIMER 6) software (Clarke and Warwick, 2001). Significant differences in diversity indices with regard to sampling stations and seasons were tested using one-way ANOVA in Statistical Package for the Social Sciences

(SPSS) version 22.0. The similarity in the assemblage of actinomycetes between the stations was estimated by Multi-dimensional scaling using Bray-Curtis similarity measure (Clarke and Ainsworth 1993) and was statistically verified using cluster analysis in PRIMER 6. Principal coordinate analysis (PCoA) was performed to understand the association between environmental variables and actinobacterial assemblages. The average taxonomic distinctness (AvTD or Δ^+) was estimated from presence or absence data (Clarke and Warwick, 1998), and funnel plots were constructed to measure the variation in the taxonomic distinctness (Λ^+) at different sites. Environmental influence on the dominant actinomycetes species was assessed with canonical correspondence analysis (CCA) using log-transformed data (terBraak, 1994). Monte Carlo permutation tests were conducted to identify the significance of each environmental parameter on the species assemblage on square-root transformed data using PAST ver. 3.26 (Paleontological Statistics Software).

Results indicated that all other indices differed significantly among the diversity indices except for Pielou's evenness ($P<0.01$) among stations. However, such a variation between seasons ($P>0.05$) was not noticed during the study. Kadalundi had the maximum species diversity (25), followed by Ponnani (19), Payyoli (14) and Kotooli (13) and the least were recorded from Thikkodi (11) and Dharmadam (7). Species abundance and other diversity indices also followed a similar pattern. Duncan's Multiple Range Test (DMRT) results indicated there exists a significant ($P<0.01$) difference in the diversity and distribution of actinomycetes based on the sediment characteristic of the regions, i.e., mangrove sediments >nearshore sediments > seaweeds. This was further confirmed by the MDS and cluster analysis wherein the species assemblage were grouped under six major clusters, each representing the six stations. Hence the

actinomycetes diversity in Northern Kerala was quite diverse related to the sediment characteristics of the region. Therefore, the strains of actinomycetes that were isolated and characterized could be tested on locally available bacterial strains for their antifouling activities. Sampling was done during 2017-18, and biofouling bacteria were isolated from the biofilms collected from the immersed surfaces of boats, ropes, oysters and other hard materials in the Ponnani fishing harbour. Out of the 28 biofouling bacterial isolates, 13 isolates showed positive results for biofilm formation. Among them, seven isolates that showed substantial adherence property were selected for further analysis. The selected biofouling bacteria were identified to come under the genus *Bacillus*, *Aeromonas*, *Staphylococcus* and*Serratia* based on phenotypic, cultural and biochemical characteristics. Exopolysaccharides (EPS) was analyzed quantitatively to ascertain the rate of biofilm production. Among the seven strains, the two strains (PPL7 and PPV2) showed high EPS production; hence, these strains were subjected to molecular taxonomy. Molecular characterization was carried for identification of the species following using the 16S rRNA gene. The 16S rRNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with forward primer-F243 (5'-GGATGAGCCCGCGGCCTA-3') and reverse primer R513GC (5'-CGGCCGCGGCTGCTGGCACGTA-3'). The complete 16S rRNA gene was sequenced by using an ABI 377 automated DNA sequencer. Results of the Basic Local Alignment Search Tool (BLAST) search program at the National Centre for Biotech Information (NCBI) showed that the strain PPL7 to be *Bacillus subtilis* (MT348573)and PPV2 as *Bacillus licheniformis*(MT348574).

Out of the 30 species of actinomycetes isolated, 18 isolates that showed better enzymatic activity were subjected to screen their antifouling property against selected

biofouling bacteria. Eighteen (18) isolates showed better enzymatic activity were subjected to crude ethyl acetate extraction and tested antagonistic activity against one or more biofouling bacteria and noted. The actinomycetes showed more significant inhibition of over 30 mm was selected, and their activity was then tested under different pH levels (pH 4-9). Of the seven strains, four showed high tolerance to a wide range of pH, and these four isolates were then explicitly tested against PPL7 and PPV2 strains of *Bacillus subtilis*and *B. licheniformes*, respectively. The results showed that four strains of actinomycetes (K2M6, KML4, APP56 and APP59) effectively reduced the biofouling properties of these two strains of bacteria. To identify the strains to species-level molecular characterization was carried out. These four strains were identified through barcoding using 16S rDNA and the NCBI Genebank accession numbers.The results of the molecular analysis showed that the four strains (K2M6, KML4, APP56 and APP59) be *Streptomyces variabilis*(MT913027), *S. griseorubens*(MT913026), *S. rubrogriseus*(MT317112) and *S. spiroverticillatus* (MT317114).

Among these four strains of actinomycetes, one strain, *S. griseorubens*(KML4), showed better antifouling properties than the other three strains.Isolation, characterization and partial purification of biomolecules from these strain*S. griseorubens*(KML4) were carried out in the study. The crude ethyl acetate extract of the pure culture of the isolates of KML4 was subjected to gas chromatography-mass spectrometry (GC-MS) analysis using a Thermo Scientific TSQ 8000 Triple Quadrupole MS and TG-5MS fused silica capillary column (30m, 0.25mm, 0.1mm film thickness). The peaks of the obtained compounds in the gas chromatography were subjected to mass spectral analysis. The spectra were then analyzed from the library data, NIST-MS search (version 2.0). Results showed the presence of six major peaks (above 60%

probability). The final purification of all the six major active fractions was carried out by reverse-phase high-performance liquid chromatography (HPLC) (ThermoFisher Scientific, DionexUltiMate 3000) using a C18 column (2.1 mm×150 mm, 3.0 µm, 120A) at a constant temperature of 40 °C. The flow rate was maintained at the rate of 1 mL/min, and the eluate was detected using a UV detector. The prominent peak compounds were collected separately and concentrated, and 30 µL of each extract was tested for ascertaining the antifouling potential against *Bacillus subtilis* PPL7 and *Bacillus licheniformis* PPV2 strains. The results indicated that the compound Ergotaman-3',6', 18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl) ($C_{33}H_{37}N_5O_5$), which have proven antimicrobial property effectively retarded the growth of strain PPL7, while Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methyl propyl) ($C_{11}H_{18}N_2O_2$) which is a known antifungal molecule retarded the growth of strain PPV2. Hence Present study depicts Actinomycetes, especially *Streptomyces* spp., as a good source for producing bioactive metabolites. The study also provides the first account of the diversity of actinomycetes and their potential for exploring new biomolecules from the sediments and seaweeds of the northern coast of Kerala. This study has identified two potential antimicrobial compounds that have not been used so far for antifouling. Knowledge generated from this work would pave the way for developing environmentally friendly molecules to replace the toxic/biocidal compounds being presently used in antifouling paints. This study also highlights four strains of *Streptomyces*, more importantly, *S. griseorubens*KML4 isolated from mangrove region of Northern coast of Kerala having potential for future research and development of novel metabolites.

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