MOLECULAR SCREENING OF MANGROVE DERIVED ACTINOBACTERIA ALONG THE COASTAL BELT OF COCHIN AS A SOURCE OF AROMATIC POLYKETIDES

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ABSTRACT

Actinobacteria is found to have a potent metabolic activity against pathogens. The present study explores the biosynthetic potential of actinobacteria from the mangrove sediments of Cochin. Samples were collected from five different mangrove areas of Cochin, Vallarpadam Island, Panambukadu, Kovilakathumkadavu, Bolgatty and Cherai. A collection of 22 different actinobacteria was obtained which differ in their colony morphological appearance. The antibacterial effects of these strains were screened qualitatively and quantitatively. In qualitative screening, 22 different strains were assessed for their antibacterial activity against different human pathogens, namely *Escherichia coli, Klebsiella sp. Staphylococcus aureus* and *Proteus sp.* by cross streak method. It could be seen that 50 % isolates were active against at least one of the test organisms and 21.31 % strains exhibited a broad spectrum activity against almost all of the test bacteria except *Klebsiella sp.* The activities of the isolates were quantified by agar well diffusion method. Solvent extraction with ethyl acetate was done to obtain the crude extracts which were further tested for zone of inhibition.The isolates, S1-1, S1-2, S1-3, S1-4, S1-5, S2-2, S2-3, S2-5, S3-2, S3-3, and S5-2 were found to show significant antibacterial activity. 10 potent isolates were selected and subjected to molecular screening for their ability to synthesize aromatic polyketides based on amplification of Ketosynthase α domain of type II PKS. Among the 10 isolates only one isolate (S1-1) had shown positive amplification. The positive isolate was characterized based on partial 16s rRNA gene analysis. The result revealedthat it belongs to the *Streptomyces sp.* BLAST and phylogenetic analysis showed 97.22 % identity to *Streptomyces violascens* but the identity could be established only on characterization of the full gene. Thus the molecular data highlights the importance of *Streptomyces* isolates in antibiotic production. The antibacterial activity and the PKS gene based approach can be applied together for efficient screening of isolated strains for pharmaceutical value and related compounds.

Keywords: Actinobacteria, mangrove, Ketosynthase domain, antibacterial, type II PKS gene, *Streptomyces sp.*

1. INTRODUCTION

Antibiotics are truly referred as the 'wonder drugs' for their virtual success against pathogenic microorganisms (Demain, 1999). The introduction of antibiotics not only helped in the treatment of infections, but also has a major role in decreasing mortality and morbidity. The first antibiotic discovered by Sir Alexander Fleming in 1928 (Fleming, 1980) facilitated the discovery of many other secondary metabolites with similar properties. The antibiotic research from the discovery of Fleming to our days has been a reverting and continuously changing adventure. As a result of intense research of more than 50 years, these days' ten thousands of natural products that are derived from microbial sources are established. After the revolution in the "golden era", in the forties and early fifties, when almost all groups of important antibacterial antibiotics (tetracycline, cephalosporin, aminoglycosides, and macrolides) were discovered, the success story had continued (Raja and Prabakarana, 2011).

Antibiotics are produced by bacteria, actinobacteria, fungi imperfecti, basidiomycetes, algae, lichens, green plants, and also from animal sources. But majority of antibiotics is derived from microorganisms especially from the species *Actinomycete*s (Bérdy, 1995). Actinobacteria are formerly referred to as Actinomycetes or Ray fungi are Gram-positive, saprophytic bacteria, with widespread distribution in nature (Selvakumar et al., 2014). Actinobacteria are the mainstay of the antibiotics industry and hold a significant role in producing variety of drugs that are extremely important to our health and nutrition (Magarvey et al., 2004). Of all known drugs, 70 % have been isolated from actinobacteria and out of which 75 % and 60 % are used in medicine and agriculture respectively (Miyadoh, 1993; Tanaka and Omura, 1993). Most of the important drugs are expensive and have side effects to the host, some microbes have no successful antibiotics and others are developing multidrug resistance. Pathogenic microorganisms have evolved sophisticated mechanisms to inactivate antibiotics caused the rapid emergence of multi drug resistance underlines the need to look for new antibiotics (Alanis, 2005; Sharma et al.,2011).

Screening of microbial species is an important aspect as there is a remarkable source for the production of structurally diverse secondary metabolites that possess pharmaceutically relevant biological activities (Berdy, 2005). It has long been an

observed fact that the search for the new secondary metabolites from microorganisms in general has been confounded because different strains belonging to the same species are capable of producing different secondary metabolites (Waksman and Bugie, 1943). Actinobacteria and their bioactive compounds show antimicrobial activity against various pathogens and multi drug resistant pathogens e.g. vancomycin resistant enterococci, methicillin resistant *Staphylococcus aureus, Shigella dysenteriae, Klebsiella sp., Escherichia coli, Pseudomonas aeruginosa etc.* (Saadoun et al., 1999; Selvameenal et al., 2009; Servin et al., 2008; Singh et al., 2012).

Search for new antibiotics effective against multidrug resistant pathogenic bacteria is presently an important area of antibiotic research. Although, considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most versatile propitious source for new antibiotics (Koehn & Carter, 2005; Baltz, 2006; Peláez, 2006; Bull and Stach, 2007). It is also becoming increasingly clear that un- explored and under-explored habitats are a rich source of novel actinobacteria which have the capacity to produce interesting new bioactive compounds. Marine is a vital source of novel microorganisms, which does not exist in terrestrial environment (Baskaran et al., 2012). Although the exploitation of marine actinobacteria as a source for discovery of novel secondary metabolites is initiated at an early stage, numerous novel metabolites have been isolated in the past few years (Lam, 2006). A few new secondary metabolites such as staurosporinone, salinosporamide A, lodopyridone, arenimycin, marinomycins and proximicins isolated from marine actinobacteria from 2005 to 2010 and they are of particular interest due to their rarity and potent and diverse bioactivity (Subramani et al., 2012). The mangrove ecosystem is a largely unexplored source for actinobacteria with the potential to produce biologically active secondary metabolites (Chaudhary, 2013). Microbial diversity of the mangrove ecosystem is one of the promising research areas which need extensive exploration (Curits et al., 2002; Das et al., 2006). The ecosystem existing between terrestrial and marine environment is an ideal habitat that supports rich and diverse group of microorganisms (Arifuzzaman et al., 2010). The secondary metabolites produced by mangrove plants are toxic to residing microbes, need to be degraded or detoxified for their survival. In this situation, microbes are under high pressure to sustain and eventually adapt to the existing conditions for their survival and evolve to produce

novel bioactive secondary metabolites (Sengupta et al., 2015). There are evidences that compounds with unique structures and potential medicinal use have been isolated from mangrove actinobacteria in recent years which include alkaloids, benzene and cyclopentenone derivatives, dilactones, macrolides, 2-pyranones, sesquiterpenes, salinosporamides, xiamycins and novel indolocarbazoles (Xu et al., 2014). Compounds uncovered from the mangrove rare actinobacteria are uniquely structured and lead directly to the advancement of novel antibiotics that are active against antibiotic-resistant pathogens (Lam, 2006). Many new compounds identified from the rare actinobacteria are proven effective in clinical and pharmaceutical industries (Azman et al., 2015). In Kerala, mangroves are distributed in unique varieties. The Cochin has a rich source of mangroves which spread along the coastal areas of Vypin, Vallarpadam, panambukadu islands which is less explored for studies.

Polyketides are large group of secondary metabolites that have notable variety in their structure and function. Polyketides exhibit a wide range of bioactivities such as antibacterial, antifungal, anticancer, antiviral, immune-suppressing, anti- cholesterol and anti-inflammatory activity (Katsuyama et al., 2007). Polyketide-based pharmaceuticals are some of our most important medicines. Biosynthesis of polyketides is very complex because the process involves multifunctional enzymes, Polyketide synthases (PKS). In *Streptomyces*, there are three types of PKSs (type I, type II, and type III) (Lal R, 2000). Among these, the type II Polyketide synthases (type-II PKSs) are responsible for producing aromatic polyketides which include the important classes of antibiotics, anthracyclines, angucyclines, aureolic acids, tetracyclines, tetracenomycins, pradimicin-type polyphenols, and benzoisochromanequinones. Some examples of aromatic polyketide produced by *Streptomyces* are actinorhodin, doxorubicin, jadomycin B, oxytetracycline, mithramycin, tetracenomycin C, and benastatin A (Okamoto et al., 2009).

Searching for novel and potent chemical entities is continuously the goal of natural product research. A little is known about the actinobacterial diversity of mangrove sediments of Kochi, which could be an endless resource for novel antimicrobials that has not been properly exploited.

2. REVIEW OF LITERATURE

2.1. Antibiotics

Once expected to be eliminated as a public health problem, infectious diseases remain the leading cause of death and disability-adjusted life years (DALYs) worldwide (Oxford University Press, 1993). Dramatic changes in society, technology, and the environment, together with the diminished effectiveness of certain approaches to disease control, usher in an era wherein the spectrum of infectious diseases is expanding, and many infectious diseases once thought to be controlled are increasing. The term "emerging infectious diseases" refers to diseases of infectious origin whose incidence in humans has either increased within the past two decades or threatens to increase in the near future (National Academy Press, 1992). Mortality as a result of infectious diseases represents one-fifth of global deaths (WHO, 2012). An antibiotic is an agent that either kills or inhibits the growth of a microorganism (Dorlands, 2010). The origin of antibiotics is ancient and antibiotic biosynthetic genes and resistance- conferring genes began to evolve millions of years ago (Wright & Poinar 2012). At the very low concentrations in which antibiotics are thought to be naturally present, they can act as signaling molecules and as such trigger transcription responses important for environmental survival (Yim et al., 2006; Goh et al., 2002). A competitive role is only achieved once the antibiotic concentration is high enough to inhibit the growth of surrounding microorganisms (Sengupta et al., 2013). Transient high antibiotic concentrations in nature made it necessary for antibiotic producing organisms to harbor resistance genes needed for self-protection and stimulated the evolution of resistant genes in neighboring bacteria. The success of antibiotics against diseases caused by bacteria is a great achievement in modern medicine. However, bacteria are becoming resistant and less responsive to antibiotic treatment when it is really needed (Bush et al., 2011). Antibiotic resistance is the ability of certain strains of bacteria to develop a tolerance to specific antibiotics to which they were once susceptible (Anderson et al., 2010). The concept of resistance development towards antibiotics is not new, but went largely unnoticed until human usage of highly concentrated doses of antibiotics in medicine began to select for bacteria with high- level resistance phenotypes. Indeed, once antibiotics were used to treat bacterial infections it did not take long for the first highly resistant strains to develop. Strains

resistant to the first generation of antibiotics, including penicillin G and streptomycin were isolated before or shortly after the drugs were introduced to the market (Madigan et al., 2006; Wright, 2007). During "pre-antibiotic era", antibiotic producing and antibiotic resistant bacteria coexisted in their natural habitats without facilitating the process of selecting for deadly resistant human pathogens, so that those remained susceptible to most antibiotics found in nature (Sengupta et al., 2013). The effectiveness of antimicrobial therapy in the 1930s was remarkably successful and led to a significant decrease in human morbidity and mortality (Martinez, 2009). The natural products labeled under secondary metabolites are synthesized through simple to complex mechanism in natural habitats and play a momentous role in the management of various disorders because of their biological activity (Mahmood et al., 2013). Most of the recent antibacterial are semisynthetic and are modifications of various natural compounds (Von Nussbaum et al., 2006). The pharmaceutical industries are investing a lot to enter into the natural product business and trying hard to commercialize their research products. The research activities relating to antibiotics from natural source other than microorganisms has thus also occupied a prominent place and as a result few valuable products have been discovered and marketed very successfully. It has tremendous opportunities to bring new molecules and compounds which can fight against resistant organisms (Ameh et al., 2012). Generally, the microbial natural products appear as the most promising source of the future antibiotics that society is expecting (Fernando, 2006). A hybrid substance is a semi synthetic antibiotic, where in a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties (Vijayakumar et al., 2015). Members of the microbial group actinobacteria are a rich source of bioactive compounds, notably antibiotics, enzymes, enzyme inhibitors, and pharmacologically active agents, and about 75 % of the known commercially and medically useful antibiotics are produced by *Streptomycetes* (Sujatha et al., 2005). Beijerinck (1900) and Eriko (2002) established that actinobacteria occur in great abundance in the soil and have a great role in the management of microbial stability with the production of antibiotic substances. Available Antibiotics of Actinobacteria are Penicillin, Cephalosporin, Tetracyclines, Macrolides, and Quinolone (Maharajan et al., 2012).

2.2. Actinobacteria

Actinobacteria are filamentous Gram-positive bacteria, characterized by a complex life cycle belonging to the phylum actinobacteria, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria (Ventura et al., 2007). Actinobacteria are widely distributed in both terrestrial and aquatic ecosystems, mainly in soil, where they play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants, animals and fungal materials. They are also important in soil biodegradation and humus formation as they recycle the nutrients associated with recalcitrant polymers, such as chitin, keratin, and lignocelluloses, (Goodfellow and Williams 1983, McCarthy and Williams 1992, Stach and Bull 2005) this produces several volatile substances like geosmin responsible of the characteristic "wet earth odor" (Wilkins, 1996) and exhibit diverse physiological and metabolic properties, for example the manufacture of extracellular enzymes (McCarthy and Williams 1992, Schrempf, 2001). The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000 of which 10,000 are produced by actinobacteria, thus representing 45 % of all bioactive microbial metabolites discovered (Berdy, 2005). Among actinobacteria, approximately 7,600 compounds are produced by Streptomyces species (Berdy, 2005). Several of these secondary metabolites are potent antibiotics. As a result of which *streptomycetes* have become the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Berdy 2005). Progress has been made recently on drug discovery from actinobacteria by using high-throughput fermentation and screening, combinatorial biosynthesis and mining genomes for cryptic pathways, to generate new secondary metabolites related to existing pharmacophores (Baltz, 2008). Actinobacteria are of tremendous economic importance as the secondary metabolites produced by them includes antibiotics, other medicinal, toxins, pesticides and animal and plant growth factors (Demain and Fang, 1995). The best known of the secondary metabolites produced by actinobacteria are the antibiotics. The secondary metabolites produced by actinobacteria serve as the sources of life saving environments. These have a broad spectrum of biological activities; e.g. antibacterial, antifungal, antiviral, antiparasitic, immunosuppressive, antitumor, enzyme inhibitory and diabetogenic Cancer transplant rejection and high cholesterol (Bérdy, 2005; Farnet and Zazopoulos, 2005). Members of actinobacteria group, in addition are producers of clinically useful antitumor drugs such as

Anthracyclines (Aclarubicin, Daunomycin and Doxorubicin), Peptides (Bleomycin and Actinomycin D), Aurelic acids (Mithramycin), Enediynes (Neocarzinstatin), Antimetabolites (Pentostatin), Carzinophilin, Mitomycins and others. actinobacteria products are not able not only for their potent therapeutic activities but also for the fact that they frequently possess the desirable pharmacokinetic properties required for clinical development (Farnet and Zazopoulos, 2005). The actinobacteria exist in various habitats in nature and widely distributed in a diverse range of aquatic ecosystem, including mangroves, sediments obtained from deep sea etc. it has been demonstrated in a comparative survey that actinobacteria population is largest in soils of surface layer and gradually decreases with as depth increased; individual actinobacteria strains are present in all soil layers.

2.2.1. Morphology

Actinobacteria display the greatest morphological differentiation among gram-positive bacteria; however, the cell structure of actinobacteria are typical prokaryotes and totally different with fungi. They are most abundant organisms that form thread-like filaments in the soil (Qinyuan Li et al., 2016). Actinobacteria produce initial micro colonies composed of branching system filaments that after 24-48 hours fragment into diptheroids, short chain and coccobacillary forms. The cell wall of actinobacteria is a rigid structure that maintains the shape of the cell wall of actinobacteria that maintains the shape of the cell ad prevents bursting of the cell due to high osmotic pressure. The wall consists of a large variety of complex compounds including peptidoglycan, teichoic and teichuronic acid and polysaccharides. The peptidoglycan consists of glycan (polysaccharides) chains of alternating N-acetyl-d- glucosamine (NAG) and N-acetyl-muramic acid (NAM) and diaminopimelic acid (DAP), which is unique in prokaryotic cell walls. Teichoic and teichuronic acid are chemically bonded to peptidoglycan (Vijayakumar et al., 2012). Actinobacteria are aerobic, spore forming, belonging to the order actinobacteria characterized with substrate and aerial mycelium growth. The mycelium is prostrate, i.e. vegetative, and growing in the substrate, or aerial, when a special mycelium is produced above the vegetative growth (Kämpfer, 1998) (fig.2.1). Some actinobacteria can form complicated structures, such as spore, spore chain, sporangia, and sporangiospore. The growth and fracture modes of substrate mycelium, the position of spore, the number of spore, the surface structures of spore, the shape of sporangia, and whether

sporangiospore have flagella or not are all important morphological characteristics of actinobacteria classification(Qinyuan Li et al., 2016). The mycelia was colorless to white, chalky reed, grey or olive in colour (Oskay et al., 2004).

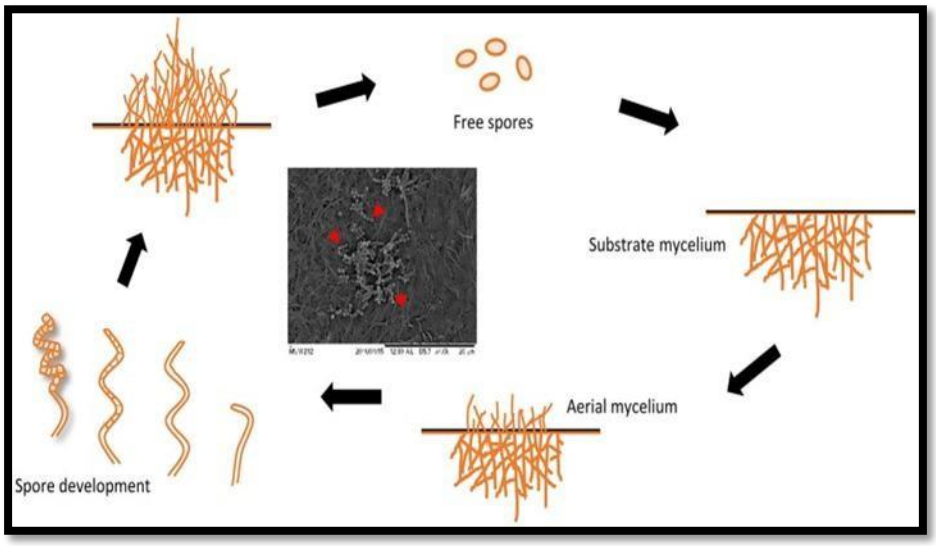

Fig. 2.1. Development and Organization of the Aerial Mycelium and substrate mycelium

Actinobacteria were high in guanine and cytosine content (≥ 55 %) and they were one of the leading Phyla of bacteria found on almost all natural substrates (Hogan, 2010; Usha et al., 2011). The growth of marine actinobacteria were characterized by tiny, compact, soft to leathery colonies adhering to the agar medium, the surface of colonies of actinobacteria were either flat or elevated (Sathi et al., 2001; Muiru et al., 2008). The surface of the colonies of actinobacteria were smooth with minute hyphae at its fringes were observed under low resolution microscope. Some colonies of actinobacteria had smooth surfaces while some other their surfaces were much folded (Mutitu et al., 2008). The surface of the colony was usually dry with pointed or elevated appearance, with or without mycelium (Muiru et al., 2008). The cell wall composition of actinobacteria varied greatly among different groups and was of considerable taxonomic significance. Four major cell wall types were distinguished in these filamentous bacteria on the basis of the three features of peptidoglycan composition and structure (Sharma, 2014). These features were diaminopimelic acid isomer on tetra peptide side chain position 3, presence of glycine in interpeptide bridges and sugar content of peptidoglycan (Lechevalier, 1970).

2.2.2. Substrate mycelium

As known as vegetative mycelium or primary mycelium, the substrate mycelium grows into the medium or on the surface of the culture medium. The main function of the substrate mycelium is the absorption of nutrients for the growth of actinobacteria. Under the microscope, the substrate mycelia are slender, transparent, phase-dark, and more branched than aerial hyphae. The figure illustrated in figure 2.2.The single hyphae is about 0.4 to 1.2 μm thick; usually do not form diaphragms and fracture, capable of developing branches. Minority groups (such as Nocardia), rudimentary to extensively branched like the roots, substrate hyphae often fragment in situ or on mechanical disruption into coccoid to rod-shaped, nonmotile elements when grown to a certain stage (Qinyuan Li et al., 2016).

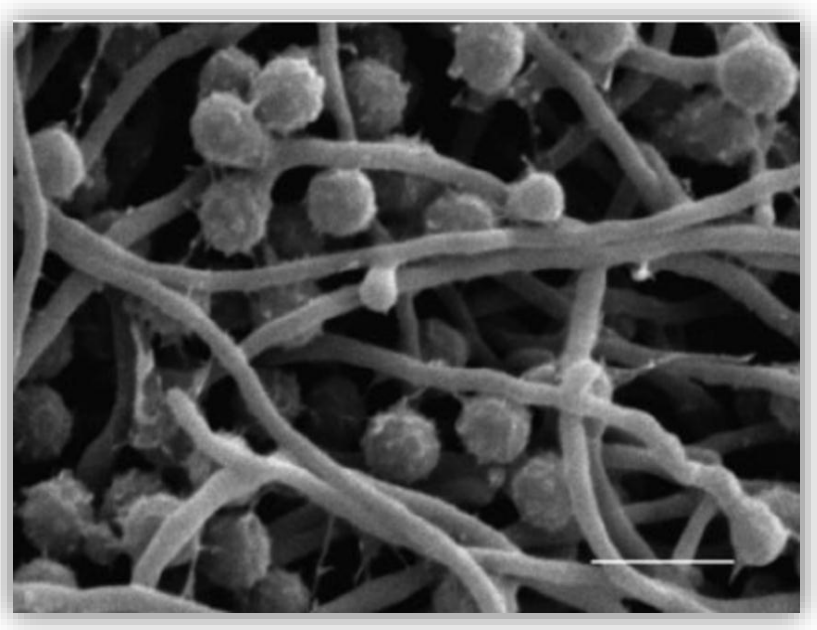

Fig.2.2. Scanning electron micrograph of substrate mycelium

2.2.3. Aerial mycelium

Aerial mycelium is the hyphae that the substrate mycelium develops to a certain stage, and grows into the air (fig. 2.3). Sometimes, aerial hyphae and substrate mycelia are difficult to distinguish. Aerial hyphae are coarse, refractive, and phase- bright. The hyphae of the aerial mycelium are characterized by a fibrous sheath, except the genera Pseudonocardia and Amycolata (Warwick et al., 1994). Ultramicroscopic, it is composed of fibrillar elements and short rodlets, forming a

characteristic pattern. The fibrous sheath is also present on sporulation aerial hyphae,causing the different surface ornamentations of the spore (Wildermuth et al., 1971).

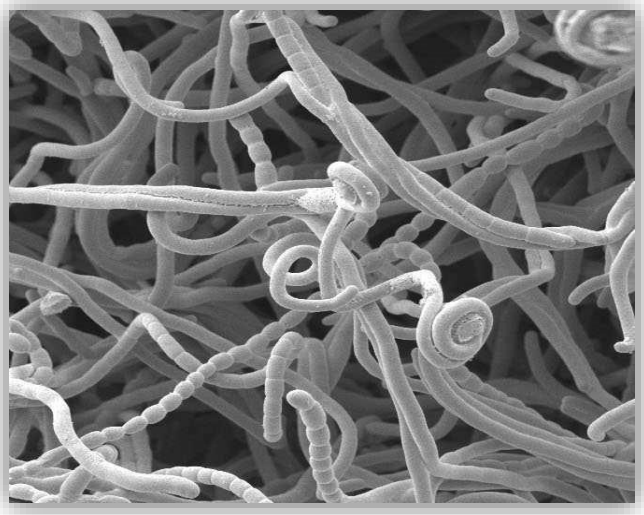

Fig.2.3. Scanning electron micrograph of aerial mycelium

2.2.4. Spore chain

Actinobacteria grow to a certain stage, differentiated in its aerial hyphae, can form reproductive hyphae called spore-bearing mycelium. Indeed, this type of spore formation occurs in most actinobacteria genera (Wildermuth et al., 1971). Spore chains can be divided morphologically respecting their length and number of spore: di- or bisporous with two spores, oligosporous with a few spores, and poly-sporous with many spores (figure 2.4). Actinobacteria spore chain length, shape, position, color are the important basis for classification (Lechevalier et al., 1989). The monosporous is the mode of single spore production. This form occurs in various suprageneric groups, represented by several well-known genera, such as Micromonospora, Thermomonospora, Saccharomonospora, and Thermoactinomyces. The spores are arranged either directly on the aerial hyphae or on very short side branches. The spore formation is initiated by lateral budding along an aerial hypha, producing short side branches (Qinyuan Li et al., 2016).

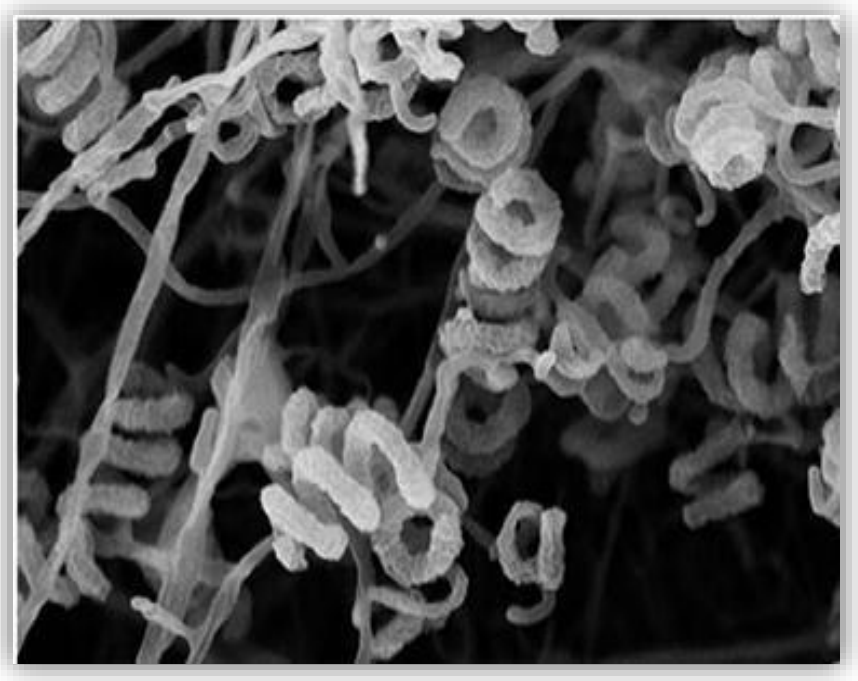

Fig.2.4. Scanning electron micrograph of spore chain

2.2.5. Spore

The division of a hyphae and the production of a spore start with the formation of a cross-wall. In general, there are three kinds of methods of actinobacteria sporulation process (Fig. 2.5): (i) when substrate hyphae are fragmented, the septum, which is known as a split septum, may occur and form spore, like the genus Micromonospora. (ii) Spores are formed by septation and disarticulation of pre- existing hyphal elements with a thin fibrous sheath. The spore wall is formed, at least in part, from wall layers of the parent hypha; this is termed as homothallic development (Locci R et al., 1987) and was found to be typical for many other spores actinobacteria, like the genus *Streptomyces.* (iii) Globose spores are formed in aerial and substrate mycelium and product spore wall, such as some strains of Thermoactinomyces. The spores are classical endospores with all the properties of bacterial endospores, relative to the formation process, ultrastructure, and physiology. Aside from the mycelial growth, spore formation is the most important morphological criterion that can be used to recognize actinobacteria. Conventionally, the formation of spores is restricted to the morphological group of sporoactinobacteria, where sporulation takes place in well-defined parts of the mycelium. It is known that a

number of different genes are involved in spore formation (Chater et.al, 2006,Hardisson, 1985).

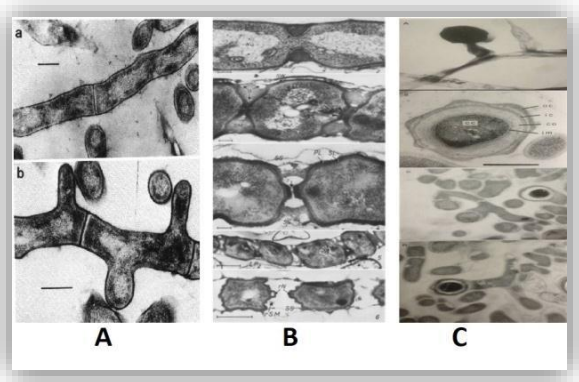

Fig.2.5. models of spore formation

The morphological characteristics of actinobacteria due to gene regulation are generally quite stable, and it is an important basis for classification. The development and formation of some structures, like aerial mycelium, spore, and sporangia, are affected by culture conditions. In some media, strains produce a lot of sporangia or spore, while in other media have little or none (fig. 2.6) (Shirling EB, 1966).

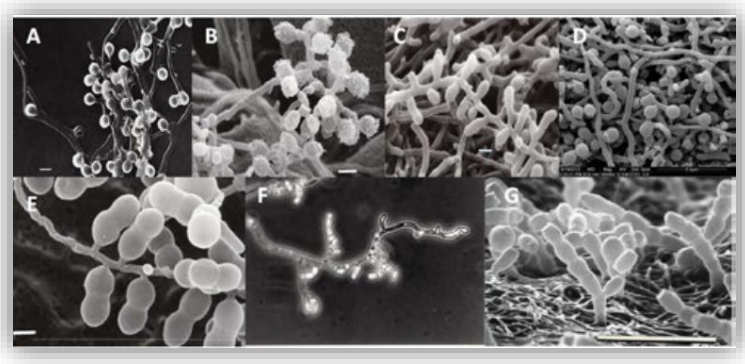

Fig.2.6. Microgram of single spore production and spores in short chains

2.3. Secondary Metabolites of actinobacteria

Actinobacteria played an important role among the marine bacterial inhabitants, because of its diversity and capacity to produce novel secondary metabolites of great therapeutic and commercial value (Amador et al., 2003; Hopwood, 2007; Dasari et al., 2011). Actinobacteria are biotechnologically valuable bacteria which are well exploited for secondary metabolites (Balagurunathan and

Radhakrishnan, 2010). Screening, isolation and characterization of promising strains of actinobacteria producing potential secondary metabolites has been a major area of research by many groups worldwide for many years (Hacène et al., 2000; Laidi et al., 2006). Among various genera of Actinomycetes; Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora and Actinoplanes are the major producers of commercially important biomolecules (Solanki et al., 2008). Actinobacteria are physiologically diverse bacteria as evidenced by their production of numerous extracellular enzymes and other metabolic products (Kekuda et al., 2010). Many actinobacteria produced melanoid pigment and other pigments like phenazines, phenoxazinones and prodiginines (Rahman et al., 2000). The production of secondary metabolites in actinobacteria is tightly regulated and responds to external stimuli from the environment (Genilloud et al., 2018). Bioactive metabolites are products of primary and secondary metabolism of different organisms such as plants, animals, fungi, bacteria. Secondary metabolites have diverse and unusual chemical structures, and often having a low molecular mass. Many secondary microbial metabolites show antibacterial, antifungal, antiviral, antitumor, antiprotozoal, hypocholesterolemic and other activities (Solecka et al., 2012). They are commonly used in medicine, veterinary practice, agriculture and industry. About 14,000 compounds of microbial origin exhibit antimicrobial (antibacterial, antifungal, antiprotozoal) activity. At present, antibacterial activity of secondary metabolites is of high interest. It is triggered by the extensive need for the discovery of new antibiotics against the widespread of multidrug resistant clinical strains and the therapeutic failure in treatment of resulting infections. In this period the discovery of antitumor, antiviral and non-antibiotic-enzyme inhibitory-metabolites, had just started (Anderson and Wellington, 2001; Salami, 2004). The production of secondary metabolites in actinobacteria is tightly regulated and responds to external stimuli from the environment. This regulation is the result of different classes of pathway-specific regulatory elements involving two-component systems, extra-cytoplasmic sigma factors or pathway specific regulators (Liu e al., 2013). The flow chart is depicted in (fig. 2.7)

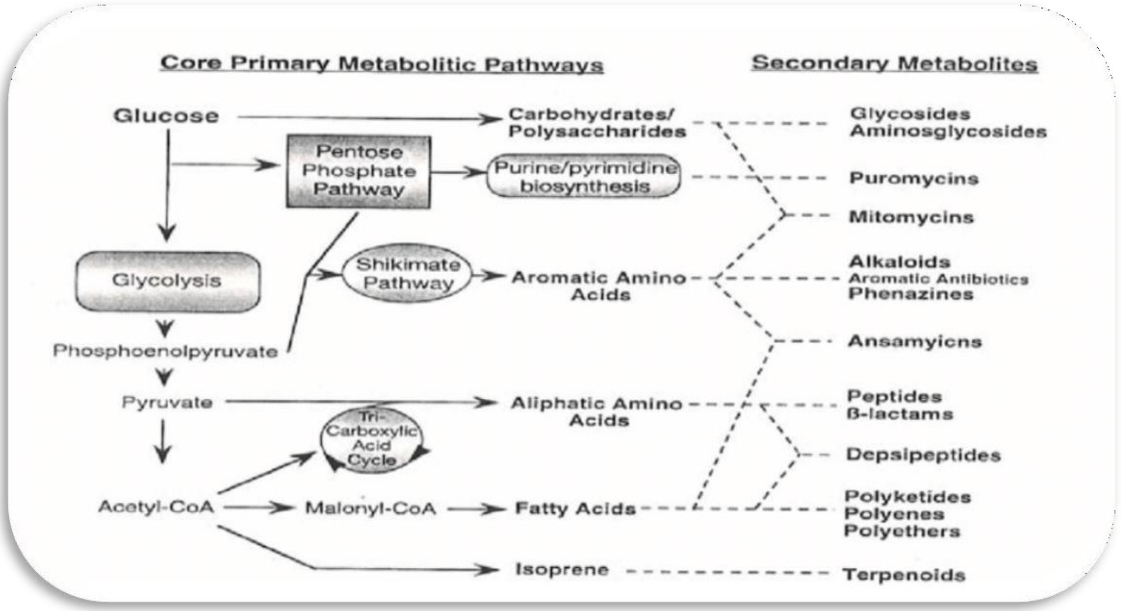

Fig.2.7. Primary metabolic pathways leading to the formation of secondarymetabolites (August et al., 1999)

2.4. The Mangrove Ecosystem of Actinobacteria

The marine environment is a treasure accretion of resources. Marine actinobacteria differ in physiological, biochemical and molecular characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds (Rosmine et.al, 2016). Mangroves are specialized marine environment which widely distributed along the coastlines and are rich in bacterial flora (Sharma et.al, 2014). Mangrove ecosystems are found all over the world in tropical and subtropical regions. Biodiversity is prevalent in the tropical estuarine system, particularly in the intertidal forested vegetation known as Mangrove (Mooney et al., 1995), which covers about 240 x 103 km^2 (Lugo et al., 1990; Twilley et al., 1992). The mangrove vegetation possesses many structural and physiological peculiarities and is composed of species with strongly marked characterized grouped under “true mangroves”. There are also plants with less strongly marked characteristics, which are known as semi-mangrove (Tansley & Fritch, 1905; Kiranmayi et al., 2012). Mangroves are considered as one of the most productive terrestrial ecosystem which can produce 29-75 tons/ha of biomass (Palot and Jayarajan, 2007) Mangroves are invaluable treasure of our biodiversity with immense ecological and economic significance. Despite its important role in maintaining the ecological balance and providing livelihood for the local communities (Hema et al.,

2015). The mangroves dominate almost 1/4th of world's tropical coastline. The world's total mangrove area which spans 30 countries including various island nations is about 1,00,000 km^2 (Deshmukh and Balaji, 1994). According to Ramachandran and Mohanan (1987) until a few centuries ago, backwaters of Kerala were fringed with rich mangrove vegetation. An estimate, based on the authentic record of Blasco (1975) indicated that there were about 70,000 ha of mangroves in Kerala. According to Kjerfve (1997), these wetland ecosystems are among the most productive and diverse in the world, and more than 80 % of marine catches are directly or indirectly dependent on mangrove and other coastal ecosystems worldwide. The importance of mangroves was realized from 19th century onwards. However, the significance of mangrove ecosystem got prime importance among the policy makers and people after the Tsunami of the year 2004. On understanding the unique features and immense services provided by the ecosystem, different researchers initiated research on mangroves worldwide (Hema, 2013).

Fig.2.8. Mangrove ecosystem

2.5. Mangrove Ecosystem of Vypin

Puthuvypeen is an upcoming major Industrial area in Kochi in the Indian state of Kerala. It is a western suburb of Kochi City and is a part of Vypin Island which is 24 km long and 2.6 km wide. Vypin Island has a total area of 87.85 km (Sahadevan et al., 2017). The island is situated on the western side of Ernakulam District with

Kodungallur Strait on the North, Cochin backwaters and Cochin Port on the South, River Periyar and Kochi city on the East and Arabian Sea on the West. The island is connected to the city by three bridges known as “Goshree” Bridges. The ecology of Vypin Island is unique, endowed with large canals extending over 50 km and a network of small canals emerging there from (Chatturvedi, 2005). Ernakulam district in Kerala particularly witnessed massive destruction of mangroves for various development projects in the last couple of decades (Hema, 2014). The destruction of mangroves was mostly for alternate development activities such as national projects, residential and commercial complexes, shrimp/fish ponds, roads and railway lines. Earlier, 90 percent of mangroves in Kerala were destroyed either for paddy cultivation, coconut orchard or for land reclamation (Ramachandran, 2005). The remaining scattered patches of mangroves are now found in Vypin, Vallarpadam, Malippuram, and Mangalavanam in the north zone and Kumbalam, Panangad, Chellanam and Kumbalangi in the south. The major species recorded in these areas are Avicennia officinalis, Rhizophora mucronata, Excoecaria agallocha, Acanthus ilicifolius, Bruguiera gymnorrhiza (Badaruddin, 1992).

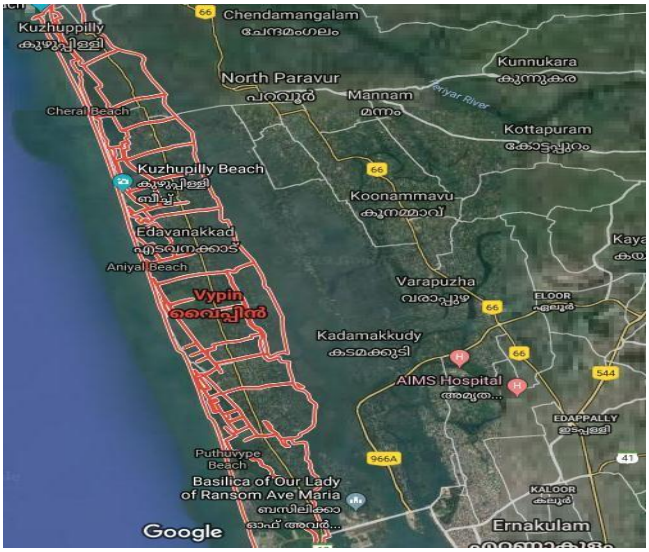

Fig.2.9. Vypin area

2.5.1. Vallarpadam

Vallarpadam Island is located close to the mouth of the Cochin estuary, within five kilometers of the city of Ernakulam. It is a part of mulavukad panchayat, a 7.3 km^2 area that includes both the islands of mulavukad and Vallarpadam. The island lies below sea level and has a wall constructed around the entire shoreline to prevent

the tides from inundating the Island (Klobe, 2002). Here, the mangroves are constituted by an assortment of medium trees and shrubs that are adapted to grow in saline coastal sediment habitats. The massive root system of mangroves is efficient at dissipating wave energy (Massel SR, 1999). The mangrove swamps protect the coastal areas from erosion and storm surge, especially during hurricanes and tsunamis (Mazda Y, 2005).

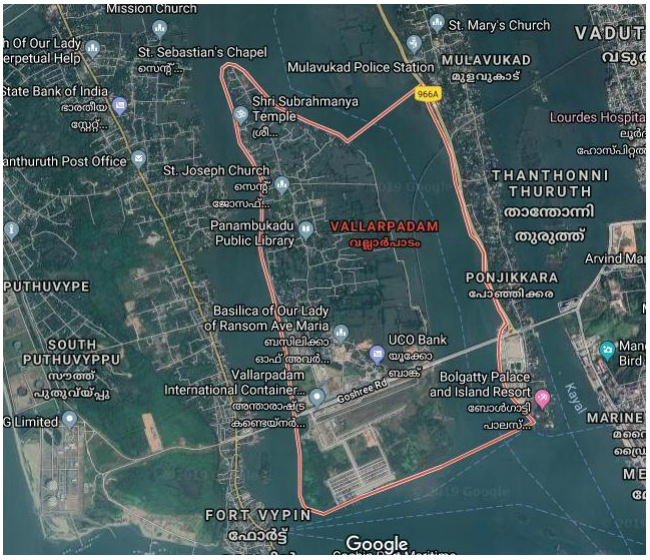

Fig.2.10. Vallarpadam Island

2.5.2. Panambukadu

Panambukadu is a small island in Vypin block. This island is surrounded by the estuarine waters of Cochin backwater. The island has an area of 9.27 sq., km. Except for the human dwellings; the Island is occupied by traditional prawn cum fish farms fringed by mangroves. This station is coming under the preview of Goshree project proposal of district administration.

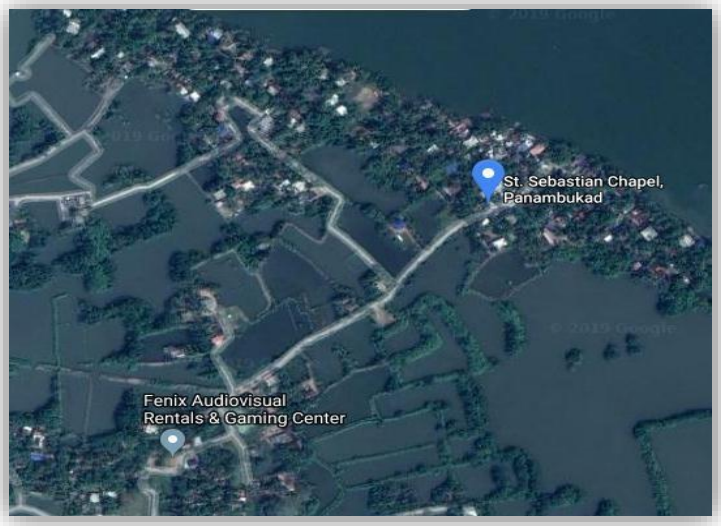

Fig.2.11. Panambukadu Island with mangrove forest

2.5.3. Kovilakathumkadavu

Kovilakathumkadavu, which is situated towards the northern end of Vypin Island near Munambam. This area is lying parallel to the Lakshadweep Sea with a direct tidal influence Lying on two sides of the Pallipuram are backwater. There are many channels and creeks fringed with luxuriant mangrove vegetation. Isolated species could also be seen in detached water bodies and reclaimed lands along the coastal tract (Sebastian et al., 2005).

Fig.2.12. Mangrove forest of Kovilakathumkadavu

2.5.4. Bolgatty

Bolgatty Island is a picturesque island located near Fort Kochi in Ernakulam district. It is famous for its eponymous palace along with being a growing cosmopolitan hotspot in the area.

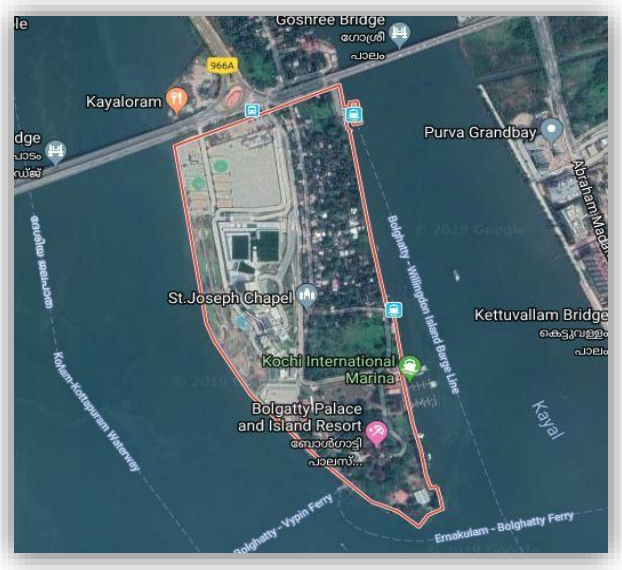

Figure.2.13. Bolgatty Island

2.5.5. Cherai

Cherai is the smallest town located in north side of Vypin Island. This town connects to Cochin, North paravur and kodungalloor. It is a region in Kochi Taluk, a suburb of the city of Kochi, in the state of Kerala, India. It is at a distance of about 25 km from the High Court Junction, Kochi. Cherai has the longest beach in Kochi - theCherai beach. The beach is located towards the centre-north of the Vypin Island.

Fig.2.14. Mangrove view at Cherai location

2.6. Polyketide synthase

Polyketides are large group of secondary metabolites that have notable variety in their structure and function (Moore, 2001). A large number of biologically active molecules are synthesized by polyketide synthase (PKS) pathways. Polyketides are structurally diverse secondary metabolites that have already found widespread application as pharmaceuticals (Ketela et al., 1999) in particular as antibiotics. Industrially important polyketides include rapamycin (immunosuppressant), erythromycin (antibiotic), lovostatin (anticholesterol drug), and epothilone B (anticancer drug). Polyketides, large group of secondary metabolites, are known possessing remarkable variety not only in their structure and but also in their function (Moore et al., 2001; Rokem et al., 2007). Polyketides exhibit a wide range of bioactivities such as antibacterial (e.g., tetracycline), antifungal (e.g. Amphotericin B), anticancer (e.g., doxorubicin), antiviral (e.g., balticolid), immune-suppressing (e.g., rapamycin), anti-cholesterol (e.g., lovastatin) and anti-inflammatory activity (e.g., flavonoids) (Funabashi et al., 2008). The majority of polyketides are made by micro-organisms (bacteria and fungi), so until now a very successful approach has been to search in the soil and in the oceans for undescribed and uncultured microbes, and then to isolate, identify and test the molecules housed inside them. As only a small percentage of soil bacteria have ever been grown in the laboratory and most marine micro-organisms remain wholly uncharacterized, this strategy should continue to reveal novel bioactive compounds for many years to come (Rouhi 2003).

In the late 1980s, inspired by the pioneering work of Professor Sir David Hopwood on the genetics of the biosynthesis of actinorhodin (Malpartida & Hopwood 1984), Peter Leadlay at the University of Cambridge and Leonard Katz, then at Abbott Laboratories in Chicago, began searching for the genes encoding for the assembly of erythromycin A in the soil bacterium *Saccharopolyspora erythraea*. In 1990, both groups published the sequences of the erythromycin genes (Cort´es et al. 1990; Tuan et al. 1990; Donadio et al. 1991), an advance that has already begun to transform polyketide drug development. Since their remarkable discovery, researchers have uncovered the genetic instructions for making over 50 additional polyketides. These sequences have come primarily from *Streptomyces* and related *Saccharopolyspora* species, but also from myxobacteria (e.g. Silakowski et al. 2001; Gerth et al. 2003). The sequencing of the erythromycin genes revealed that nature has adopted an assembly-line process for polyketide biosynthesis, in that the number of chemical steps required to make the molecule matches the complement of enzymatic domains in the pathway (Weissman, 2004). Biosynthesis of polyketides is very complex because the process involves multifunctional enzymes called Polyketide synthases (PKSs). The mechanism of PKS is similar to fatty acid synthase (FAS) which includes acyltransferase (AT) that has a role in catalyzing the attachment of the substrate(e.g., acetyl or malonyl) to the acyl carrier protein (ACP), ketosynthase (KS) which catalyzes condensation of substrates attached in ACP. For the subsequent steps, polyketide intermediate is processed by ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) as shown in Figure 2.15.

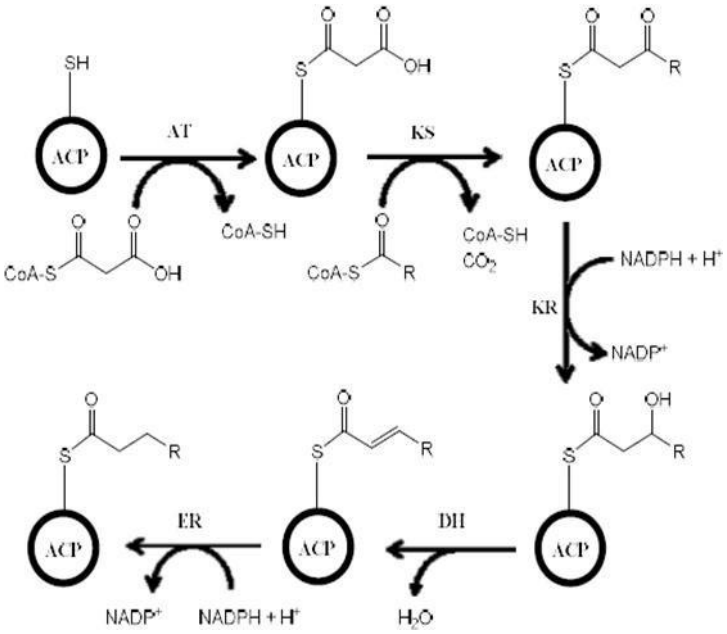

Fig.2.15. Scheme of reaction occurred in polyketide synthases (PKSs).

In Streptomyces, there are three types of PKSs (type I, type II, and type III).

2.6.1 Polyketide Synthases Type I

The type I polyketide synthases (type-I PKSs) involve huge multifunctional proteins that have many modules containing domains, in which a particular enzymatic reaction occur. Each module has responsibility to perform one condensation cycle in a non-iterative way. Because this system works with some modules, hence it is also called as modular PKS. The essential domains exist in each module are acyltransferase (AT), keto synthase (KS) and acyl carrier protein (ACP) that collaborates to produce β-keto ester intermediate. In addition, the other domains that may be present in the module are β-ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) which are responsible for keto group modification. In the process of producing polyketide, the expanding polyketide chain is transferred from one module to other module until the completed molecule is liberated from the last module by a special enzyme (Rokem et al., 2007; Staunton et al., 2001; Lal et al., 2000). Furthermore, type-I PKSs are responsible for producing macrocyclic polyketides (macrolides). Macrolide belongs to polyketide compound characterized by macrocyclic lactone ring containing between 12 and 16 atoms which has various bioactivities such as antibacterial, antifungal, immunosuppressant and anticancer. As an antibacterial agent, macrolide works by inhibiting protein synthesis by binding to the 50S ribosomal subunit and

blocking translocation steps of protein synthesis (Mazzei, 1993). Some examples of macrolides produced by *Streptomyces* are rapamycin, FK506, spiramycin, avermectin, methymycin, narbomycin and pikromycin (Schwecke et al., 1995). (Figure 2.16)

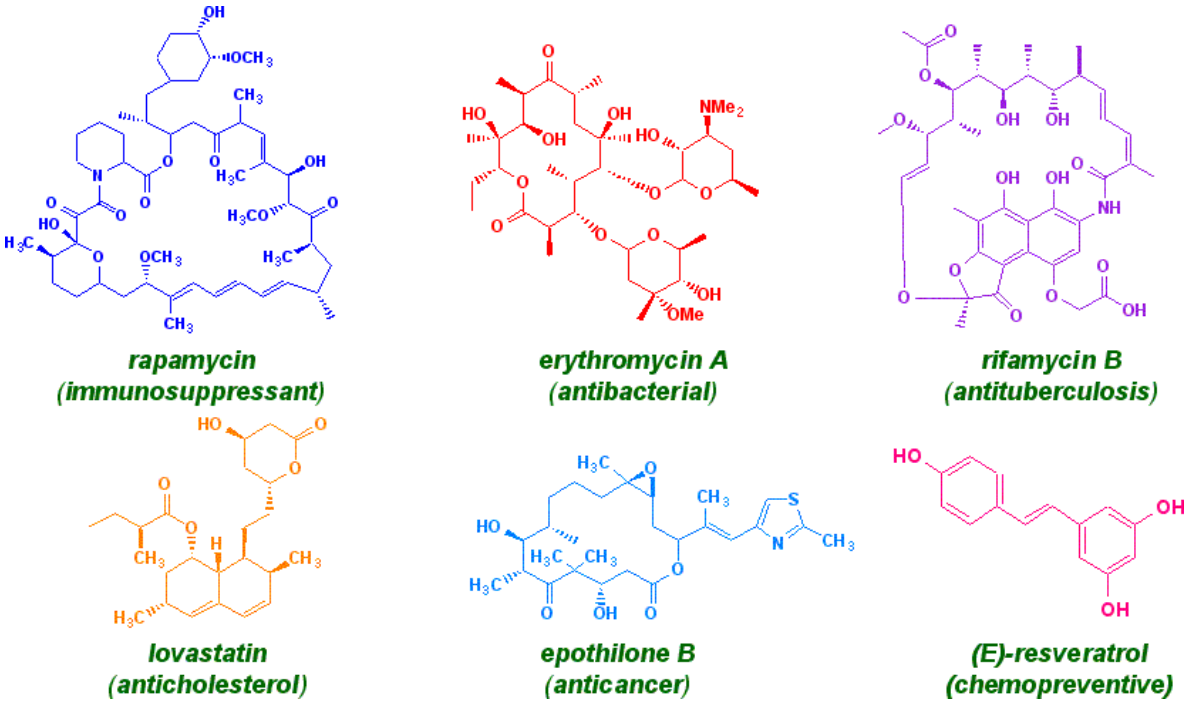

Fig.2.16. Some of macrolides produced by Streptomyces.

2.6.2 Polyketide Synthases Type II

The type II polyketide synthases (type-II PKSs) are responsible for producing aromatic polyketide. Based on the polyphenolic ring system and their biosynthetic pathways, the aromatic polyketides produced by type-II PKSs are classified into three groups, i.e. anthracyclines, angucyclines, aureolic acids, tetracyclines, tetracenomycins, pradimicin-type polyphenols, and benzoisochromanequinones. Some examples of aromatic polyketide produced by Streptomyces are actinorhodin, doxorubicin, jadomycin B, oxytetracycline, mithramycin, tetracenomycin C, and benastatin A (Hertweck et al., 2007). Unlike type-I PKSs that involve huge multifunctional proteins that have many modules containing domains and perform the enzymatic reaction in a non-iterative way, the type-II PKSs have monofunctional polypeptides and work iteratively to produce aromatic polyketide (Hertweck et al., 2007). However, like the type-I PKS, the type-II PKSs also comprise acyl carrier protein (ACP) that functions as an anchor for the nascent polyketide chain. In addition

to possessing ACP, the type-II PKSs also consist of two ketosynthases units (KSα and KSβ) that work cooperatively to produce poly-β-keto chain. KSα unit catalyze condensation of the precursors, on the other hand, the role of KSβ in the type-II PKSs is as a chain length-determining factor. The three major systems (ACP, KSα and KSβ) are called 'minimal PKS' that work iteratively to produce aromatic polyketide (Zhang et al., 2017). The other additional enzymes such as ketoreductases, cyclases and aromatases cooperate together to transform the poly-β-keto chain into the aromatic compound core. Furthermore, the post-tailoring process is conducted by oxygenases,glycosyl and methyl transferases (Chan et al., 2009).

2.6.3 Polyketide Synthases Type III

Unlike the type-I and type II PKSs, the type-III PKSs do not utilize ACP as an anchor for the production of polyketide metabolite (Shen et al., 2003). In this case, acyl-CoAs are used directly as substrates for generating polyketide compounds. In order to create polyketides, this system contains enzymes that construct homodimers and catalyzes many reactions such as priming, extension, and cyclization in the iterative way (Yu et al., 2012). With this fact, the type-III PKSs are the simplest structure among the other type PKSs (Nakano et al., 2009).

2.6.4. Importance of Type II Polyketides

Aromatic polyketides, which are synthesized by type II polyketide synthase (PKS), exhibit a wide array of biological activities including antibacterial, antitumor, antiviral and enzyme inhibitory activities and afford some of the most common antibiotics and anti-cancer drugs currently in clinical use, e.g. tetracyclines and anthracyclines. Anthracyclines rank among the most effective anticancer drugs ever developed (Weiss, 1992). The first anthracyclines were isolated in the 1950s and were named rhodomycin and cinerubicin. A few years' later daunomycin and doxorubicin were discovered (Brockmann et al., 1950). In analogy to type II bacterial and plant fatty acid synthases (FASs), type II PKSs are comprised of several individual enzymes (Hopewood, 1997).

In spite of its importance, type II polyketide biosynthesis has been rarely concerned. In the future, continuous efforts are still needed to characterize the assembly machinery and catalytic logic of aromatic polyketides at the genetic, structural, and biochemical levels. With the development of gene sequencing technology, combined with optimized heterologous expression systems, cDNA will be an essential source of novel aromatic polyketides. On the other hand, biosynthetic engineering mediated by gene combination from different pathways or activation of the cryptic gene clusters will continue to play an important role in developing new antibiotics with improved activities as well (Zhuan Zhang et al., 2017).

3. SCOPE OF INVESTIGATION

It is indisputable that new drugs, notably antibiotics, are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life threatening infections and risk undermining the viability of healthcare systems. Searching for novel and potent chemical entities is continuously the goal of natural product research. Natural products of microorganisms are potential source of bioactives that have been extensively exploited to develop next generation anti- infective drugs proposed by pharmaceutical companies.

Filamentous bacteria belonging to the order Actinomycetes, especially *Micromonospora* and *Streptomyces* strains, have a unique and proven capacity to produce novel antibiotics hence the continued interest in screening such organisms for new bioactive metabolites. Screening and isolation of potent actinobacteria for novel antimicrobial secondary metabolites is achievement in recent years.

It is also becoming increasingly clear that un- and under-explored habitats are a rich source of novel actinobacteria which have the capacity to produce interesting new bioactive compounds. The exploration of marine actinobacteria received greater attention due to their complex biosynthetic pathways and potential implications on the development of anti-cancer agents and anti-infective to combat multi-resistant strains. The mangrove ecosystem has made its way into the researchers' view because it is nutritionally versatile and is a largely unexplored source for actinobacteria for bioactive metabolites. A little is known about the actinobacterial diversity of mangrove sediments of Kochi, which could be an endless resource for novel antibiotics that has not been properly exploited.

Traditionally, the activity-based screening method has been a useful method to identify new natural products. However, this method has increasingly encountered problems from sources that are not readily accessible. In recent years, a large number of biosynthetic gene clusters have been cloned. Consequently, new screening methods are emerging in natural product research through exploring the common biosynthesislogic of many important classes of natural products.

Polyketide synthases (PKSs) are modular proteins involved in the biosynthesis of complex bioactive molecules diverse structural complexities. The tailoring of

catalytic domains and AA sequence of these domains are drastically changes with natural bioresources and therefore, the nature and chemical structure of end product is varied between/within the species. The genes encoding PKS are usually clustered with their auxiliary and regulatory elements on the genome, and their products are classified into types I, II, and III depending on their domain organization. Bacterial aromatic polyketides are a pharmacologically important group of natural products synthesized by type II Polyketide synthases (type II PKSs). Isolation of novel aromatic polyketides from microbial sources is currently impeded by molecular screening based on type II PKS.

With this view, the present study "Molecular screening of mangrove derived actinobacteria along the coastal belt of Cochin as a source of aromatic polyketides" with the following objectives:

1. Isolation and purification of actinobacteria from the mangrove growing areas of Cochin

Sediments from five different mangrove growing areas were collected and subjected to pour plate method. The actinobacteria colonies were picked and purified

2. Evaluation of the antimicrobial activity of the various isolates

The antibacterial activity of the different actinobacteria isolated from the mangrove sediments were evaluated qualitatively by cross streak method and quantitatively by well diffusion assay of the crude organic extract against potenthuman pathogens.

3. Molecular screening for type II polyketides

The isolates which showed efficient antibacterial activities were further screened or their ability to synthesize aromatic polyketides by molecular screening for type II Polyketide synthases.

4. Molecular characterization of the positive isolate

The positive isolate for the presence of type II polyketides were further characterized for their taxonomic position by analyzing their 16s rRNA sequence

4. MATERIALS AND METHODS

4.1 Study Area:

Five different sites of Mangroves growing areas were identified along the coast ofCochin.

Fig.4.1. Mangrove Ecosystem of Cochin

Kerala lies towards the South - West coast of India, a segment barred by theWesterly Ghats It extends between the latitudes 8" 18' and 12' 48' north and longitudes71" 53' and 77" 24' east with an area of about 38863 sq. kms (Ganeadharan, 1999).The area under wetland in Kerala estimated using satellite remote sensing data is127930.07 ha, out of which inland wetlands cover 31199 57 ha (Anonymous, 2000~). After conducting a detailed survey about Cochin, five mangrove growing places were identified in which fisheries and aquaculture are more prevalent. Each station was varying in their environmental conditions.

4.1.1. Station 1- Vallarpadam

The first station was selected for mangrove soil collection from Vallarpadam. It is one among the group of islands in Cochin. Mangroves of this area protect the coastline from natural calamities like tsunami etc. They stabilize coastline, promote coastal accretion and serve as natural barriers against torrential storms.

4.1.2. Station 2- Panambukadu

The second station was selected for mangrove soil collection is from panambukadu. It is a Village in Vypeen Block in Ernakulam District of Kerala State, India. It belongs to Central Kerala Division. This island is surrounded by the estuarine waters of Cochin backwater. Except for the human dwellings, the island is occupied by traditional prawn cum fish farms fringed by mangroves.

4.1.3. Station 3- Kovilakathumkadavu

The third station was selected for mangrove soil collection is from kovilakathumkadavu. Kovilakathumkadavu, which is situated towards the northern end of Vypin Island near Munambam. This place is thickly populated area and mangrove ecosystem of this region is subject to population pressure.

4.1.4. Station 4- Bolgatty

The fourth station was selected for mangrove soil collection is from Bolgatty Island. It is one of the islands that form part of the city of Kochi, Kerala, India. Vypin Island and Vallarpadam Island lie on its west side. Patches of mangroves are growing here and there. Highly restricted occurrence of the mangroves of this area is directly attributed to the gross interference of man's activities.

4.1.5. Station 5- Cherai

The fifth station was selected for mangrove soil collection is from smallest town located in north side of Vypin Island. Cherai has the longest beach in Cochin. Patches of mangrove species are growing along both banks of field which is use for traditional prawn cum fish farms fringed by mangroves.

4.2. Sample Collection

Soil samples were collected from the 5 specified regions for the isolation of actinobacteria. The samples were collected in the month of October from top of mud or soil profile, where most of the microbial activity takes place. Soil samples were collected by using clean, dry and sterile polythene bags. The site selection was done by taking care of the point where widely varying characteristics such as, the organic matter, moisture content, particle size and color of soil, are possible so as to avoid contamination as far as possible. Five soil samples were collected different areas.

Samples were stored in ice boxes and transported to the laboratory where they werekept in a refrigerator at 4°C until analysis.

4.3. Isolation of actinobacteria from soil samples

4.3.1. Media and culture conditions

Actinomycetes Isolation Agar (AIA): [Gms / Litre: Sodium caseinate 2, L-Asparagine 0.1, Sodium propionate 4, Dipotassium phosphate 0.5, Magnesium
sulphate 0.1, Ferrous sulphate 0.001, Agar 15 and Final pH (at 25°C) 8.1±0.2(Himedia, Mumbai) is used for isolation and propagation of Actinobacteria.

4.3.2. Isolation of Actinobacteria by pour plate method

The samples were subjected to serial dilution up to the 10^{-3} dilution and 1 ml of each dilution was inoculated in duplicate plates of the AIA media. After incubation, all plates incubated at 37^{o}C in the incubator for 7 days. The isolation of actinobacteria is done by serial dilution followed by pour plate method (Barker, 1998). After incubation, plates were examined for the appearance of actinobacteria colonies. Total number of colonies in each set of plates was scored and recorded.

4.3.3 Purification of Actinobacteria

The typical actinobacteria colonies contaminated by other microorganisms were purified by quadrant streak plate method until pure cultures were obtained. The actinobacteria colonies were picked up with a sterile loop inside laminar flow and were streaked on to the Kenknight & Munaier's agar and further incubated. Kenknight and Munaiers medium is used for isolating actinobacteria species from soil samples (Subba, 2015).The colonies were subcultured and maintained on Kenknight & Munaier's agar for further studies.

4.4. Colony characterization of the isolates

Pure actinobacteria isolates were spot inoculated on Actinomycetes Isolation Agar medium (AIA). The plates were incubated at 37°C for 6 days and results were observed. Morphological characters and pigmentation of isolates was studied on AIA.

4.5. Screening of actinobacteria for antibacterial Activity

The test bacterial pathogens included in antibacterial activity namely *Escherichia coli, Klebsiella sp. Staphylococcus aureus* and *Proteus sp*. were used as target organisms.

4.5.1. Qualitative screening of Actinobacteria

The antibacterial activity of the pure isolates of actinobacteria was tested preliminarily by cross streak method (Katz, 2008). Actinobacteria isolate were streaked across the diameter on Luria Bertani agar plates. After incubation at 37ºC for 3 days, overnight cultures of *Escherichia coli, Klebsiella sp. S. aureus* and *Proteus sp*. were streaked perpendicularly to the central strip of actinobacteria culture including control plate without actinobacteria. All plates were again incubated at 37ºC for 24 hours and the results were observed.

4.5.2. Quantitative screening of Actinobacteria

Secondary screening of actinobacteria was performed by agar well diffusion method. Agar well diffusion method is widely used to evaluate the antibacterial activity of plants or microbial extracts (Magaldi et al., 2004).

4.5.2.1. Mass production and extraction of antibiotic

The 22 isolates were incubated in Kenknight & Munaier's broth for 7 days. After 7 days, centrifugation was done at 10,000 rpm for 10 min. The supernatant were taken and equal volumes of ethyl acetate were added to each isolates. The extracts were evaporated to dryness and the crude extracts were stored in a refrigerator at 4°Cfor future use (Chessbrough, 2000).

4.5.2.2. Antibacterial activity of crude extracts using disc method

The test bacterial strains (100 µL) were inoculated on Luria Bertani agar plates by pour plate method. The wells are made using the well cutter and 50µL of crude ethyl acetate extract was added to each wells and incubated for 24hrs at 30°C. After incubation, the zone of inhibition (ZOI) was measured and expressed as centimeter (Mohanraj et al., 2011). Bioactivity was determined by subtracting the ZOI of control from the ZOI of test (Shantikumar, 2006). The best 10 isolates were selected on the basis of high antibacterial activity and molecular screening was done for Polyketide synthase type II gene.

4.6. Molecular screening of type II Polyketide synthase gene

4.6.1. Isolation of Microbial DNA

1.5 ml of overnight culture of purified actinobacteria samples were taken to Eppendorf tube using cut tips and centrifuged at 4000rpm for 3min. The supernatant were discarded and pellet were dissolved in 467 µL TE Buffer, 30 µL of 10% SDS and 2 µL of proteinase K. It was incubated at 37ºC for one hour. Then, equal volume of chloroform isoamyl alcohol was added and mixed well and centrifuged at 4000rpm for 2min and the supernatant was collected. It was repeated thrice. 0.6^{th} volume of isopropanol and $1/10^{th}$ volume of 5 M sodium acetate as added and incubated in freezer. Then, it was centrifuged at 10,000 rpm for 10 min. To the pellet 1 ml of 70 % ethanol was added and centrifuged at 10,000 rpm for 10 min. The pellet was air driedand dissolved in minimum volume of autoclaved water (Sambrook et al., 1989).

4.6.2. Purification and Quantification of DNA

The isolated crude DNA was made up to 200 µL and 20 µL of RNAase A (1mg/ml) was added and it was then incubated for one hour at 37ºC. 3.7 µl of proteinase K (20 mg/ml) was added and incubated for 30 min at 37ºC. Equal volume of phenol chloroform was added, inverted and centrifuged at 12,000 rpm for 5 min. The top aqueous layer was collected, 3M sodium acetate (pH 5.8) of $1/10^{th}$ volume and 2.5 volume of absolute ethanol was added to it and incubated at -20ºC for 30 min. It was then centrifuged at 12000 rpm for 5 min and air dried. The pellet was dissolved in 20 µL of autoclaved water (Ogram, 1987). The quantification is done by using a spectrophotometer which is able to determine the average concentrations of the nucleic acids DNA or RNA present in a mixture, as well as their purity (Haque, 2003). The reading was taken at 260 nm for the calculation of the concentration of nucleic acid in the sample and absorbance was measured against the blank.

Agarose Gel Electrophoresis was done and the gel was visualized under UV transilluminator (Infinity capture software, Vilbert Lourmat, Cedex, France).

4.6.3. Primer Designing

Certain parameters were considered for the designing of type II polyketide synthase gene. The optimal length of primer is 20 bp, GC content is 60 % and Tm is 72 %. Based on these parameters, specific primers for type II polyketide synthase gene was designed using the software, FAST PCR based on the conserved regions of

Ketosynthase α domain of type II PKS from seven aligned sequence retrieved from Genbank. Table 4.1. represents the accession numbers of sequences used for designing primers

Table 4.1. Accession numbers used for primer designing for Type II PKS

SL NO.	Accession no.
1	KX707878.1
2	KX708205.1
3	KX708435.1
4	KX707909.1
5	KX708288.1
6	FR845719.1
7	CP003720.1

4.6.4. Amplification of Polyketide synthase gene type II (PKS)

The PCR conditions were optimized for the amplification of PKS gene using the positive strain for type II PKS, *Amycolaptis rifamycina* with the designed primers (Table 4.2). PCR reactions were performed in a 20 µl reaction mixture (Table 4.3.) in thermal cycler (Eppendorf, Germany). The PCR was performed under the following cycling program: initial denaturation for 10 min at 94°C, 30 cycles of denaturation for 30 s at 94°C, annealing for 60s at 54°C, extension for 90 s at 72°C and final extension for 1 min at 72°C. The amplified product was visualized by Agarose gel electrophoresis under UV transilluminator.

Table.4.2. primers and sequences

Primer name	Sequence(5'-3')	Expected product size (bp)
KSN1F	TGCTTCGACGCCATGAAGGC	500
KSN1R	ACGTAGTCCAGGTCGCACTC	

Table 4.3. PCR reaction of PKS type II gene

PCR Reaction mixture PCR mixture	Volume (µl)
10X PCR buffer	2 µl
10mM dNTPS	1 µl
10Pm forward primer (KSN1F)	2 µl
10Pm reverse primer (KSN1R)	2 µl
5U/µl Taq polymerase	0.5 µl
Template DNA	3 µl
25mM Mgcl2	1.2 µl
Autoclaved water	8.3 µl
Final volume	20 µl

4.7. Characterization of positive Isolate

4.7.1. Amplification of 16s rRNA sequence

The positive isolate showing amplification for Type II PKS characterization of 16S rRNA gene, PCR amplification was done with two universal primers 8F and 1429R (Table 4.4). The reaction and cycling condition was same as that for the amplification of PKS II except for the annealing temperature, 60°C. After amplification, PCR product was visualized in 1.2 % agarose gel electrophoresis.

Table 4.4. Primers and sequences

Primer name	Sequence(5'-3')	Expected product size (bp)
8F	AGAGTTTGATCCTGGCTCAG	1000
1429R	CGGCTACCTTGTTACGACTT	

4.7.2. Gel elution of PCR product

The PCR amplified product for 16s r RNA gene was eluted out from the gel using Gel DNA Elution kit (Genei, Bangalore) following manufacture's protocol. The amplified product was run on 1.5 % agarose gel to separate the DNA band was excised from the gel and was transferred to a pre weighed micro centrifuge tube. The net weight of the gel was calculated and 2.5 times volumes of sodium iodide were added to it. For solubilizing the gel it was incubated at 55ºC for 10 min. 15 µl of glass solution silica was added to it mixed well and incubated at room temperature with

occasional mixing. The sample was then centrifuged at 12000 rpm for 30seconds and the supernatant was discarded. To the pellet obtained 200µl of wash buffer was added mixed well and centrifuged at 12000 rpm for 30s. This step was repeated twice and the pellet was kept at 55°C for 10min to remove traces of wash buffer. The pellet was resuspended in 35µL of autoclaved water and incubated at 55°C for 5 min. It was then centrifuged at 12000rpm for 30seconds and supernatant was collected in another fresh tube. To obtain the second elute 15µl of autoclaved water was added to the pellet and resuspended. This was incubated at 55°C for 5min. It was centrifuged at 12000 rpm for 30s and the supernatant was collected in a fresh tube. The eluted product was checked in 1.5 % agarose gel and stored at -20°C. The gel was visualized under UV transilluminator (Infinity capture software, Vilbert Lourmat, Cedex, France). The eluted product was observed as bright band.

4.8. Sequencing and sequencing analysis

4.8.1. Sequencing Analysis

The eluted product was sent for automated sequencing (Scigenome, Cochin). Sequence data were compiled with the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and examined for sequence homology with the archived 16S rDNA sequences from GenBank at http:// www.ncbi.nlm.nih.gov/nucleotide, employing the BLAST search program.

4.8.2. Construction of Phylogenetic Tree

Multiple sequences homologous to the query sequence obtained from GenBank were aligned with CLUSTAL W. Phylogenetic analyses were performed according to the neighbor joining (NJ) methods using free online tool https://www.genome.jp/tools-bin/clustalw

5. RESULTS AND DISCUSSION

The search for novel antibiotics has gained urgency because of increased occurrence of multi-drug resistant human pathogens (Witte, 1999). Marine actinobacteria have been recognized as an important and untapped resource for novel bioactive compounds. (Donia and Haman 2003; Anand et al., 2006). Indian mangroves have a rich diversity of soil-dwelling organisms which include micro, meio and macro forms (Goyal, 2009). They are regarded as highly productive ecosystems and abode to unexplored microbial diversity including actinobacteria (Hong et al., 2009).

The results emanated from the present study in identifying efficient antibiotic producer from the mangrove sediments of Kochi are summarized below.

5.1 Collection of mangrove sediment samples and isolation of actinobacteria

Soil sediments were collected from five different mangrove growing areas of Vallarpadam Island, Panambukadu Island, Kovilakathumkadavu, Bolgatty and Cherai situated in Ernakulam, Kerala. Fig. 5.1 depicts the various sediment samples collected. The characteristics of sediment samples are presented in Table 5.1. The sediment samples were then subjected to pour plate method and the suspected colonies of actinobacteria were picked and purified by streaking. Actinobacteria manifest good growth in actinobacteria isolation agar (AIA) medium. All five soil samples showed actinobacteria growth within 6-8 days after pour plate method. In this study, a total of 22 different actinobacteria from Vallarpadam (6 isolates), Panambukadu (5 isolates), Kovilakathumkadavu (4 isolates), Bolgatty (2 isolates) and Cherai (5 isolates) were obtained. Rao et al., (2012) reports a similar study based on activity of actinobacteria from mangrove soil from local area Visakhapatnam which led to the isolation of 30 actinobacteria isolates.

5.2. Colony characterization of actinobacteria

The characteristics of the colony of the 22 different isolates were studied by spot inoculating on AIA medium. Different isolates showed varied colony morphology, texture and pigmentation which are depicted in (Fig.5.2). Table 5.2 represents the colonycharacterization of the selected isolates.

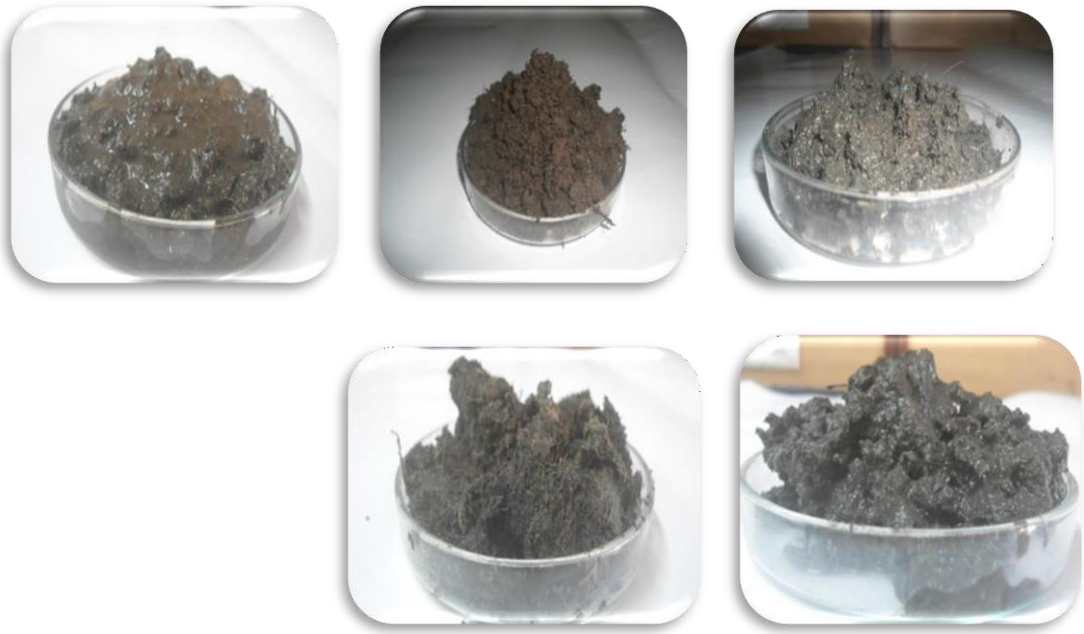

Fig. 5.1. The various sediment samples of Cochin from A. Vallarpadam B. Panambukadu C. Kovilakathumkadavu D. Bolgatty E. Cherai

Table 5.1. Characteristics of sediment samples

Soil Samples	Place	Characteristics	No. of isolates
S1	VALLARPADAM	loamy in nature	6
S2	PANAMBUKADU	black dry soil in nature	5
S3	KOVILAKATHUMKADAVU	water logged mud	4
S4	BOLGATTY	vertisol in nature	2
S5	CHERAI	water logged mud	5

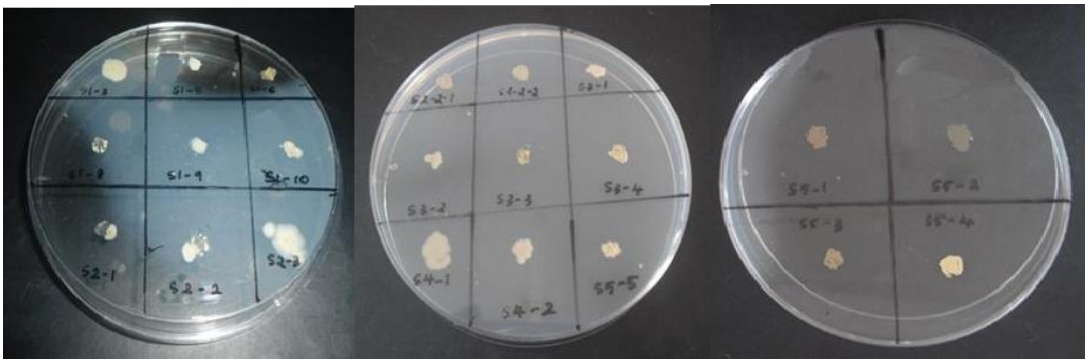

Fig. 5.2. Colony morphology of 22 Actinobacteria isolates

Table 5.2. Colony characterization of 22 actinobacteria isolates

Serial no.	Actinobacteria isolates	Colony characteristics
Sample 1	S1-1	Yellowish white with chalky texture
	S1-2	White with Powdery nature
	S1-3	Pale yellow with Butyrous nature
	S1-4	Yellow colour which is embedded into the agar except the rough surface
	S1-5	Whitish yellow with Powdery nature
	S1-6	Pale yellow with Butyrous nature
Sample 2	S2-1	Pale yellow with Powdery nature
	S2-3	White and Puffy in nature
	S2-4	Tan with chalky texture
	S2-5	Yellow brown with chalky texture
Sample 3	S3-1	Tan colour with leathery appearance
	S3-2	Light brown with leathery appearance
	S3-3	Pale yellow with Powdery nature
	S3-4	White with Powdery nature
Sample 4	S4-1	Yellow with Butyrous nature
	S4-2	Ceramic yellow with Leathery nature
Sample 5	S5-1	Coffee brown with Powdery nature
	S5-2	Brown with Powdery nature
	S5-3	Light brown with Chalky texture
	S5-4	Pale yellow with Butyrous nature
	S5-5	Sandy brown with Leathery nature

5.3. Anti-bacterial screening of Actinobacteria against pathogenic bacteria

The antibacterial effect of each of the isolated actinobacteria were tested against four test organisms which were reported to be pathogenic namely *Escherichia coli, Klebsiella sp. Staphylococcus aureus* and *Proteus sp*. The assay was done by two approaches: qualitative and quantitative screening.

5.3.1 Qualitative screening

It has been well established that most actinobacteria exhibit antibacterial activity. The actinobacteria were initially screened to determine their ability to produce antibacterial compounds by the cross streak method (Egorov, 1985). In the initial screening, the active isolates exhibited different inhibitory patterns against the test organisms. *E. coli* and *Proteus sp.* were inhibited by 15 isolates (68%) showing good activity, 7 isolates (31%) showed moderate activity against all test organisms, 5 isolates (22%) showed weak activity against *Klebsiella sp., S. aureus* and *Proteus sp.,* and 2 (9%) showed no antagonistic activity against test organisms (Table 5.3). Lertcanawanichakul & Sawangnop, (2011) claim that the cross streak method was suitable for a preliminary assessment of the antagonistic effects of microbial species. Chaudhary et al., (2013) reports the antagonistic activity of 31 actinobacteria isolates against 12 pathogenic microorganisms.

5.3.2. Quantitative Screening

The 22 isolates were cultured in Kenknight & Munaier's medium for a period of 7 days. 10 ml of broth of each isolate were later extracted with ethyl acetate and evaporated to get the crude extract. The concentrated crude extract was used to study their inhibitory role. Agar well diffusion method was used for quantitative screening by calculating the zone of inhibition (ZOI) presented by crude ethyl acetate extract of 22 different isolates against the pathogens with ethyl acetate as control. The results are summarized in Table 5.3. It was shown that all actinobacteria isolates were found to inhibit at least one of the four pathogenic strains tested. Among the strains tested, S1-1, S2-2, S2-3, S5-2, S1-2, S1-4 showed promising antibacterial activity against the tested pathogens followed by (Table 5.3). The zone of inhibition is maximum in case of the pathogen *E.coli* and *S. aureus* while the minimum against *Klebsiella sp.* This indicates that the potential of these isolates against *S. aureus* can lead to the further investigation towards multi- drug resistant *Staphylococcus aureus* (MRSA) and other strains. Tara et

al. (2009) reported similar antibacterial activity of *Nocardia alba* from mangrove soil against *E.coli, Proteus mirabils, P. vulgaris, K. pneumonia, K. oxytoca, Staphylococcus aureus, Bacillus subtilis, Shigella species, Salmonella typhi* and *Salmonella paratyphi A.* Based on this assay 10 isolates S1-1, S1-2, S1-3, S1-4, S1-5, S2-2, S2-3, S2-5, S3-2, S5- 2 have been selected which showed high antibacterial effect against the test organisms for molecular screening.

Table 5.3. Qualitative screening of actinobacteria isolates

SERIAL.NO	CULTURE NO.	*E.coli*	*Klebsiella sp.*	*S. aureus*	*Proteus sp.*
1	S1-1	+++	+	+++	++
2	S1-2	++	+	-	++
3	S1-3	++	-	+++	++
4	S1-4	+	+	-	+
5	S1-5	++	-	+	+++
6	S1-6	+	-	+	++
7	S2-1	+	-	+	-
8	S2-2	+++	-	-	+++
9	S2-3	++	+	-	+++
10	S2-4	-	-	+	+
11	S2-5	-	-	-	-
12	S3-1	-	-	+	-
13	S3-2	-	-	+	-
14	S3-3	-	-	+	-
15	S3-4	-	-	+	+
16	S4-1	-	+	+	+
17	S4-2	-	+	-	-
18	S5-1	-	-	-	-
19	S5-2	++	-	++	-
20	S5-3	+	-	-	-
21	S5-4	-	-	+	-
22	S5-5	-	+	-	++

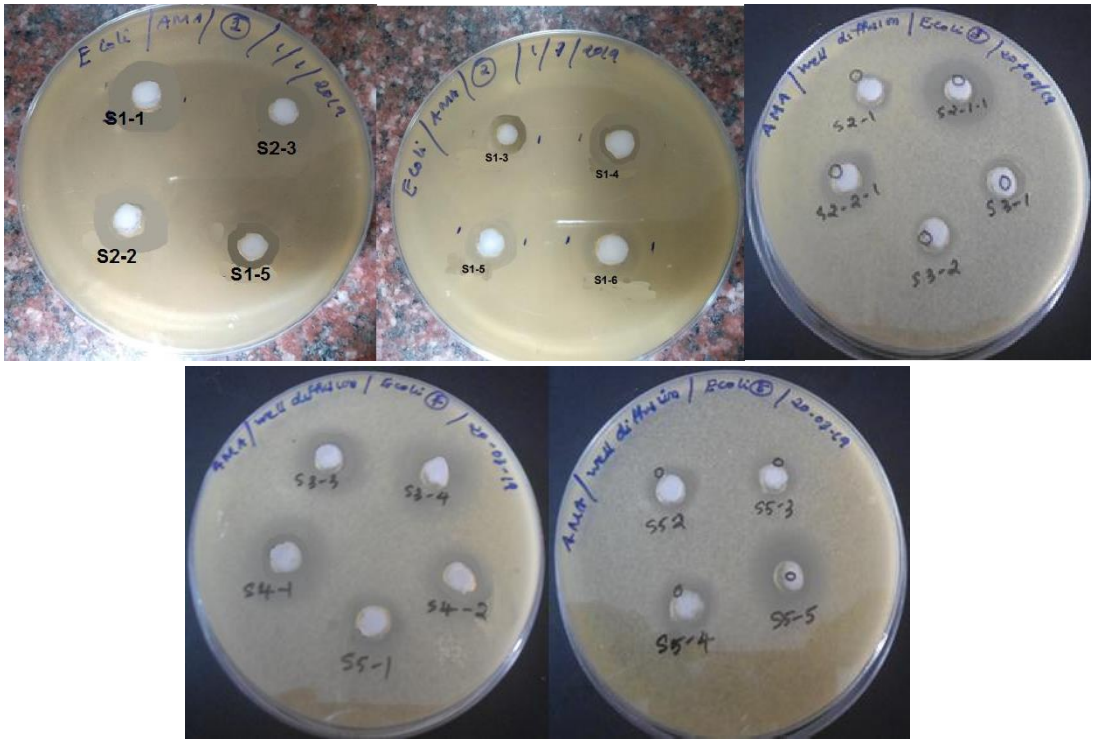

Fig. 5.3.1. Anti-microbial activity of 22 isolates against *E.coli*

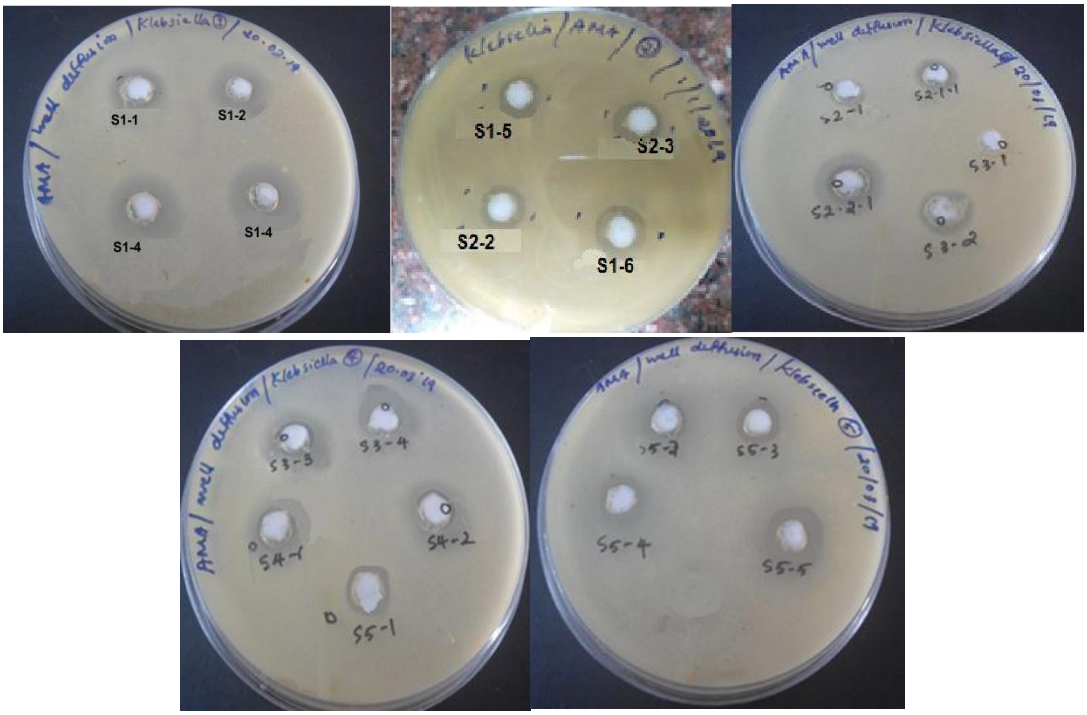

Fig. 5.3.2. Anti-microbial activity of 22 isolates against *Klebsielia sp.*

Fig. 5.3.3. Anti-microbial activity of 22 isolates against *Proteus sp.*

Fig. 5.3.4. Anti-microbial activity of 22 isolates against *Staphylococcus aureus*

Table 5.3. Well diffusion of different actinobacteria isolates against bacterialstrains

Serial no.	Culture no.	*E.coli*	*Klebsiella sp.*	*S. aureus*	*Proteus sp.*
		Zone of inhibition	Zone of inhibition	Zone of inhibition	Zone of inhibition
1	S1-1	**1.8**	1.2	1.25	**1.45**
2	S1-2	**1.3**	**1.6**	0.75	0
3	S1-3	**1.6**	**1.3**	1.45	**1.65**
4	S1-4	**2.1**	**1.3**	0.15	**1.35**
5	S1-5	**1.5**	**1.5**	0.55	**1.65**
6	S1-6	**2**	**1.8**	0.25	**1.95**
7	S2-1	1	0.4	1.05	0.45
8	S2-2	1.1	**1.7**	0.95	0.75
9	S2-3	**1.5**	**1.4**	0.95	0.85
10	S2-4	1	0.7	1.05	1.075
11	S2-5	1	1	0.85	**1.275**
12	S3-1	0.9	0	0.45	0.55
13	S3-2	0.4	0.9	0.95	**1.35**
14	S3-3	0.7	1.1	0.5	**1.375**
15	S3-4	0.5	0.6	1.45	1.175
16	S4-1	0.8	1.1	0.85	1.175
17	S4-2	0.9	0.7	0.55	0.95
18	S5-1	0.8	0.4	0	1.15
19	S5-2	1.2	0.6	0.95	**1.375**
20	S5-3	0.6	0.4	0.95	0.45
21	S5-4	1.1	0.5	0.65	0.85
22	S5-5	0.8	1.1	0.95	0.45

5.4 Molecular screening for type II Polyketide synthase

A substantial fraction of the metabolites produced by *Streptomyces* and related actinobacteria consist of a Polyketide or a polypeptide scaffold. Most peptide-derived metabolites are synthesized by NRPSs (Marahiel, 1997), while two major routes exist for Polyketide synthesis in actinobacteria (Hopwood, 1997): one employs type I enzymes (PKS-I), also known as modular PKSs; and the other uses type II systems (PKS-II), mostly responsible for the synthesis of aromatic polyketides. Evidences show that only a few aromatic polyketide producers are being reported and that the complete realm of these microorganisms remains to be explored. Furthermore, studies on type II PKSs and their polyketides have been performed on a limited number of genomes. So, this study focuses on screening based on the actinobacteria type II. A group of 10 isolates were selected for molecular screening of type II PKS based on the *in vitro* antibacterial activityagainst the pathogenic test bacteria.

5.4.1 Preliminary evaluation of the PCR screening method

According to the conserved regions of ten representatives of ketosynthaseα domain of type II PKS, a pair of degenerate primers was designed. To test the specificity of the primer pair in PCR detection of polyketide synthase genes, a positive strain, *Amycolaptis rifamycina* for type II PKS was used. The PCR conditions were optimized and as expected, the PCR product consistent with the predicted size of about 550bp was obtained.

5.4.2. Detection of PKS II genes from selected actinobacteria

Genomic DNA of the 10 selected isolates were extracted and purified and found to be of good quality (Fig. 5.4). The primer set designed before (KSN1) was used for the subsequent PCR detection of the PKS II gene in the selected 10 isolates. Amplification products of the expected size (about 500 bp) were obtained only from one of the selected isolate (S1-1) (Fig. 5.5.) Metsä-Ketelä et al. (1999) reported an efficient approach to predict the genetic novelty in antibiotic production by molecular screening for minimal PKS gene. A 613 bp fragment internal to KSα gene of Streptomyces sp. JAJ13 was amplified using degenerative primers.

5.5. Amplification and sequencing of 16s rRNA gene

The isolate (S1-1) which gave amplification for type II PKS was identified by 16S rRNA gene sequencing using the primers 8F and 1492R isolates. Analysis of 16S rRNA sequences is one of the most powerful methods to determine higher taxonomic relationships of actinobacteria (Lee, 2012). Good band were observed in the gel showing amplified gene. The band size for primers 8F/1492R is approximately 1,200 bp by comparing with the 1 kb marker (Fig. 5.6).

The amplified 1,200 bp product was successfully eluted using Gel DNA Elution kit (Genei, Bangalore) (Fig. 5.7.). The eluted product along with the same primers was sent to Scigenome, Kochi for sequencing.

5.5.1. 16S rRNA gene analysis and species identification

The identification was based upon pair wise alignment and phylogenetic study with the already existing sequences in the database. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank database by using the multi sequence advanced BLAST comparison tool that is available in the

website of National Centre for Biotechnology Information. The study confirmed that the sequenced product is 16s rRNA gene. BLAST analysis showed that S1-1 belongs to genus *Streptomyces.* The highest 16S rRNA sequence similarity value of 97.22 % with was obtained to species of *Streptomyces violaceous*. Several previous reports from different geographical locations around the world have described the occurrences of Streptomyces in different mangrove habitats. In the Indian context, 150 *Streptomyces* strains were isolated from Muthupet mangroves in Tamil Nadu (Sathya and Ushadevi, 2014). Several *Streptomycetes*, viz., *Streptomyces alboniger*, *Streptomyces violaceus*, *Streptomyces moderatus* and *Streptomyces aureofasciculus* were also reported from the Vellar estuary on the southeast coast of India (Sivakumar et al., 2007).

Phylogenetic analysis of the 16srRNA gene sequence of S1-1 with the corresponding sequences of the close representative strains of *Streptomyces sp*. based on BLAST analysis from the GenBank database showed that this strain formed a distinct phyletic line with clade encompassed by *Streptomyces violaceous* (Fig. 5.8) . The taxonomic studies showed that S1-1 were highly potential to be assigned as novel species based on the phylogenetic and pairwise comparison of 16S rRNA gene sequences with the type strains. This demands the full 16s rRNA gene characterization of this isolate.

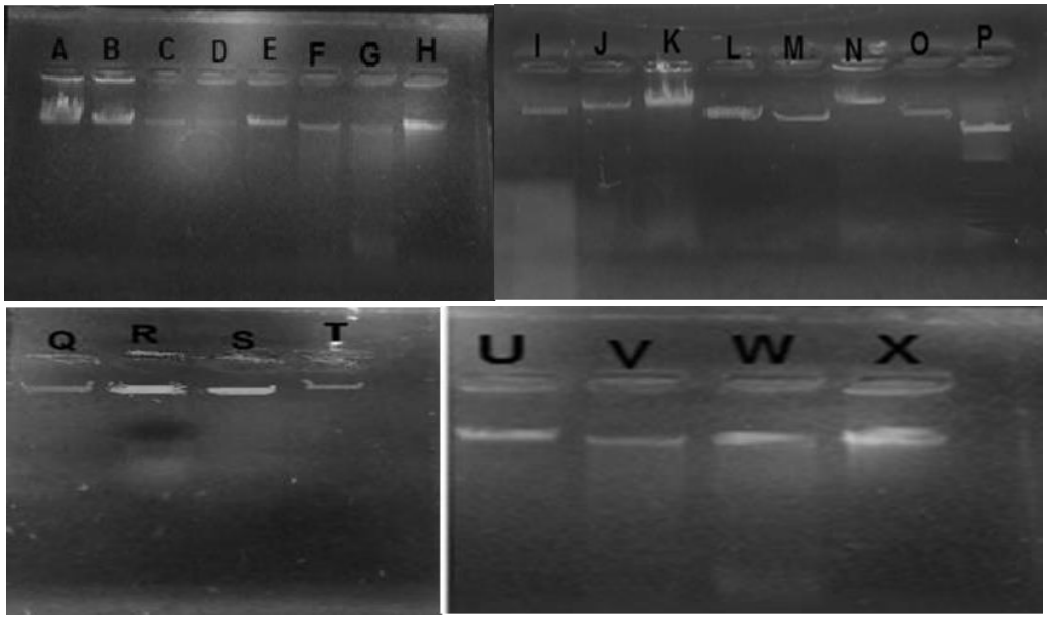

Fig. 5.4. Microbial DNA samples of 22 isolates from A to X

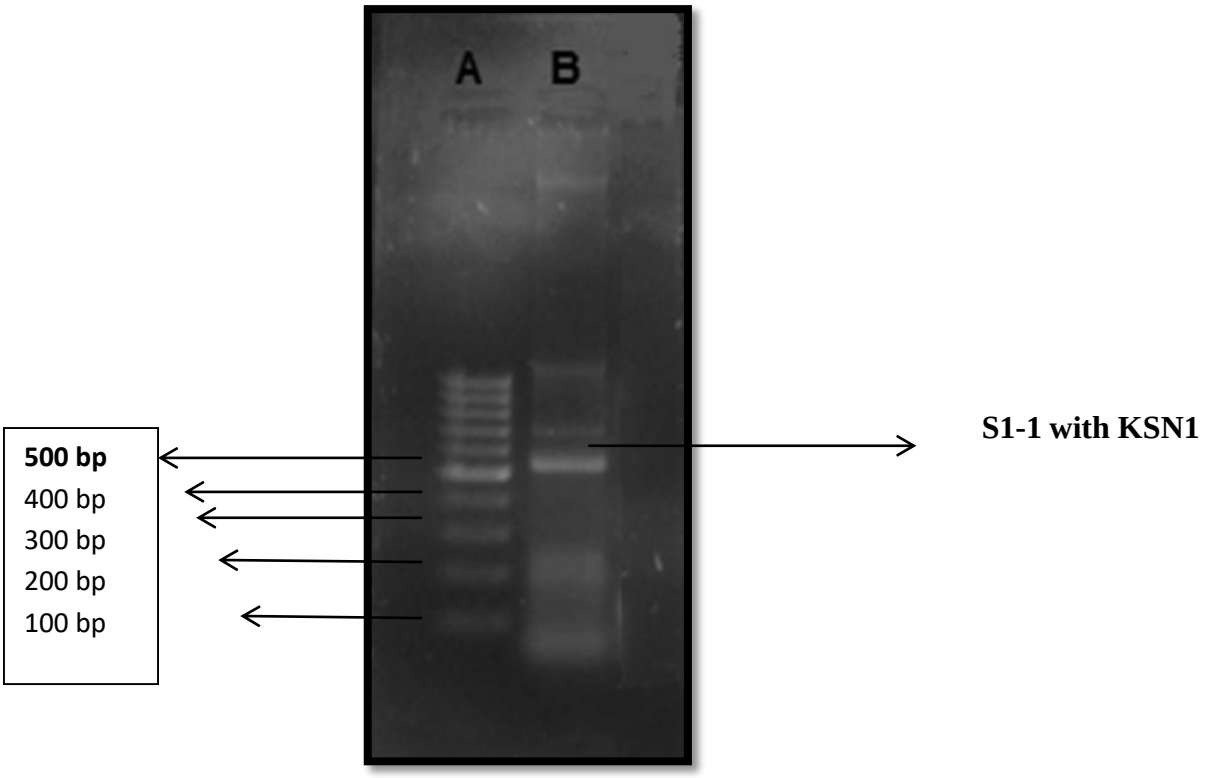

Fig. 5.5. Gel Documentation of S1-1 (Well 2) KSN1 Primer with 100 bp marker

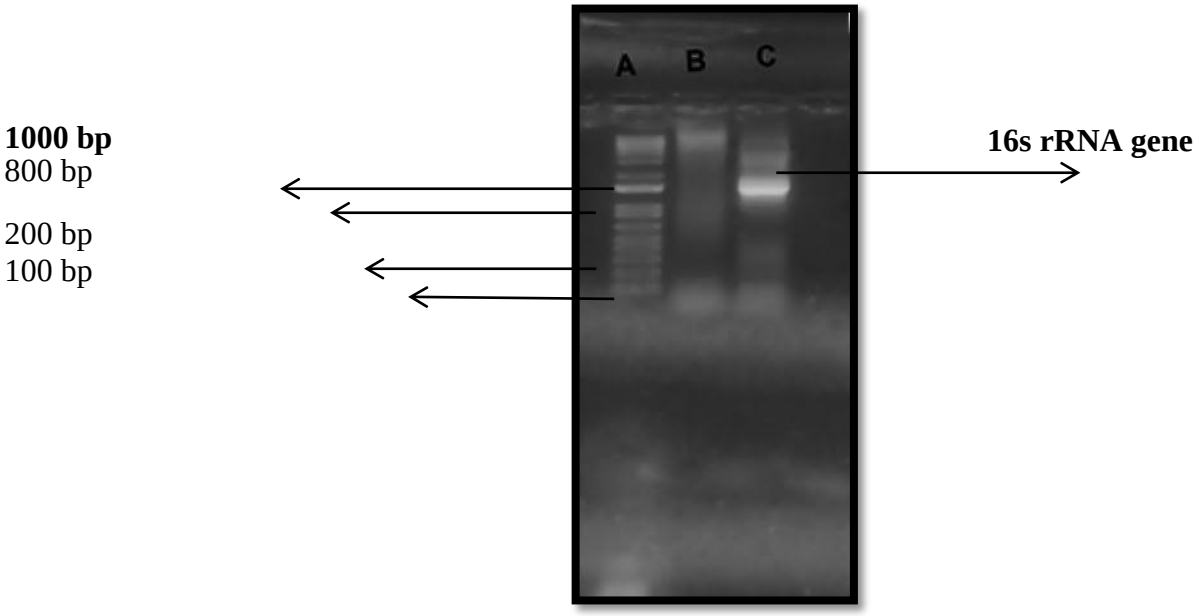

Fig. 5.6. Gel documentation of 16S rRNA gene

Eluted product

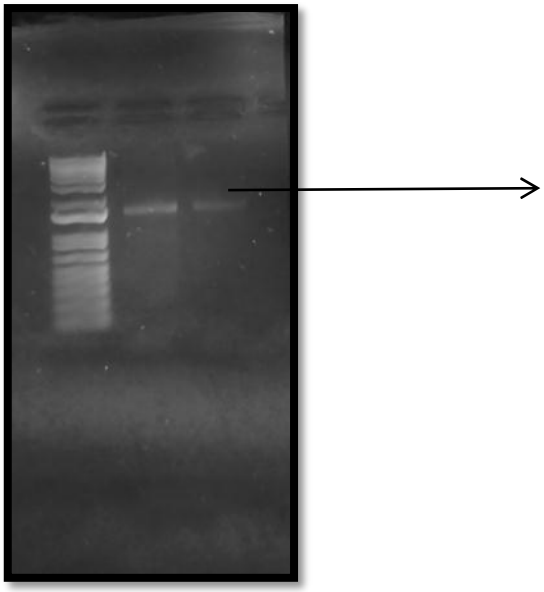

Fig. 5.7. Gel documentation of eluted product

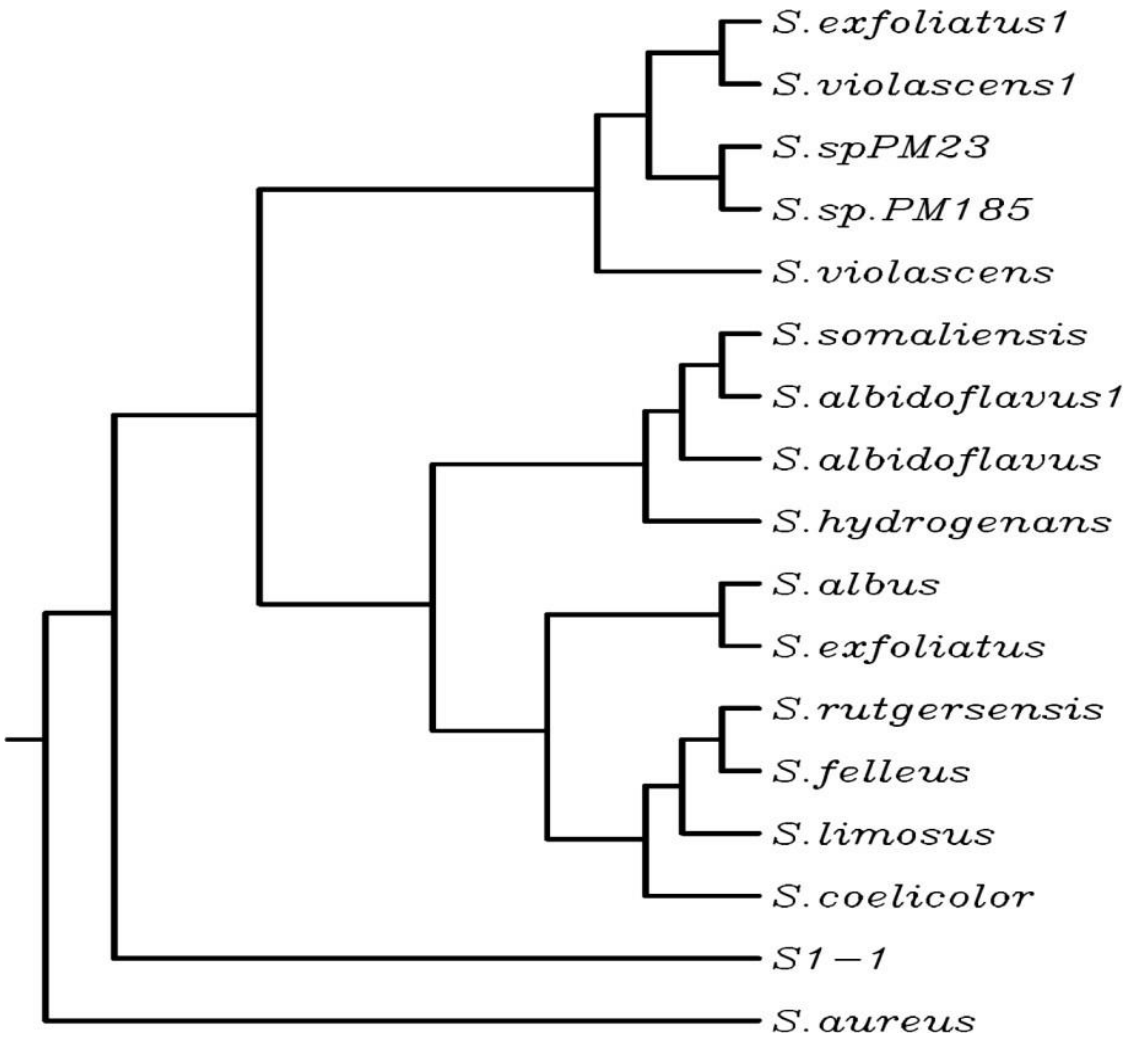

Fig. 5.8. Phylogenetic analysis of 16srRNA gene of S1-1 with representative isolates

6. CONCLUSION AND FUTURE PERSPECTIVES

New broad spectrum antimicrobial agents are urgently needed to combat frequently emerging multi drug resistant pathogens. Actinobacteria, the most talented group of microorganisms isolated from unexplored regions of the world may be the ultimate solution to this problem. Marine actinobacteria are known to produce many secondary metabolites and its diversity in Indian marine ecosystem is being explored. This study was undertaken with an aim of isolating antibiotic producing actinobacteria from unexplored ecosystem of the mangroves of Cochin and selecting the potent strains. In our research, it could be observed that the regular distribution of actinobacteria was different for different mangrove soil sites. The subsequent bioassays showed that the 22 different strains possess varied antibacterial activity against the various human pathogens screened. Our present study evidently revealed the antibacterial potential of actinobacteria isolated from mangroves of Cochin. Hence, further characterization of antibacterial compounds produced by these actinobacteria could solve the emerging problem of antibiotic resistance.

Wide spectrums of the characterized bioactive compounds were the products of PKS gene clusters that expressed co-ordinately. Moreover, the importance of type II PKS gene for the expression for aromatic polyketides having significant antibacterial activity in marine actinobacteria has been established. We found that PCR based pre-screening of type II PKS genes is one of the effective approaches for the detection of effective aromatic polyketide producer. Only one among the potent isolates (S1-1) gave positive result for aromatic polyketides. This forms rapid method for preliminary classification of unidentified bacterial strains on the basis of the genetic presence of type II PKS and from the pharmaceutical industry point of view, such classification is more useful than traditional activity based assay. Further, the functional gene analysis of type II PKS will give new insights of the novelty and applicability of the product. In addition, the amplified region can be used as a homologous hybridization probe in the cloning of new interesting type II PKS gene clusters. It was revealed from the characterization of partial sequence of 16s rRNA gene that the positive isolate belonged to the genus *Streptomyces*, though the species identity could not be established which emphasizes the need of full gene characterization for reporting its novelty.

The work could also be extended in exploring the collection of actinobacteria for other secondary metabolite biosynthetic pathways, type I PKS and NRPS for novel antibiotics. Further studies on the characterization of the isolates, purification of the antibiotic substance and study of its biological activities like antitumor, antiviral etc., and elucidation of its production pathways can expand the dimensions of the present research. The focus on these sediment derived actinobacteria could contribute new biodiversity and structurally unique bioactive compounds with unique mode of action to drug development pipelines and thus serve as a powerful tool in guiding future bioprospecting efforts. Actinobacteria thus identified seem to be a promising source of new and interesting natural products that will be further explored for their biotechnological applications.

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APPENDIX – I

i. National Technology Day-One day Seminar on 'Indigenous Technologies for the growth and development of Kerala' organized by SCMS Institute of Bioscience and Biotechnology Research & Development in collaboration with Kerala State Council for Science, Technology and Environment (KSCSTE), Cochin, Kerala on 12th May 2017.

ii. International Conference of SciCon Series on Current Trends in Biosciences (CTBIO)- 2017 organized by Scire Science, Cochin, Kerala during 21-23 August 2017.

iii. Two day workshop on 'An Insight into Analytical Instruments in Research' organized by Industry Institute Partnership Cell of the Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences &Technology, Thiruvananthapuram during 17-18 November2017.