ACTINOMYCETE ISOLATES FROM ARABIAN SEA AND BAY OF BENGAL: BIOCHEMICAL, MOLECULAR AND FUNCTIONAL CHARACTERIZATION

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To the Lord Almighty…

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Certificate

*This is to certify that the thesis entitled "**Actinomycete Isolates from Arabian Sea and Bay of Bengal: Biochemical, Molecular and Functional Characterization**" is an authentic record of the research work carried out by **Smt. Deepthi Augustine** under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements of the degree of **Doctor of Philosophy in Microbiology** of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

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Declaration

*I hereby declare that the thesis entitled "**Actinomycete Isolates from Arabian Sea and Bay of Bengal: Biochemical, Molecular and Functional Characterization"** is a genuine record of research work done by me under the supervision and guidance of **Dr. Rosamma Philip** in the Department of the Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.*

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This is to certify that all relevant corrections and modifications suggested by the audience during the presynopsis seminar and recommended by the doctoral committee of ***Smt. Deepthi Augustine*** *has been incorporated in the thesis entitled* ***"Actinomycete Isolates from Arabian Sea and Bay of Bengal: Biochemical, Molecular and Functional Characterization."***

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1

General Introduction

1.1 Introduction

For decades, microbial natural products have been one of the major resources for discovery of novel drugs. Among the potential sources of natural products, bacteria have been proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds discovered. Of the 22,000 known microbial secondary metabolites, 70% are produced by actinomycetes, and two thirds of them are contributed by the genus *Streptomyces* (Subramani and Aalbersberg, 2012).

Actinomycetes are Gram positive, filamentous bacteria, with high G+C content (69-78%) in DNA exhibiting highly differentiated developmental cycle (Williams et al., 1989), inhabiting a wide range of habitats. Unlike bacteria, actinomycetes are unique in their morphology with extensive branching substrate and aerial mycelium bearing chain of arthrospores. The substrate mycelium and spores can be pigmented, which makes them most colourful and attractive among microbes. On agar plates they form lichenoid, leathery or powdery colonies. They possess cell wall characteristic of bacteria and filamentous nature of fungi. They are recognized as prolific producers of secondary metabolites with diverse biological activities. Actinomycetes

contain about 40 families and over 170 genera and about 2000 species have been validly described and published (Harwani, 2013).

In the early 1950's, with the isolation of antibiotic actinomycin from the soil actinomycete *Streptomyces antibioticus*, stimulated extensive screening of terrestrial actinomycetes for novel drugs. Over the past 70 years, indiscriminate screening of actinomycetes has led to the re-isolation of known bioactive compounds from the terrestrial environment. Hence it became crucial that actinomycetes from unexplored habitats be pursued as a source of novel secondary metabolites (Fenical and Jensen, 2006; Hamedi et al., 2013).

Over billions of years, oceans have been regarded as the origin of life on earth. Oceans include the largest range of habitats, hosting the most life forms. The complexity of marine environment, with variations in salinity, pH and temperature acts as powerful selective force, which has led to microbial evolution. The evolution prompted the marine microorganisms to generate multifarious enzymes and secondary metabolites to adapt to the complicated marine environments. It was reported that large numbers of actinomycetes are undoubtedly washed from shore into the sea (Goodfellow and Haynes, 1984). Accordingly, members of the genus *Thermoactinomyces* are useful markers of terrestrial wash in and give information about the distribution and survival of terrestrial microorganisms in marine environments (Goodfellow and Haynes, 1984). Few natural product studies have assessed the taxonomic novelty of the marine strains, that have yielded exciting new molecules (Fehling et al., 2003; He et al., 2001) and have suggested that targeting the marine taxa would yield novel metabolites. Marine microorganisms, particularly actinomycetes, have evolved the greatest genomic and metabolic diversities. Therefore, effort should be directed towards exploring marine actinomycetes as a source for the discovery of novel secondary metabolites (Lam, 2006).

In recent years, actinomycetes isolated from the marine environment (sediments, sponges, tunicates, neuston etc.) have attracted considerable attention (Lane and Moore, 2011; Liu et al., 2010). Any appropriate exploitation of the chemical diversity of these microbial sources relies on proper understanding of their biological diversity and other related key factors that maximize the possibility of successful identification of novel molecules.

1.2 Distribution of marine actinobacteria

Marine actinomycetes have been isolated all around the globe ranging from shallow coastal sediments to the deepest sediments from Mariana trench. It appears that they are widely distributed throughout the ocean and found in intertidal zones (Goodfellow and Williams, 1983), seawater, animals (Ramesh and Mathivanan, 2009; Ramesh et al., 2006), plants (Castillo et al., 2005), sponges, sea weeds (Bull and Stach, 2007; Goodfellow and Fiedler, 2010; Sun et al., 2010; Zhang et al., 2008), and in ocean sediments (Das et al., 2008; Jensen et al., 2005a; Thornburg et al., 2010; Xiao et al., 2011). The ocean floor has been recently demonstrated as an ecosystem with many unique forms of actinomycetes requiring seawater for their growth (Fenical and Jensen, 2006; Jensen et al., 2005a, Jensen et al., 2007).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). Metagenomic analysis of sea water column has also identified considerable number of actinomycete taxa (Venter et al., 2004). Actinomycetes have been isolated from unique marine environments, such as the marine organic aggregates of Wadden Sea (Kim et al., 2004) and deep sea gas hydrate reservoirs, where they were found to be the major components of

the microbial communities. Actinomycete phylotypes comprised up to 30-40% of clone libraries obtained from hydrate bearing sediments from the Gulf of Mexico (Parkes and Wellsbury, 2004) and the Nankai Trough near Japan (Inagaki et al., 2003).

Novel actinomycete groups have been found in the Great Barrier Reef sponges *Rhopaloeides odorabile*, *Pseudoceratina clavata* and *Candidaspongia flabellate,* and the Mediterranean sponges *Aplysina aerophoba* and *Theonella swinhoei* (Burja and Hill, 2001; Hentschel et al., 2002; Kim et al., 2004; Webster et al., 2001). Unusual actinomycetes, belonging to Micrococcaceae, Dermatophilaceae and Gordoniaceae, have also been isolated from sponges (Hill, 2004). Novel bioactive metabolites have been obtained from these actinomycetes isolated from sponges (Jensen et al., 2005c).

Cultivation of actinomycetes sampled from sediments (Kim et al., 2004; Takami et al., 1997) has expanded the diversity that has been detected and extended the data available for actinomycete classification and biogeography (Maldonado et al., 2005) and for the estimation of actinomycete numbers (Stach et al., 2003). This has also led to more novel marine actinobacteria being isolated and recognized (Magarvey et al., 2004).

1.3 Diversity of actinomycetes in the marine environment

Actinomycete biodiversity is a goldmine for the biotechnology industry as it offers countless new genes and biochemical pathways to probe for commercially valuable bioactive molecules. Bio prospecting efforts focusing on the isolation and screening of actinobacteria from ocean habitats (Magarvey et al., 2004; Mincer et al., 2002) have added new biodiversity to

the order *Actinomycetales* and revealed a range of novel natural products of pharmacological value (Engelhardt et al., 2010). As marine environment is extremely different from terrestrial habitat, it is well documented that actinomycetes adapted to marine environment exhibit an unique metabolic diversity and enzymatic potentialities (Nakashima et al., 2005).

Researchers around the world have concentrated to isolate and identify the actinobacteria from different marine habitats. Earlier it was reported that *Streptomyces* was the dominant genera in marine sediments while *Nocardia* and *Micromonospora* constitute about 20% of total isolates (Grein and Meyers, 1958). Contrary to these results, *Micromonospora* was found as the dominant genus in marine sediments (Jensen and Fenical, 1991; Mincer et al., 2002).

Actinomycetes isolated from marine sediments in neritic zone in the sea around Japan were compared with terrestrial isolates. Actinomycetes were identified by amplification of 16S rRNA gene sequences as belonging to genera *Streptomyces* and *Micromonospora*. Marine isolates of both genera were found to have higher sodium chloride (NaCl) tolerance than terrestrial ones. Around 37% of *Streptomyces* and 26% of *Micromonospora* that were isolated from the marine environment could tolerate up to 12% and 5% NaCl, respectively. It was suggested that the frequency of microorganisms with antimicrobial properties increased in the medium supplemented with seawater (Chiaki et al., 2010). Recently, among the 116 actinomycetes collected from marine sediments of the Yellow Sea, 56 grew slowly and appeared after 2–3 weeks of incubation. Phylogenetic analysis based on 16S rRNA gene of six representative isolates with different morphological characteristics identified the strains as belonging to the family Streptomycetaceae, Micrococcaceae and Nocardiopsaceae respectively (Zhang et al., 2012).

The Bay of Bengal has been targeted as a potential source of marine derived bacterial bioactive compounds by several investigators. Predominance of *Streptomyces* was also reported from marine sediments along Indian coast (Kokare et al., 2004; Lakshmanaperumalsamy, 1978; Sivakumar, 2001). Earlier it was reported that 72% of actinomycetes isolated from sediments of Bay of Bengal belonged to genus *Streptomyces* and 9% to genus *Micromonospora* (Peela et al., 2005). The diversity of actinomycetes in marine sediments of Bay of Bengal was investigated and *Streptomyces* was reported as the dominant genera with antifungal and cytotoxic activity (Ramesh and Mathivanan, 2009). Screening of marine sediment samples near the coast of Andaman Islands in the Bay of Bengal resulted in the isolation of numerous marine actinomycetes which belonged largely to the genus *Streptomyce*s. Sediment samples from Alibag, Janjira and Goa was remarked as potent sources for the isolation of bioactive actinomycetes (Kokare et al., 2004). Most of the isolates from sediment samples were identified as *Streptomyce*s. Diversity of bioactive actinomycetes investigated along the Puducherry coast of Bay of Bengal, India, revealed that out of 50 actinomycetes isolated from the marine sediments, majority belonged to *Streptomyces* (Suthindhiran and Kannabiran, 2010). Fifty three rare actinomycete strains isolated from mangrove sediments of Karwar identified by morphological and chemical properties belonged to the following genera *viz*., *Micromonospora*, *Microbispora*, *Actinoplanes*, and *Actinomadura* (Naikpatil and Rathod, 2011). Actinomycetes isolated from various sources especially sponges, sediments and seawater of different coastal regions of Arabian Sea identified *Streptomyces* sp. as the dominant and potential strain against pathogens (Ravikumar et al., 2010; Santhi et al., 2010; Gulve et al., 2012).

Phylogenetic diversity of actinomycetes cultured from coastal multi pond solar saltern in Tuticorin, India was investigated. Sequencing and analysis of 16S rDNA from chosen representative isolates displayed the presence of members affiliated to actinobacterial genera: *Streptomyces*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora* and *Nonomuraea*. The genus *Streptomyces* was found to be the dominant among the isolates. Furthermore, rare actinomycete genus *Nonomuraea* was isolated for the first time from Indian solar salterns (Jose et al., 2012). *Nocardiopsis* spp. are distributed ubiquitously in the environment (Kroppenstedt and Evtushenko, 2002). The genus *Nocardiopsis*, a widespread group in phylum actinobacteria, has received much attention owing to its ecological versatility, and ability to produce a rich array of bioactive metabolites. Its high environmental adaptability might be attributable to its genome dynamics, which can be estimated through comparative genomic analysis targeting microorganisms with close phylogenetic relationships but different phenotypes (Li et al., 2013).

1.4 Marine actinobacteria as a source of secondary metabolites

Several reviews describing biologically active molecules isolated from marine actinomycetes have been published (Bull and Stach, 2007; Fenical and Jensen, 2006; Goodfellow and Fiedler, 2010; Mayer et al., 2011). Apparently, the trend of discovery of novel compounds from marine actinomycetes and their characterization is gaining momentum, which is not surprising considering the exhausted pipelines for discovery of new antibiotics and constant need for improved treatment of cancer, as well as chronic diseases.

Until recently, most antibiotics of microbial origin came from terrestrial bacteria belonging to one taxonomic group, the order *Actinomycetales* (Bull et al., 2005; Lam, 2006). Among the different microorganisms, actinomycetes are undoubtedly the largest producers of secondary metabolites. Of the presently known microbial metabolites, 45% (approximately 10,000 compounds) were isolated from various *Actinomycetale*s. Of these compounds, 75% were from *Streptomyces* and 25% from rare actinomycetes. The representation of rare actinomycete metabolites in the 20th century was only 5% (Berdy, 2005). However, *Micromonospora*, *Actinomadura*, *Actinoplanes*, *Streptoverticillium*, *Nocardia*, *Saccharopolyspora* and *Streptosporangium* spp. are increasingly playing a significant role in the production of a wide spectrum of antimicrobial metabolites and antibiotics (Lam, 2006). The rare actinomycetes produce diverse and unique, unprecedented, sometimes very complicated compounds exhibiting excellent bioactive potency and usually low toxicity (Kurtboke, 2010).

Actinomycetes are physiologically diverse bacteria as evidenced by their production of numerous extracellular enzymes and other metabolic products (Kekuda et al., 2010). Biodegradation by actinomycetes plays an integral role in recycling of materials in nature. As a result of their great metabolic diversity, actinomycetes have great biotechnological potential for the production of pharmaceuticals and as bioremediators. Actinomycetes are reservoirs of many enzymes including cellulases, xylanases, amylases, lipases, collagenases, proteases, chitinases, ligninases etc. The secondary metabolites produced by actinomycetes have a broad spectrum of biological activities eg. antibacterial (streptomycin, tetracycline, chloramphenicol), antifungal (nystatin), antiviral (tunicamycin), antiparasitic (avermectin), immunosuppressive (rapamycin), antitumor (actinomycin, mitomycin-C,

anthracyclines), enzyme inhibitory (clavulanic acid) and insecticidal activities. They also synthesize and excrete pigments viz., melanins, carotenoids, phenazines etc., with pharmacological activities especially antitumour, antimicrobial and antioxidant activities.

However, judging from the data by Mayer et al. (2011), only a small portion of the discovered marine natural products with antimicrobial, anti-inflammatory, anticancer and neuromodulating activities originate from actinomycetes. The latter is probably due to the traditional approach to the search for medicines in the sea, which for many years have focused primarily on sponges, tunicates, corals and cyanobacteria. Considering the fact that many of the biologically active compounds isolated from marine fauna are probably synthesized by symbiotic or associated bacteria (Konig et al., 2006), the focus is currently shifting towards isolation of such bacteria and their sustainable cultivation.

Streptomycetes are an economically important group of organisms among actinomycetes and they are the pivotal source for wide range of biologically active compounds (Berdy, 2005). About three quarters of all the known commercially and medicinally useful antibiotics (Kieser et al., 2000) and several agriculturally important compounds (Okami and Hotta, 1988) were obtained from streptomycetes. Streptomycetes have been shown to have the ability to synthesize antibacterial (Berdy, 2005; Ramesh et al., 2009), antifungal (Han et al., 2009; Prapagdee et al., 2008), insecticidal (Pimentel-Elardo et al., 2010), antitumor (Hong et al., 2009; Lam, 2006), anti-inflammatory (Renner et al., 1999), antiviral (Sacramento et al., 2004), antifouling (Xu et al., 2010), anti-infective (Rahman et al., 2010) as well as many other agents such as enzyme inhibitors (Hong et al., 2009) and vitamins

(Atta, 2007) and hence, they are widely recognized as industrially important microorganisms (Higginbotham and Murphy, 2010).

Few potential bioactive *Nocardiopsis* have been reported from marine sediment samples of Indian coast. i.e., a protease-producing, crude oil degrading marine *Nocardiopsis* sp. NCIM 5124 (Dixit and Pant, 2000) and bio surfactant producing marine *Nocardiopsis* sp. (Khopade et al., 2012; Selvin et al., 2009) which is a clear proof of existence of potential *Nocardiopsis* sp. along Indian coast. A sponge associated actinomycete, *Nocardiopsis dassonvillei* MAD 08 showing activity against multidrug resistant pathogens have been reported (Selvin et al., 2009).

1.5 Systematics in biodiscovery

Systematics helps in providing a road map to genes and hence products and new chemical entities are likely to be discovered in novel organisms. Very powerful tool for discovering novel enzymes of possible biotechnological interest is the exploration of marine microorganisms with unique physiological traits. This approach can complement the enormous amount of data on gene diversity in marine environments offered by metagenomics (Trincone, 2011).

Biodiscovery is hampered by the shortage of professional systematists, particularly those with expertise in actinobacterial taxonomy, and of natural product chemists (Bull and Stach, 2007). A similar decline in the study of microbial physiology gives cause for further concern in the post genomics era. Actinomycetes taxonomy can be a very important and helpful tool in natural product screening. Also the postulated paradigm shift in biotechnology points out the importance of taxonomy in this important industrial field of research

(Bull et al., 2000). Natural product diversity is still the pool for new compounds filling the pipeline for drug discovery (Newman and Laird, 1999). As the number of isolates which have to be screened to detect new biologically active metabolites is rising, it is even more important to work with strains which have never been used for screening processes (Goodfellow and O'Donnell, 1989). This can be achieved by isolating microorganisms from unusual habitats, trying to cultivate organisms which are difficult to handle or isolate new species and genera (Baltz, 2008). In all these cases the taxonomic characterization of isolates plays an important role.

1.6 Relevance of the Present Study

Although terrestrial samples have been screened extensively,only a small fraction of the actinomycete taxons have been discovered (Baltz, 2007, 2005). Taxonomic characterization of actinomycetes producing novel metabolites is an important step in any screening program. In many instances, however, taxonomic study of the microorganism is initiated, only when the metabolite it produces is of great interest, i.e., when a description of the producing microbe is needed for the patent application. Identification of isolates to the species level will often give the researcher a clue as to whether or not a metabolite is novel (Labeda, 1987). Regarding the Indian peninsula, the marine ecosystems of Arabian Sea and Bay of Bengal are mostly unexplored, and may provide a rich source of actinomycetes producing novel and efficient bioactive molecules. Novel antimicrobial compounds are essential to control emerging diseases in aquaculture. Shrimp farming, a major source of revenue in Asian countries, particularly India, is being plagued by devastating bacterial diseases especially vibrios. Also there is an increasing demand for ecofriendly

alternatives for combating insect pests. In this regard the present study was undertaken with the following objectives.

1.7 Objectives

- Morphological and biochemical characterization of actinomycete isolates from the Arabian Sea and Bay of Bengal.
- Molecular identification and phylogenetic analysis of the marine actinomycete isolates.
- Functional characterization of the actinomycete isolates for exploring its bioactive potential for biotechnological applications.

1.8 Outline of the Thesis

The thesis is comprised of seven chapters. Chapter 1 gives a general introduction to marine actinomycetes; Chapter 2 gives an account on the morphological, biochemical and physiological characterization of marine actinomycetes. Comprehensive description of molecular identification and phylogenetic analysis of actinomycetes is dealt with in Chapter 3. The antimicrobial property with special reference to antivibrio activity is described in Chapter 4. Chapter 5 explores the melanin production ability of marine actinomycetes, characterization of melanin and evaluation of its bioactivity. Chapter 6 illustrates the study on chitinolytic *Streptomyces* as antifungal and insecticidal agents. Summary and Conclusion of the study is presented in Chapter 7, followed by References and Appendices.

2

Morphological, Biochemical and Physiological Characterization of Marine Actinomycete Isolates

2.1 Introduction

Successful and effective exploitation of marine microbes relies on assessing their diversity in the marine environment. Diversity is considered as a key factor by pharma industries in the line of drug discovery process. Taxonomy is the key to explore biodiversity. Characterization studies indeed helps to understand the unexplored features of the organism, ecological, physiological and biochemical aspects. The characterization of these microbes is as important as studying their existence in the natural environments (Hirsch et al., 2010). Even though a large number of actinomycetes from marine environment have been screened for bioactive compounds, only a few have been characterized. It is essential to understand the taxonomy and ecology of secondary metabolites producing actinomycetes to facilitate the exploration of the different strains for biotechnology (Adegboye and Babalola, 2012). Understanding and interpretation of knowledge derived from conventional studies conducted since the discovery of streptomycin, on the ecology, taxonomy, physiology and metabolism of actinomycetes is essential for

emergence of novel discoveries. Continuously advancing molecular tools will provide powerful platform to reveal taxonomical and chemical identities of previously undetected bioactive actinomycetes (Kurtboke, 2010).

The Indian peninsula harbours its own diverse habitats which support the growth of various actinobacterial communities in specific microbial niches. Hence in India, actinobacterial diversity has been an important source for natural product discovery (Ballav et al., 2012). Streptomycetes, the largest taxon of actinobacteria, well known as a rich source of novel bioactive compounds have been subjected to intensive isolation and screening which has led to over speciation and taxonomic chaos within the genus (Guo et al., 2008).

2.1.1 The Polyphasic taxonomy

Over the years, numerous methods have been applied with the aim of identifying, characterizing, and discriminating streptomycete strains. The primary aim of such investigations has been to develop the most rapid and accurate method for proving the novelty of newly isolated strains (Hain et al., 1997).

Polyphasic characterization integrates both phenotypic and genotypic traits and the term ‘polyphasic’ was first introduced to refer to classifications based on a consensus of all available methods: single character tests, as well as multiple character tests, including both phenotypic and genotypic data (Colwell, 1970). Polyphasic taxonomy was interpreted as, at the higher levels of phylum, division, class, order and family, taxa are ordered in phylogenetic terms (based on sequence analysis), but at lower levels, at species and perhaps genus levels, taxa are established as phenetic groups based on a consensus of phenotypic data (Young, 2001).

Genes and genomes do not function on its own and can only display their potential within the cell as the basic unit of evolution and hence taxonomy. Genes and genomes can only unfold their potential within a cell, and it is the phenotype that in combination with the natural selection 'drives' evolution in a given environment (Kampfer and Glaeser, 2012). Hence interplay between genetic and phenotypic datasets provides a sound basis for appreciating and describing the diversity of prokaryotes and has the potential to become the foundation of a more stable, in depth taxonomy of the prokaryotes (Tindall et al., 2010).

2.1.2 Taxonomy of Actinomycetes

Interest in systematics of actinomycetes gained momentum with the extensive isolation and screening of actinomycetes since the discovery of antibiotics actinomycin and streptomycin from *Streptomyces antibioticus* and *Streptomyces griseus* (Waksman and Woodruff, 1941). The need for patenting of novel natural products led to an over classification of the genus and standard identification criteria and type strains were needed to prevent over speciation of the genera (Anderson and Wellington, 2001).

The methods used for characterization of actinomycetes have evolved through several phases over the years, from morphological observations, to subsequent classifications based on numerical taxonomic analyses of standardized sets of physiologic and biochemical characters and, most recently, to the use of molecular phylogenetic analyses of gene sequences.

Various approaches in the systematics of actinomycetes are given below.

2.1.2.1 Traditional approach

Traditional or conventional approaches for classification make use of morphological, physiological, and biochemical characters. The classical method described in the identification key is used in the identification of actinomycetes (Nonomura, 1974; Shirling and Gottlieb, 1966). The problems facing streptomycete systematists were addressed in a series of cooperative studies, the most notable of which was the International *Streptomyces* Project (ISP). The ISP study like earlier attempts to classify streptomycetes was largely based on a limited number of features with heavy emphasis on morphology and pigmentation. Chromogenicity of aerial mycelium is considered an important character for grouping and identification (Pridham and Tresner, 1974) of actinomycetes. Carbohydrate utilization test play a prominent role in the taxonomic characterization of actinomycete strains (Pridham and Gottlieb, 1948). Chemotaxonomy is the study of chemical variation in organisms and the use of chemical characteristics in the classification and identification. It is one of the valuable methods to identify the genera of actinomycetes. Studies have established that actinomycetes have a cell wall composition akin to that of Gram-positive bacteria, and also indicated that the chemical composition of the cell wall might furnish practical methods of differentiating various types of actinomycetes (Cummins and Harris, 1956). Traditionally, chemotaxonomy is still considered useful in actinomycete taxonomy, but it is not always reliable as several genera exhibit similar chemical properties. These methods are not routinely used, as the technique is cumbersome, time consuming and considered to have limited diagnostic value (Wang et al., 1999).

The first comprehensive numerical taxonomic study of the genus Streptomyces was undertaken by Williams et al. (1983a) who assigned the

type strains of *Streptomyces* into 23 major, 20 minor (2-5 strains) and 25 single membered clusters based on phenetic tests (Labeda et al., 2012). The minor and single membered clusters were considered as species and major cluster as species groups. The largest species-group is *Streptomyces albidoflavus* (cluster 1) Their phenetic characteristics included yellow grey pigmentation, no melanin production, smooth spores which grow in straight chains and resistance to a number of antibiotics including penicillin, lincomycin and cephaloridine. Many of the strains were patented as antibiotic producers (Goodfellow et al., 1992). The circumscription of most Streptomyces species described in Bergey's Manual of Systematic Bacteriology, volume 4 (Williams et al., 1989) is based on the numerical taxonomic survey of Williams et al. (1983a). Different tests were also used to define the major clusters, including an increased number of antibiotic resistances. The variability of antibiotic resistance within taxonomic groups defined was demonstrated using the identification matrix (Huddleston et al., 1997).

Serological methods (Ridell et al., 1986), phage typing (Korn-Wendisch and Schneider, 1992) and protein profiling (Ochi, 1995; Taguchi et al., 1996) have also been used in characterization and assigning an unknown isolate to the genus of Streptomycetes. However these methods have difficulties to discriminate the species at the intragenus level (Anderson and Wellington, 2001).

2.1.2.2 Molecular Approach

Molecular systematics, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies, but has achieved a new status following the introduction of nucleic acid

sequencing techniques (O'Donnell et al., 1993). Significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and actinomycetes (Yokota, 1997).

2.1.3 Actinomycete genera in marine sediments

Preliminary works regarding the diversity of marine actinomycetes, reported that species of *Mycobacterium*, *Actinomyces*, *Nocardia*, *Micromonospora* and *Streptomyces* have been isolated from the marine sediments (Grein and Meyers, 1958; ZoBell, 1946; ZoBell and Upham, 1944). It is a well known fact that *Streptomyces* genera is the most dominant in any actinomycete population (Alexander, 1977; Ellaiah and Reddy, 1987). Contrary to these results, Jensen et al. (1991) and Mincer et al. (2002) reported *Micromonospora* as the dominant genus in marine sediments. *Streptomyces, Micromonospora* were reported as most common genera from various sites in Atlantic and Pacific Oceans and metal contaminated sediments of Baltimore, inner harbor (Fiedler et al., 2005; Ravel et al., 1998). Maldonado et al. (2005) contradicted that *Micromonospora-Rhodococcus-Streptomyce*s group seems to be ubiquitous in cultured actinobacteria from marine environments.

An investigation on the biodiversity of actinomycetes in Norwegian shallow water sediments revealed *Streptomyces* and *Micromonospora* as the predominant genera by macro and micro morphological and chemo taxonomical criteria. Rare actinomycete genera, viz., *Rhodococcus* (43.5%), followed by *Nocardiopsis* (17.9%), *Streptosporangium* (15.2%), *Nocardia* (1.5%), *Knoellia* (5.4%), *Actinomadura* (2.2%), *Pseudonocardia* (1.6%), *Glycomyces* (1.1%) and *Nonomuraea* (0.6%) were also identified from the shallow water sediments (Bredholdt et al., 2007). Contrary to these results, actinomycete strains isolated from marine sediments and sponges collected in

the Trondheim Fjord, Norway classified based on growth, morphology and 16S rRNA gene analysis revealed the largest clade of seven isolates belonging to *Nocardiopsis* genus followed by *Streptomyces* and *Micromonospora* (Engelhardt et al., 2010)

Marine actinobacterial research in India have been reviewed and marine actinobacterial biodiversity and its potentials has been documented (Sivakumar et al., 2007). The study on isolation and characterization of actinomycetes from west coast of India showed that majority (47%) of the isolates belonged to the genera *Streptomyces* (Remya and Vijayakumar, 2008) followed by the rarer genera *Glycomyces*, *Nocardiopsis* (11%), *Nocardia* etc. Various genera such as *Streptomyces*, *Actinopolyspora*, *Actinomadura*, *Nocardiopsis*, *Micromonospora* and *Actinomyces* are reported as actinobacterial diversity in water and sediment samples from the marine environment of Tamil Nadu (Manivasagan et al., 2009). Recently, in a comparative study of marine sediment actinomycetes from Pulicat estuary, Muttukadu estuary and Ennore estuary (Tamil Nadu), majority of the isolates were assigned to the genus *Streptomyces* (60%), followed by *Actinopolyspora* (35%), and genus *Nocardiode*s (5%) (Chacko et al., 2012).

The actinomycete strain VITSVK5 identified as *Nocardiopsis* sp., isolated from the south east coast of India supported the evidence that marine sediment sample is an important source of potential non streptomycete genera (Vimal et al., 2009). The relevance of the non-streptomycete group of actinomycetes in this regard can also be demonstrated that many genera such as *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharothrix*, *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Saccharopolyspora* and *Streptosporangium* also produce many interesting antibiotics (Genilloud et al., 2011).Streptomycetes, the ubiquitous genera within the class actinobacteria are especially prolific and

can produce around 80% of the total antibiotic products with other genera trailing numerically (Hopwood et al., 2000).

2.1.4 Dominant genera - *Streptomyces*

Streptomycetes, the most widely exploited group among actinomycetes is included in the suborder, *Streptomycineae*, family Streptomycetaceae (Kampfer, 2006). They have a type 1 cell wall and G + C content of around 69 to 78 mol % (Farris et al., 2011).

At present, over 500 species of *Streptomyces* have been described by Euzeby (2008). The odour of the moist earth is largely the result of the production of volatile substances such as geosmin by *Streptomyces* (Jiang et al., 2007). They are flexible nutritionally and can aerobically degrade complex substances such as pectin, lignin, chitin, keratin, latex and aromatic compounds (Shi et al., 2011). *Streptomyces* are best known for their synthesis of a vast array of antibiotics, some of which are useful in medicine and agriculture (Watve et al., 2001). The family previously consisted of ten genera including *Actinopycnidium*, *Elytrosporangium*, *Actinosporangium*, *Kitasatoa*, *Chainia*, *Kitasatospora*, *Microellobosporia*, *Streptacidiphilus*, *Streptomyces* and *Streptoverticillium*. The development of numerical taxonomic systems, which utilized phenotypic traits helped to resolve the inter-generic relationships within the family Streptomycetaceae and the changes resulted in the genus *Streptomyces* being the sole member of the family Streptomycetaceae (Kim et al., 2003; Logan, 1994; Stackebrandt et al.,1997).

2.1.5 Nocardiopsis

The genus *Nocardiopsis* first described by Meyer (1976) typically exhibit a branched substrate mycelium that fragments into rod shaped and

coccoid elements and abundant aerial hyphae that frequently form a zigzag morphology. They are frequently isolated from habitats with moderate to high salt concentrations such as saline soil or marine sediments (Al-Tai and Ji-Sheng, 1994; Al-Zarban et al., 2002; Evtushenko et al., 2000) and salterns (Chun et al., 2000). The closest relatives of the genus *Nocardiopsis* are members of the genera *Actinomadura*, *Thermomonospora*, *Streptosporangium*, and *Microtetraspora*. Chemotaxonomically, *Nocardiopsis* species fall into two groups: those that synthesize mainly menaquinones with highly saturated isoprenoid side chains, *Nocardiopsis dassonvillei* subsp. *dassonvillei*, *N. alba*, *Nocardiopsis kunsanensis*, *N. prasina*, *N. lucentensis*, *Nocardiopsis trehalosi, N. tropica* and *N. halophila*; and others, including *N. halotolerans*, *N. dassonvillei* subsp. *albirubida*, *N. listeri* and *N. synnemataformans*, that contain non saturated isoprene units (Kroppenstedt and Evtushenko, 2002). The results of a 16S ribosomal DNA sequence analysis are generally consistent with the available chemotaxonomic, phenotypic, and DNA DNA hybridization data (Rainey et al., 1996). Differentiation of *Nocardiopsis* species is currently based on the color of the mycelium and the results of comparative physiological tests (Kroppenstedt, 1992).

2.1.6 Biogranulation property

Actinomycetes are filamentous and when cultured in shake flask cultures, the filaments entangle and aggregate to form bead like appearance or granules. These granules vary in size from tiny to large granules depending on the type of species. Even though the anaerobic granulation and granulation of fungus has been investigated by researchers, not much focus has been given to the granulation property of actinomycetes. The gross morphology of actinomycetes grown in submerged culture has received little attention, despite the importance of these organisms (Lawton et al., 1989). One of the first

descriptions of gross morphology of anaerobic actinomycete *Actinomyces israelii wa*s compact ‘bread crumb’, ‘cauliflower’, or 'puffball' colonies, which settled out in static culture (Erikson, 1940). Small bead like colonies, granules, or flakes of actinomycetes was grown in submerged culture. In a study of five *Streptomyces* spp. loose open colonies, free hyphal colonies with dense central regions, and radiating hyphae as well as hyphal ropes was described. Pellets may originate from spores coagulating together, freshly germinated spores aggregating, or by mycelial entanglement (Williams et al., 1974).

2.1.7 Antibacterial activity of marine actinomycetes

Fiedler et al. (2005) screened actinomycetes (600 Nos.) isolated from various sites of Pacific and Atlantic Oceans for the production of bioactive secondary metabolites. Abyssomicin producing actinomycete belonging to the rare genus *Verrucosisspora* was isolated during the screening process. *Streptomyces* and rare actinomycetes isolated from sediments of Trondheim Fjord, Norway exhibited antibacterial and antifungal potential (Bredholdt et al., 2007). Gentamicin producing *Micromonospora* was isolated from sediment samples of Sunshine coast in Australia during the screening process for antibiotic producing microorganisms (Glen et al., 2008).

Marine actinomycete strains isolated from coastal waters of Dhanushkodi (Ramanathapuram district, India) showed antibacterial and antifungal effect against selected human pathogens (Asha et al., 2006). Marine actinomycetes isolated from different locations of the Manakudi Estuary of Arabian Sea in Tamil Nadu, exhibited higher antagonistic activity against *Staphylococcus aureus*, *Enterobacter* sp., *Salmonella typhi*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Proteus vulgaris* (Santhi et al., 2010).

A novel polyene type antimicrobial metabolite producing actinomycete, *Streptomyces* was isolated from marine sediments of Bay of Bengal along Andhra Pradesh coast of India (Valan et al., 2012a). The lowest minimum inhibitory concentration of ethyl acetate extracts of the metabolite against *Escherichia coli* and *Curvularia lunata* were 67.5 and 125 µg/ml, respectively. Similarly antimicrobial and antioxidant potential of the actinomycete isolate *Streptomyces* sp. VITSTK7 from marine sediments collected at the Bay of Bengal coast of Puducherry, India, was evaluated. Ethyl acetate extract showed moderate antibacterial activity against selected bacterial pathogens (Thenmozhi and Kannabiran, 2012). A study on antibacterial activity of 107 marine actinomycetes isolated from near sea shore sediment and seawater from Konkan coast of Maharashtra identified *Streptomyces* sp. antagonistic to *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella aerogenes, Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* by agar well method (Gulve et al., 2012).

2.1.8 Hydrolytic Enzymes

Actinomycetes have long been credited as an important resource of enzymes and bioactive compounds. Marine actinobacteria elaborate a wide range of enzymes and secondary metabolites as an aid to their survival. The ecological features of habitat in which marine microbes thrive impact their metabolic functions enabling their bio molecular machinery (Trincone, 2011).

Marine actinomycetes due to their diversity and proven ability to produce novel metabolites and other biologically active molecules rank a prominent position as targets in screening programs (Ellaiah et al., 2004). Actinomycetes have shown degradation of starch and casein besides the production of antimicrobial agents (Barcina et al., 1987; Zheng et al., 2000).

Ecological role of marine actinobacteria, like their terrestrial counterparts, are known to be degraders of recalcitrant organic compounds. Many of the marine actinomycete derived natural products possess unique structural features rarely or never found among the compounds isolated from the terrestrial sources. It is thus not surprising that their molecular modes of action are sometimes also unique, prompting their investigation as potential leads for drug development (Zotchev, 2012). The diverse range of enzymes produced by actinomycetes are capable of catalyzing various simple to complex biochemical reactions.

Many researchers have reported the production and characterization of enzymes from *Streptomyces* and *Nocardiopsis*, mostly terrestrial, although few reports are available from marine isolates (Chakraborty et al., 2012; Goshev et al., 2005; Kavitha and Vijayalakshmi, 2011; Stamford et al., 2001; Tsujibo et al., 2003). In aquatic environments, microbial extracellular hydrolytic enzymes are the major biological mechanism for the decomposition of sedimentary particulate organic carbon and nitrogen (Brunnegard et al., 2004; Dang et al., 2009). There are reports on the multi enzyme activity of actinomycetes from marine sediments (Leon et al., 2007; Ramesh and Mathivanan, 2009).

2.1.8.1 Amylases

In a recent review on marine biocatalysts, marine amylases are gaining interest from a biotechnological perspective. Occurrence of amylases in actinobacteria is a characteristic commonly observed in *Streptomyces* (Vigal et al., 1991), although the enzyme has been isolated from other genera also. Selvam et al., (2011) reported marine actinomycetes isolated from sediments of south coast of India as producers of industrial enzymes, amylase and lipase.

Thermo stable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation (Manivasagan et al., 2013).

A novel moderately thermo stable α-amylase from marine *Streptomyces* sp. D1 has also been reported with α 1–4, α-1–6 activity, wide range of pH stability, and potential widespread application in the detergent industry (Chakraborty et al., 2009). Production of α-amylase by *Nocardiopsis* sp., an endophytic actinobacterium was studied from yam bean (Stamford et al., 2001). An actinobacterium strain AE-19 producing α -amylase, was isolated from a shrimp pond (Poornima et al., 2008). A haloalkaliphilic actinomycete, *Saccharopolyspora* sp. A9 isolated from marine sediments collected from west coast of India was found to produce a stable α-amylase (against surfactants, oxidants and detergents) (Chakraborty et al., 2011). Detergent stable and calcium ion independent α -amylase from marine *Streptomyces* A3 has wide spread applications for detergent and pharmaceutical industry where higher salt concentration inhibits enzymatic conversions (Chakraborty et al., 2012).

2.1.8.2 Proteases

Proteases are used in the detergent industry, leather industry, and also for pharmaceutical applications, such as digestive and anti-inflammatory drugs ((Demain and Zhang, 2005; Kumar et al., 2004;). In a previous report on screening of actinomycetes for proteolytic activity, *Streptomyces exfoliatus* CFS 1068 and *Streptomyces sampsonii* GS 1322 isolated from various terrestrial Indian habitats were found to possess high proteolytic activity (Jain et al., 2009). Among the actinobacteria (191no:) isolated from marine samples collected from the Bay of Bengal and associated mangrove habitats, it was found that 82% produced caseinase, 59% gelatinase and 56% produced both the enzymes (Ramesh and Mathivanan, 2009). An alkaline protease from

Nocardiopsis sp. NCIM 5124 has been purified and characterized (Dixit and Pant, 2000).

2.1.8.3 Lipases

Lipases are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol. Besides this, lipases are also efficient in various reactions such as esterification, transesterification and aminolysis (Babu et al., 2008). Also, many microbial lipases are available as commercial products, the majority of which are used in detergents, paper production, cosmetic production, food flavouring, organic synthesis and other industrial applications (Chi et al., 2009; Seiichi et al., 1991). A novel extracellular phospholipase C was purified from a marine streptomycete; its enzyme activity was optimal at pH 8.0 and 45 ºC, and hydrolyzed phosphatidyl choline (Mo et al., 2009).

In addition, actinomycetes are important for the production of enzymes, such as chitinases (eg: *Streptomyces viridificans*), cellulases (eg. *Thermonospora* spp.), xylanases (*Microbispora* spp.), ligninases (*Nocardia autotrophica*), sugar isomerases (*Actinoplanes missouriensis*), pectinases, hemicellululases and keratinases (Solans and Vobis, 2003).

2.1.8.4 Pectinases

Pectinases are a heterogenous group of related enzymes that hydrolyse the pectic substances, widely distributed in higher plants and microorganisms (Whitaker, 1992). They are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage (Sakai et al., 1993). Pectinolytic enzymes reported from actinomycetes are mostly pectate lyases (Spooner and Hammerschmidt, 1989), identified

from *Streptomyces viridochromogens* (Agate et al., 1962). Pectinolytic enzymes from actinomycetes have been used for degumming of ramie bast fibres (Bruhlmann et al., 1994). The sediment strain *Streptomyces lydicus* was found to be a potent producer of polygalacturonase (Jacob et al., 2008).

2.1.8.5 Chitinases

Amongst actinobacteria, the genus *Streptomyces* is the best studied for chitinases and is mainly responsible for the recycling of chitinous matter in nature. Several chitinolytic enzymes have been identified in several *Streptomyces* spp. including *S. antibioticus*, *S. griseus*, *S. plicatus*, *S. lividans*, *S. aureofaciens* and *S. halstedii* (Joo, 2005; Taechowisan et al., 2003). Several chitinases have been reported from *Streptomyces* (Kavitha and Vijayalakshmi, 2011) and *Nocardiopsis* sp. (Tsujibo et al., 2003). Antifungal and biodegradable properties of chitinase are useful for environmental, food technology and agriculture (Han et al., 2009). Purification, characterization of chitinase from *Streptomyces venezuelae* P10 was investigated and characterization studies revealed antifungal activity against fungal pathogens (Mukherjee and Sen, 2006). Although reisolation of known compounds is exploding, it has been predicted that only less than 10% of the Streptomycete bioactive metabolites have been discovered (Clardy et al., 2006; Watve et al., 2001).

Little is known about the diversity of actinobacteria in marine sediments of Arabian Sea and Bay of Bengal which still remains under explored. The present study was aimed at finding out the major actinomycetes in the sediments of Arabian Sea and Bay of Bengal and their bioactive potential in terms of antibacterial and hydrolytic property.

2.2 Materials and methods

2.2.1 Microorganisms used for the study

Actinobacteria (230 Nos.) isolated from the continental shelf and slope sediments of West and East coast of India and maintained in the Microbiology Laboratory of School of Marine Sciences, Cochin University of Science and Technology were used for the present study. The list of various cruises of Fisheries and Oceanographic Research Vessel (FORV) *Sagar Sampada* (Centre for Marine Living Resources and Ecology, Ministry of Earth Sciences, Govt of India) and the area of sample (sediment) collection giving details of the source of isolation of actinomycetes used in the present study is given in Appendix 1.

2.2.2 Purification of Isolates

Actinomycetes (230 Nos.) were purified by repeated streaking on nutrient agar vials and stocked in soft nutrient agar vials overlaid with sterile liquid paraffin. The working cultures were maintained in nutrient agar slants and kept refrigerated at 4°C for further studies.

2.2.3 Morphological and Cultural Characterization

The actinomycetes comprise a ubiquitous order of bacteria, which exhibit wide morphological and physiological diversity. Morphological, physiological and biochemical properties of the strains were studied as per International *Streptomyces* Project (Shirling and Gottlieb, 1966) and Bergey's manual of systematic bacteriology (Williams et al., 1989).

The isolates were streaked on to 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; seawater 1L; agar 20.0 g; pH 7.2) 3) Glycerol asparagine agar (ISP 5) (L-asparagine 1.0 g; glycerol 10.0 g; K_2HPO_4 1 g; seawater 1 L; agar 20.0 g; pH 7.2) and 4) Nutrient agar (peptone 0.5 g; beef extract 0.3 g; agar 2 g; seawater 100 ml) and the colony characteristics were noted; colour of mature sporulating aerial mycelium, substrate mycelium, macromorphology, diffusible pigment, colony reverse colour, colony texture etc. were recorded after observing the plates under the Stereomicroscope (Tresner and Backus, 1963).

2.2.3.1 Cover slip Culture technique

Coverslip culture is an important tool for studying the micromorphology of filamentous actinomycetes under undisturbed conditions. Spore chain morphology, fragmentation of substrate mycelium, aerial mycelium, shape and number of spores in spore chain etc. can be clearly studied by this technique. The isolates were inoculated in to Marine actinomycete broth and incubated at 28 °C for 1-2 days. Plates containing Casein starch peptone yeast malt extract agar medium (Casein 3.0 g; maize starch 10.0 g; peptone 1.0 g; yeast extract 1.0 g; malt extract 10.0 g; K_2HPO_4 0.5 g; sea water 1 L; pH 7.4; agar 20 g) were prepared. Sterile cover slips 3-4 were inserted at an angle of 45°C into the agar medium. A loopful of spore suspension of actinomycete was dispensed at the intersection of the medium and cover slip. The plates were incubated at 28°C for 4-8 days. The cover slips were removed at intervals of 2-4 days and were observed under high power and oil immersion objectives. Morphology of aerial mycelium, substrate mycelium, arrangement of

sporogenous hyphae, their morphology (straight, flexuous, spiral shaped) were recorded according to ISP (Nonomura, 1974; Shirling and Gottlieb, 1966).

2.2.4 Biochemical and Physiological characterization

Biochemical and physiological characterization of the individual isolates is of utmost importance to understand the basic physiology of the marine actinomycete isolates. The physiological and biochemical tests for characterization of aerobic sporogenous actinomycetes was done according to Berd (1973), with slight modifications.

2.2.4.1 Decomposition of organic substrates

Decomposition of tyrosine, xanthine, hypoxanthine, casein and esculin was considered as a basic taxonomic criterion in the characterization of actinomycetes.

a) Tyrosine: Tyrosine (0.5 g) was added to 10 ml of distilled water and autoclaved. This suspension was mixed with 100 ml of sterile nutrient agar media (peptone 5.0 g; beef extract 3.0 g; seawater 1 L; agar 20 g; pH 7.2) at 50°C, thoroughly mixed and poured into petri plates. Clearing zone around the actinomycete colony were scored as positive.

b) Hypoxanthine: Hypoxanthine (0.5 g) was added to 10ml of distilled water and autoclaved. This suspension was mixed with 100 ml of sterile basal media (nutrient agar) at 50°C, mixed well and poured into petri plates. Clearing zone around the colony were scored as positive.

c) Xanthine: To check xanthine decomposition, 0.4 g xanthine was mixed with 10 ml of distilled water and autoclaved and this suspension was added to sterile nutrient agar media (100 ml) and poured into petriplates as above. Spot

inoculation of isolates was done in the respective media and the plates were incubated at 28°C for 5-7 days. Clearing zone around the colony were scored as positive.

d) Casein: Decomposition of casein was done in skim milk agar prepared by autoclaving 10 g of skim milk in 100 ml distilled water. Two grams of agar was added to 100 ml of sea water and were autoclaved separately. Sterile skim milk and agar were mixed thoroughly and poured into petri dishes. Spot inoculation of isolates was done and the plates were incubated at 28°C for 5-7 days. Clearing of skim milk agar around the colony was recorded as positive.

e) Esculin: Esculin decomposition media was used to determine whether the actinomycete isolates have the ability to decompose glycoside esculin to esculetin and dextrose. Esculetin reacts with ferric ammonium citrate in the medium to produce a dark brown to black complex. Slants with esculin (0.1%) agar media (esculin 1 g; ferric ammonium citrate 0.5 g; peptone 10 g; seawater 1 L; agar 20 g; pH 7.2) were inoculated with loopful of actinomycete spores and control slants with the same agar base without esculin were inoculated in the same manner. The tubes were incubated at 28°C for 1-2 weeks, and blackening of the slants was recorded as positive.

2.2.4.2 Lysozyme resistance

Sterile glycerol broth (peptone 5 g; glycerol 70 ml; sea water 1000 ml; pH 7.2) was mixed with 5 ml of lysozyme solution (100 mg of lysozyme [Himedia] in 100 ml of 0.01 N hydrochloric acid sterilized by seitz filtration), and the mixture was dispensed in test tubes. These tubes and controls containing glycerol broth without lysozyme were inoculated with one drop of culture suspension with a Pasteur pipette. Readings were made weekly for 2 weeks. The result was considered positive if good growth was noted in both

tubes, negative if growth was good in the control tube but poor or absent in lysozyme broth.

2.2.4.3 Urea hydrolysis

Christensen's urea agar medium (yeast extract 0.1 g; KH_2PO_4 9.1 g; Na_2HPO_4 9.5 g; NaCl 20 g; urea 20 g; agar 20 g; phenol red 4 ml of 0.25% solution; distilled water 1000 ml; pH 6.8) was used to test the production of urease. The above ingredients except urea were dissolved in 950 ml of distilled sea water and autoclaved at 15 lbs for 15 minutes. Urea was sterilised using diethyl ether and dissolved in 50 ml sterile distilled water. This urea was added to the basal medium, dispensed into test tubes (3 ml each) and slants were prepared. Cultures were inoculated and after incubation a change of colour in the medium to pink was noted as urea hydrolysis. The actinomycetes utilize urea and liberate ammonia, making the medium alkaline, which is indicated by a pink colour in the medium.

2.2.4.4 Nitrate reduction test

Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate reduction test was carried out in nitrate broth supplemented with 0.1% potassium nitrate and Durham's tube was introduced (inverted) into the medium (Peptone 5.0 g; beef extract 3.0 g; potassium nitrate 1.0 g; seawater 1000 ml; pH 7.2). Spore suspension of isolates were inoculated into the medium and incubated for one week. Uninoculated tubes were used as negative control. Nitrate reduction was detected by adding equal volumes of reagents A and B to the inoculated tubes.

A red coloration in the medium due to formation of azo dye indicates positive nitrate reduction test. If the suspension was colorless after the addition of reagents A (α-naphthyl amine 0.5 g; acetic acid (5 N) 100 ml) and reagent B (sulfanilic acid 0.8 g; acetic acid (5 N) 100 ml) a small pinch of zinc dust was added to the medium. If the medium remained colorless after the addition of zinc powder, the test result was considered positive. If the medium turns pink after the addition of zinc powder, the result was considered negative.

2.2.4.5 Hydrogen sulfide production

Hydrogen sulphide production was detected along with nitrate reduction test by inserting sterile lead acetate strips (5%) through the mouth of the test tube in the nitrate broth. Blackening of the strips was scored as positive.

2.2.4.6 Citrate utilization test

Utilization of sodium citrate (0.1%) as sole source of carbon was detected in Simmon's citrate agar medium (Himedia) of the following composition: (sodium citrate 0.2 g; $MgSO_4.7H_2O$ 0.02 g; NaCl 15.0 g; $(NH_4)_2HPO_4$ 1.0 g; K_2HPO_4 1.0 g; bromothymol blue 0.02 g; agar 20 g; seawater 1 L; pH 6.9). Change in colour of the medium from green to prussian blue was recorded as positive.

2.2.4.7 Melanin production ability

Melanin production ability of actinomycetes was tested using Peptone yeast extract iron agar (ISP6) (Peptone 15 g; proteose peptone 5 g; ferric ammonium citrate 0.5 g; dipotassium phosphate 1 g; sodium thiosulphate 0.08 g; yeast extract 1.0 g; agar 20 g; pH 7.2; sea water 1L) and Tyrosine agar (ISP 7) (Glycerol 15.0 g; L-tyrosine 0.5 g; L-asparagine 0.5 g; K_2HPO_4 0.5 g;

$MgSO_4.7H_2O$ 0.5 g; $FeSO_4.7H_2O$ 0.01 g; seawater 1 L; agar 20 g; pH 7.2). Loopful of spores were inoculated on to the agar slants and incubated at 28°C for 5-7 days. Brown to back diffusible pigment in the medium was scored as positive. Absence of brown to black colour, or total absence of diffusible pigment, was considered as negative for melanin production.

2.2.4.8 Carbohydrate utilization test

The ability of strains to utilize and produce acid from various carbon sources was studied by the method recommended in the International *Streptomyces* Project. Utilization of carbon sources like glucose, lactose, mannitol, sucrose, arabinose, trehalose, inositol, ribose, sorbitol and xylose were tested on basal carbohydrate utilization agar (ISP 9) ($(NH_4)_2HPO_4$ 1 g; KCl 0.02 g; $MgSO_4$ $.7H_2O$ 0.2 g; agar 20 g; seawater 1 L). Fifteen milliliters of a 0.04% solution of bromocresol purple was added to each liter of basal medium, and the pH was adjusted to 7.0. Carbohydrate solution (1%) of each carbon source sterilized by seitz filtration was added to sterile basal media (Nonomura, 1974) and the media was dispensed into test tubes (3 ml) as agar slants. Basal media without supplemented carbon source was used as the control tube. The isolates were inoculated into respective carbohydrate agar slants and incubated at 28°C for 5-7 days. An acid reaction indicated by the change in colour of the carbohydrate medium from purple to yellow indicated a positive result.

2.2.5 Screening of marine actinomycetes for biogranulation property

The marine actinomycete isolates were tested for the ability to produce biogranules (self-immobilization). Spores from single colony of actinomycete isolates were inoculated into nutrient broth (50 ml) prepared with sea water.

The inoculated broth was placed on an incubator shaker set at 150 rpm, 28°C for 7 days. The culture broth was observed at regular intervals for the formation of biogranules of each strain. The size of the biogranules were measured and recorded as small (0.5-2mm), medium (2-4mm) and large (>4mm) based on the diameter of the granules.

2.2.6 Screening of marine actinomycetes for antibacterial activity

All the marine actinomycete isolates were tested for their ability to produce antibacterial activity. Spores of actinomycete cultures were inoculated into nutrient broth (50 ml) prepared with sea water, in a 250 ml conical flask. The inoculated broth was placed on an incubator shaker set at 150 rpm, 28°C for 7 days. The culture broth was centrifuged at 10,000 rpm at 4°C for 10 minutes to obtain cell free supernatant. Cell free supernatant of the culture broth was used for antibacterial assay.

The cell free supernatant of the actinomycete culture broth was tested mainly against aquaculture and a few human pathogens. The pathogens included both Gram positive and Gram negative bacteria procured from National Centre for Aquatic Animal Health, CUSAT. The pathogens selected for the study were *Vibrio harveyi* (BCCM 4044), *Vibrio parahaemolyticus* (MTCC 451), *Vibrio alginolyticus* (MTCC 4439),*Vibrio fluvialis* (BCCS 11654) *Vibrio proteolyticus* (BCCM 3772), *Vibrio cholera* (MTCC 3906) *Staphylococcus aureus* (MTCC 3061)*Bacillus cereus* (MTCC 1272) *Aeromonas hydrophila (MTCC 1739),Pseudomonas aeruginosa* (MTCC 741) *Escherichia coli* (MTCC 1610) and *Edwardsiella tarda* (MTCC 2400).

Kirby Bauer disc diffusion method was employed for testing antibacterial activity. Muller Hinton agar plates were prepared and swab inoculation of the pathogens was made on the surface to produce a lawn

culture. Sterile filter paper discs impregnated with cell free supernatant (20 µl) of the culture broth was placed on the surface of inoculated agar. The plates were incubated at 28°C for 24-48 hours and inhibition was observed as zone of clearance. Zone of inhibition was measured and recorded.

2.2.7 Screening for hydrolytic enzyme production

The marine actinomycete isolates (230 Nos.) were screened for the production of various hydrolytic enzymes, viz., amylase, lipase, protease, chitinase, aryl sulfatase, phosphatase, ligninase, pectinase, DNase and cellulase.

2.2.7.1 Amylase, Protease, and Lipase:

Nutrient agar medium (peptone 0.5 g; beef extract 0.3 g; agar 2 g; sea water 100 ml; pH 7.0) supplemented with starch (1%), gelatin (2%) and tributyrin (1%) were prepared separately for the respective enzyme assays. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 3 to 5 days and observations were made. Starch agar plates were flooded with grams iodine solution (Iodine 1 g; potassium iodide 2 g; distilled water 300 ml) and gelatin agar plates with mercuric chloride solution (15%). Appearance of clearance zone around the colonies was noted as positive and the diameter of the zone was recorded. Tributyrin agar plates were noted for a clear zone around the colonies for lipase production.

2.2.7.2 Pectinase:

Pectin Agar (pectin 0.5g; $CaCl_2.2H_2O$ 0.02g; NaCl 2g; $FeCl_3.6H_2O$ 0.001g; yeast extract 0.1g; agar 2g; seawater 100ml; pH 7.0) was used for testing the production of pectinase. The plates were spot inoculated and

incubated at room temperature (28 ± 2°C) for 5 to 7 days. After incubation, the plates were flooded with 1% cetavlon (cetyl trimethyl ammonium bromide), allowed to stand for 20-30 minutes and the zone of clearance was noted as positive.

2.2.7.3 Cellulase:

Cellulose agar (casein hydrolysate 0.05 g; yeast extract 0.05 g; $NaNO_3$ 0.01 g; cellulose powder 0.5 g; agar 2 g; seawater 100 ml; pH 7.0) was used for testing cellulase production. The plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 7 to 10 days. The zone of clearance around the colonies was noted as positive.

2.2.7.4 DNase:

The isolates were spot inoculated on DNAse agar (Tryptone 3 g; DNA 0.2 g; agar 2 g; sea water 100 ml; pH 7.0). After incubation at 28 ± 2°C for 5 days, the plates were flooded with 1 N HCL. Zone of clearance around the colonies were recorded as positive.

2.2.7.5 Aryl sulfatase:

Nutrient agar (peptone 0.5 g; yeast extract 0.1 g; ferric phosphate 0.002 g; agar 2 g, seawater 100 ml, pH 7.0) supplemented with Tripotassium phenolphathalein disulfate (PDS) was used for the production of aryl sulfatase. The plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 5-7 days. After incubation the agar plates were exposed to ammonia vapour, development of pink colour around the colonies due to the release of phenolphthalein from PDS was recorded as positive.

2.2.7.6 Phosphatase:

The marine actinomycete isolates were spot inoculated on nutrient agar basal media supplemented with 0.01% phenophthalein diphosphate. Incubated at room temperature (28 ± 2°C) for 5-7 days. After incubation the agar plates were exposed to ammonia vapour, development of pink colour around the colonies due to the release of phenolphthalein from phenolphthalein diphosphate was recorded as positive.

2.2.7.7 Ligninase:

Crawford's agar (Glucose 0.1 g; yeast extract 0.15 g; Na_2HPO4 0.45 g; KH_2PO_4 0.1 g; $MgSO_4$ 0.002 g; $CaCl_2$ 0.05 g; agar 2 g; sea water 100 ml; pH 7.0) was used as the basal medium for testing lignin degradation. The basal medium was supplemented with 0.02% methylene blue and the plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 5 to 14 days. Formation of halo zone or decolourisation of methylene blue was considered as positive.

2.2.7.8 Chitinase:

Chitinase activity of the isolates was tested by spot inoculation on chitin agar media prepared with 5% colloidal chitin in mineral basal media. The composition of the media is as follows: Colloidal chitin 5g; K_2HPO_4 0.07g; KH_2PO_4 0.03g; $MgSO_4.7H_2O$ 0.05g; $FeSO_4.7H_2O$ 0.001g; $ZnSO_4.4H_2O$ 0.0001g; $MnCl_2.4H_2O$ 0.0001g; agar 2g; seawater 100 ml pH 7.0). Incubation of the plates was done at 28°C for 7-14 days and the zone of clearance was recorded.

2.2.7.8.1 Preparation of colloidal chitin

Purified chitin (15 g) was dissolved in 50% H_2SO_4 (100 ml), and mixed by stirring in ice bath. After one hour, 2 L of distilled water was added to the dissolved chitin for precipitation. The suspension was kept overnight at 4°C, supernatant decanted and pH adjusted to neutral. Colloidal chitin collected by centrifuging at 5000 rpm for 10 minutes was stored at 4°C.

2.3 Results

2.3.1 Morphological and Cultural characterization

The actinomycete isolates exhibited good growth in ISP medium (ISP 5) and starch casein agar. Majority of the isolates grew within 3-5 days and sporulation was noticed after 5-7 days. Approximately 12% isolates were slow growing, and distinct colony appearance was noticed only after 3 days. The colony texture of isolates in the ISP media varied from powdery, cottony, and velvety to leathery colonies. The appearance of colonies ranged from concentric, wrinkled, umbonate, and chrysanthemum (radial furrows) type (Fig. 2.1). The spore mass colour of actinomycetes is considered taxonomic criterion for grouping of actinomycetes. Among the 230 marine actinomycete isolates, 134 isolates produced white/off white spore mass, 48 isolates exhibited ash/grey, 20 produced yellow spore mass colour, 19 isolates had green spore mass and 9 isolates were in pink/red series (Fig. 2.2). Only six isolates produced a diffusible pigment in almost all the culture media viz., starch casein agar, glycerol asparagine agar and yeast extract malt extract agar and nutrient agar. The colour of the substrate mycelium in starch casein agar, glycerol asparagine agar, yeast extract malt extract agar and nutrient agar were observed and varied from off white to pink, red, grey, green and yellow.

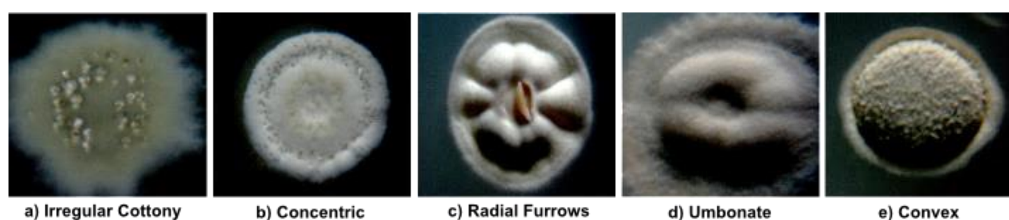


Fig. 2.1 Colony appearance of actinomycete isolates under stereomicroscope

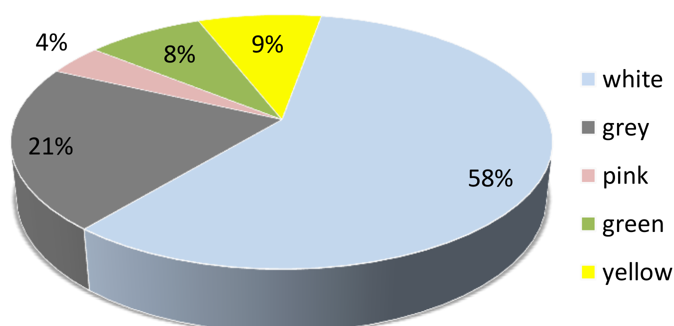


Fig. 2.2 Percentage of actinomycete isolates with different spore mass colour

The spore chain morphology of actinomycetes grown in coverslip observed under high power and oil immersion objectives revealed four types of spore chain morphology. The most prominent spore chain morphology was the spiral one, and 34% of the cultures exhibited spiral spore chain (mostly verticillate type) followed by 28% exhibiting rectiflexibiles (straight to flexuous) and 13.9% retinaculiaperti (open hooks, loops or spirals with one to two turns) spore chain morphology. (Fig.2.3 and Fig. 2.4) Remaining 23.9% of the isolates exhibited long chain of spores with zigzag fragmenting hyphae.

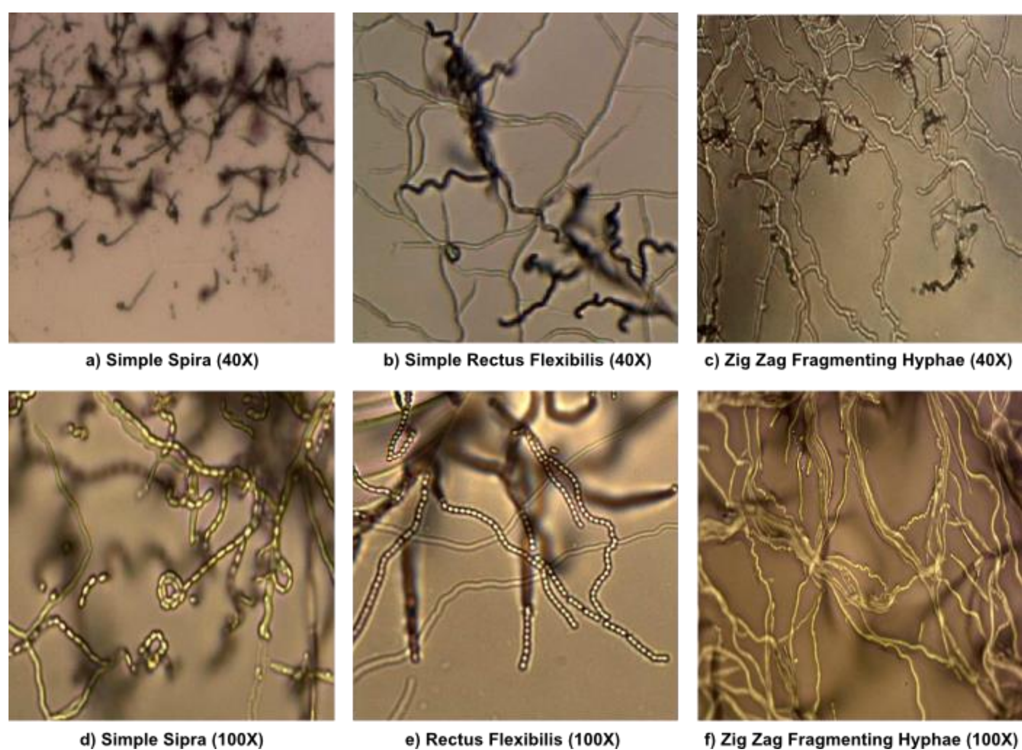


Fig. 2.3 Microscopic appearance of spore chain morphology of actinomycete isolates

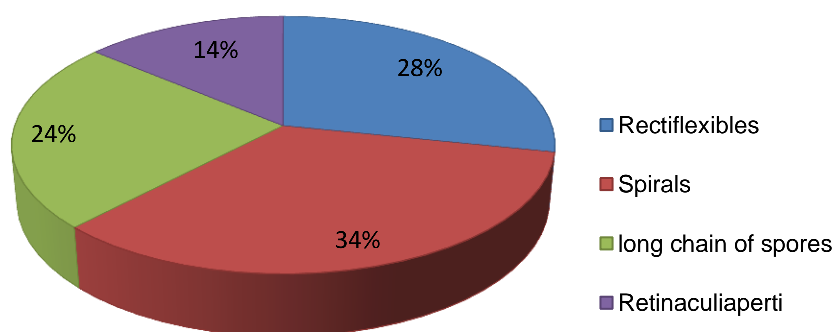


Fig. 2.4 Relative spore chain morphology of actinomycete isolates

2.3.2 Generic composition

Based on the morphological and biochemical characteristics, the marine actinomycete isolates from Arabian Sea and Bay of Bengal were classified mainly into two genera, i.e., 175 isolates with characteristic spore chain morphology as belonging to genus *Streptomyces* and 55 isolates with long chain of spores and zigzag fragmenting aerial hyphae as *Nocardiopsis* (Fig. 2.5).

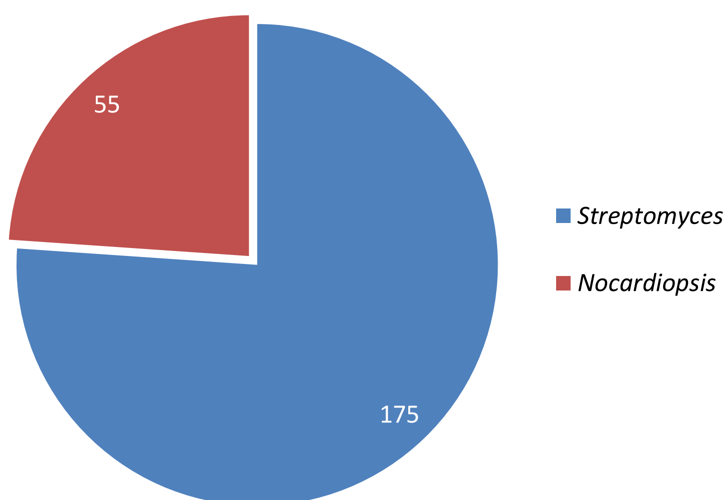


Fig. 2.5 Generic composition of actinomycetes in marine sediments of Arabian Sea and Bay of Bengal

2.3.3 Biochemical and physiological characterization

Decomposition of various substrates such as xanthine, hypoxanthine and tyrosine carried out in respective agar media revealed that of the isolated 230 strains of marine actinomycetes, 86.47% had the ability to decompose tyrosine, 77.39% isolates decomposed hypoxanthine and 67.42% decomposed xanthine. Decomposition of casein (1%) by actinomycete isolates were tested on skim milk agar. Of the total, 73.91% were able to decompose casein which was indicated by a clearing zone around the colonies. Esculin decomposition was carried out by 90.43% of the actinomycete isolates.

Melanin production ability was exhibited by only 4% isolates on peptone yeast extract iron agar and tyrosine agar. Only *Streptomyces* isolates (4%) were able to produce melanin. None of the *Nocardiopsis* isolates produced melanin. Almost all the isolates were able to grow in the presence of lysozyme, i.e. lysozyme resistant. 94.35 % were able to hydrolyse urea with the production of urease enzyme. Nitrate reduction was carried out by 69.57% actinomycete isolates, while 59.13% was able to produce hydrogen sulphide indicated by blackening of lead acetate strips and only 13.48% isolates were able to utilize citrate as the sole carbon source (Fig. 2.6).

Among the 175 *Streptomyces* isolates identified, 154 strains (88%) decomposed esculin, 117 (67%) decomposed tyrosine, 125 (71%) decomposed hypoxanthine, and 90 (51%) decomposed xanthine. Out of the 55 *Nocardiopsis* isolates, 54 (98%) decomposed esculin, 39 (71%) decomposed casein, 30 (55%) decomposed tyrosine, 53 (96%) decomposed hypoxanthine and 30 (55%) isolates decomposed xanthine. Relative biochemical profile of *Streptomyces* and *Nocardiopsis* revealed almost equivocal reactions, with

slight increase in nitrate reduction ability for *Streptomyces* compared to *Nocardiopsis* (Fig. 2.7).

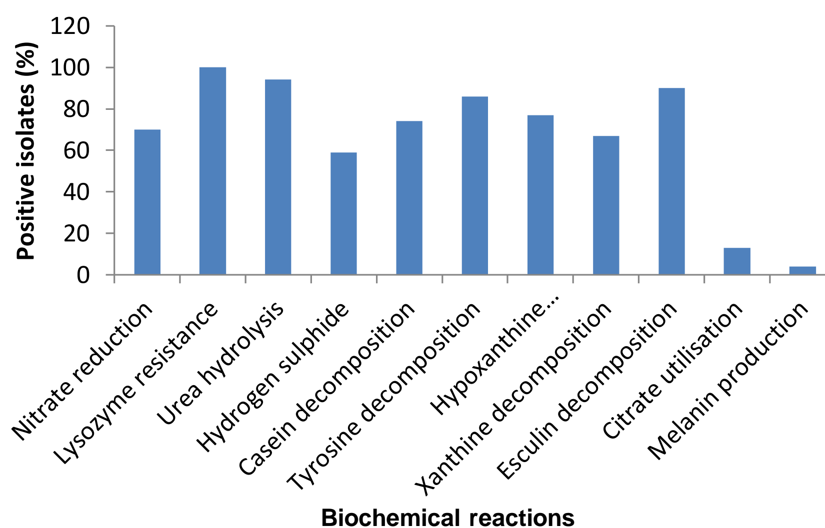


Fig. 2.6 Biochemical profile of actinomycete isolates

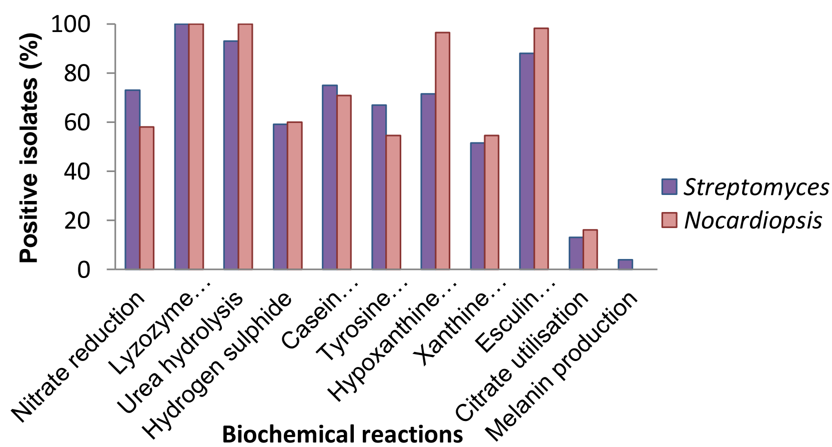


Fig. 2.7 Biochemical profile of *Streptomyces* and *Nocardiopsis* isolates

2.3.3.1 Carbohydrate utilization test

The entire carbohydrate source ranging from monosaccharides, disaccharides to sugar alcohols were utilized by actinomycete isolates. The carbon source most utilized by all the isolates was the hexose sugar glucose and the pentose sugar rhamnose. Rhamnose was utilized by 98.7% isolates followed by glucose (97.4%), trehalose (94.35%), inositol and galactose (92.61% each), arabinose (92.17%), mannitol (90.43%), sorbitol (85.22%), lactose (81.3%) and xylose (76.96%). Heavy growth and sporulation was observed for almost all cultures in media supplemented with pentose sugar rhamnose. Acid production from carbohydrates varied considerably among the isolates i.e., 81.3% of the isolates were capable of acid production from glucose followed by mannitol (74.35%), galactose (63.04%), rhamnose (58.70%), xylose (58.26%), lactose (54.78%), trehalose (53.04%), arabinose (51.3%), sorbitol (20.0%) and inositol (19.13%) (Fig. 2.8). *Streptomyces* spp. were found to be better in acid production (in terms of percentage of positive isolates) compared to *Nocardiopsis* spp. (Fig. 2.9).

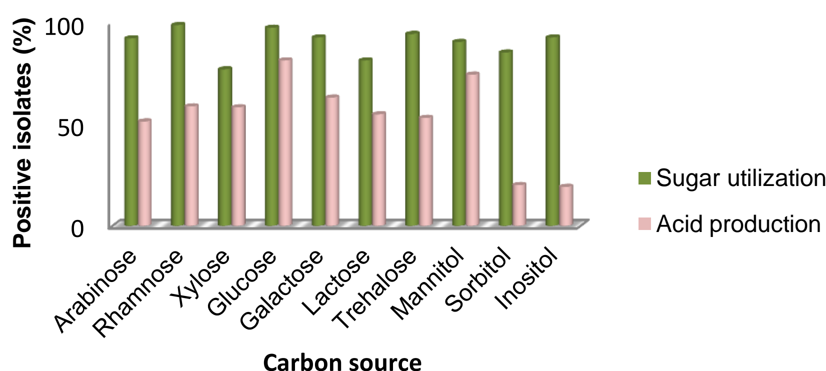


Fig. 2.8 Carbohydrate utilization profile of actinomycete isolates

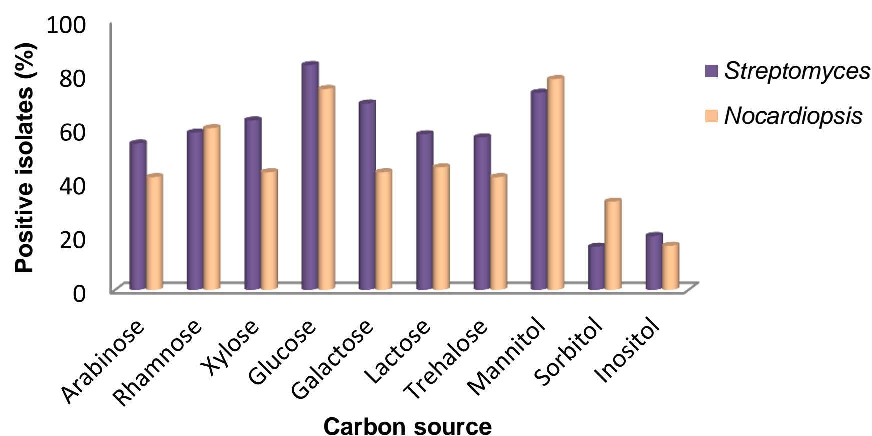


Fig. 2.9 Acid production ability of *Streptomyces* and *Nocardiopsis* isolates

2.3.4 Screening of marine actinomycetes for biogranulation property

Of the total 230 marine actinomycetes screened for biogranulation property, 82% isolates had the ability of biogranulation. 61% of the isolates produced small granules (0.5-2mm), 9% isolates produced medium sized granules (2-4mm) and 3% isolates produced large granules (>4mm). Mixed granules of different sizes were produced by 9% isolates and 18 % isolates did not produce granules (Fig 2.10).

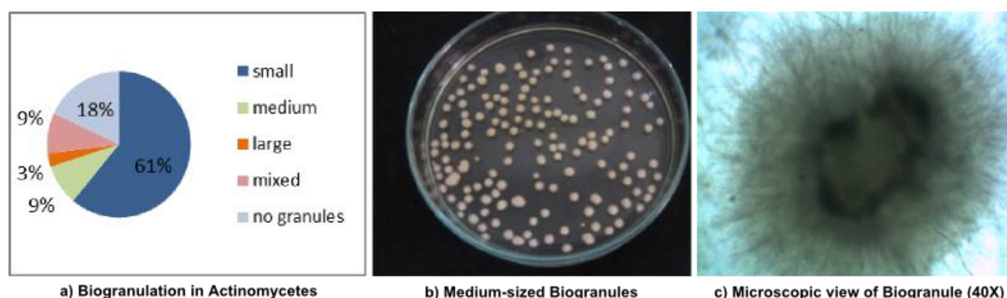


Fig. 2.10 Biogranulation in marine actinomycetes

2.3.5 Screening of marine actinomycetes for antibacterial activity

The marine actinomycete isolates were screened for antibacterial activity by disc diffusion assay. Percentage of actinomycetes which inhibited *Bacillus cereus* was 24% isolates, followed by *Staphylococcus aureus* (18.8%), *Vibrio alginolyticus* (13.4%), *Aeromonas hydrophila* (8%) and *Vibrio fluvialis* (8%). *Vibrio cholera*, *Vibrio parahaemolyticus* and *V.harveyi* was inhibited by 5% of the actinomycete isolates (Fig. 2.11). Two percent of the isolates inhibited *Pseudomona*s *aeruginosa* and none of the isolates had activity against *E. coli* and *Edwardsiella tarda*. Actinomycete M56 isolated from sediments of Bay of Bengal exhibited broad spectrum activity against Gram positive and Gram negative bacteria especially *Vibrio* spp.

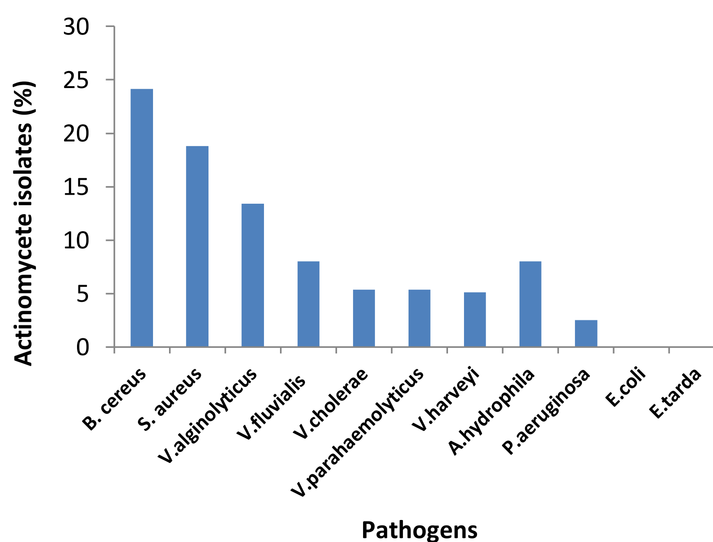


Fig. 2.11 Percentage of actinomycete isolates with antibacterial activity

2.3.6 Screening for hydrolytic enzyme potential

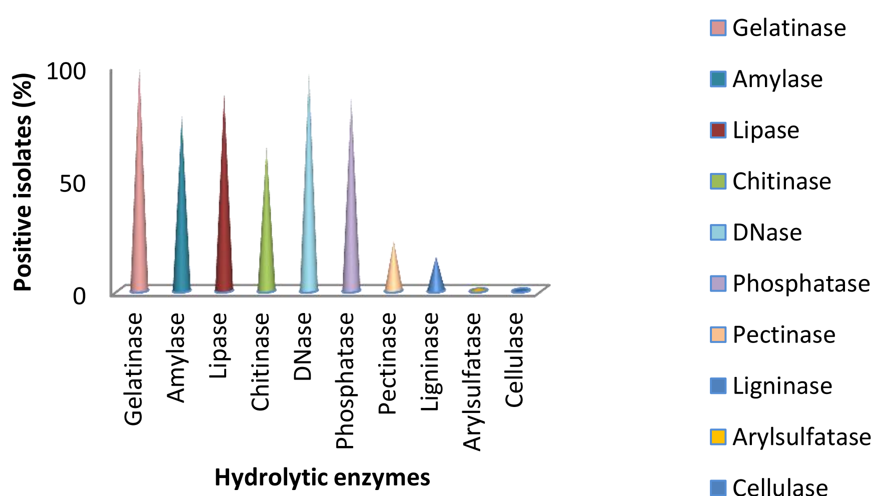


Fig. 2.12 Hydrolytic enzyme profile of marine actinomycete isolates

The actinomycetes isolated from the marine sediments invariably had the potential to elaborate a wide array of enzymes, ranging from gelatinase (99.13%) to ligninase (15.22%). DNase activity was exhibited by 96.09%, followed by lipase (86.96%), phosphatase (84.78%), amylase (76.96%), chitinase (63.48%), pectinase (22.17%), and only very few isolates showed arylsulfatase activity (1.08%). None of the strains exhibited cellulase activity (Fig. 2.12 and Fig. 2.13).

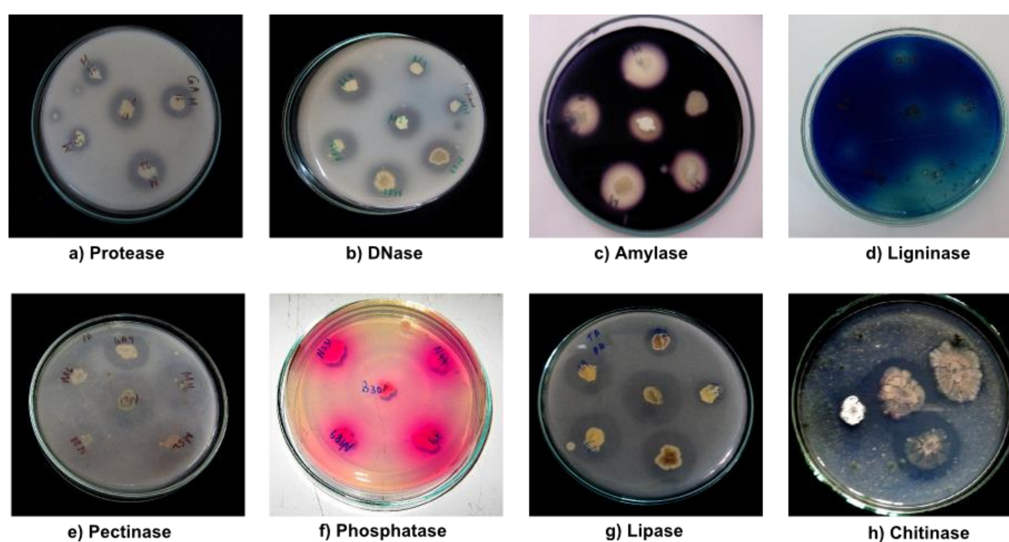


Fig. 2.13 Hydrolytic enzyme activity of marine actinomycetes on respective agar media

2.4 Discussion

Microbial systematics, physiology and natural product chemistry are underpinning disciplines in unraveling biodiscovery of marine natural products (Bull and Stach, 2007). Maximizing the novel bio discovery process lies in the fact that understanding of the actinomycete systematics from the vast ocean realm is indispensable. Little is known about the actinomycete diversity in marine sediments, which is an inexhaustible resource that has not been properly exploited (Chacko et al., 2012; Karthik et al., 2010).

Chromogenicity of aerial mycelium is considered an important character for grouping of actinomycetes (Pridham and Tresner, 1974). It was found that the actinomycete cultures, isolated from sediments of Arabian Sea and Bay of Bengal were morphologically distinct on the basis of spore mass colour, aerial, substrate mycelium, pigmentation, spore chain morphology etc. Majority of isolates were white, followed by grey, yellow, green, and pink spore mass

respectively. Out of the total isolates, 58.26% exhibited white/offwhite spore mass with dark brown colony reverse colour and 20.8% grey spore, 8.7% yellow spore mass, 8.26% green spore mass and 4% isolates had light red/pink spore mass. Similar colour series of actinomycetes was recorded by previous workers (Baskaran et al., 2011; Das et al., 2008; Patil et al., 2001; Sujatha et al., 2005). In a recent investigation on actinomycetes from soil samples of Egyptian localities, potent antimicrobial compound producing actinomycete isolates belonged to the yellow, grey and white colour series (Atta, 2012). Actinomycete strains from South Pacific coast of Philippines revealed that most (54%) of the isolates belonged to white and grey color series (Parthasarathi et al., 2012). Interestingly, grey and white pigmented (mycelial) marine actinomycetes were reported as prominent in the Bay of Bengal (Ramesh and Mathivanan, 2009) which was observed in the present study also.

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 as one of the important characteristics in the identification of *Streptomyces* and it greatly varies among the species (Tresner et al., 1961). It has been already reported that the majority of the marine streptomycete 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. The reports of the present study also agree with the spiral spore chain in majority of marine actinomycetes.

In the present study of marine actinomycetes from the continental shelf and slope sediments of south west and south east coast of India, *Streptomyces* species (76%) was found to be the dominant genera and (24%) belonged to

rare actinomycetes, mostly *Nocardiopsis*; the mature aerial mycelium showed the typical zigzag formation that fragments which is diagnostic for *Nocardiopsis* and related taxa (Labdae et al., 1984). Actinomycete populations from islands of the Andaman coast of Bay of Bengal was investigated and found that 72% isolates were streptomycetes (Peela et al., 2005) which supported the results of the present study. Similarly predominance of *Streptomyces* in marine sediments was reported in sediments collected from the coastal areas of Gokharna and Muradeshwara of Karnataka state (Attimarad et al., 2012). Preliminary investigation on the distribution and generic composition of actinomycetes in marine sediments of continental slope of Bay of Bengal reported *Streptomyces* as dominant genus followed by *Micromonospora* and *Actinomyces* (Das et al., 2008).

Anzai et al. (2008) gave valuable supporting information for the predominance of *Streptomyces* (60% to 99%) in all marine environmental samples around Nagasaki Prefecture, Japan, and reported Nocardiopsaceae as the second most predominant organism in the environmental samples of Iki coast. This report is in agreement with the present findings of *Nocardiopsis* as the prevalent culturable rare actinomycete genera next to *Streptomyces* in the marine sediments. Actinomycetes isolated from near sea shore marine environment locations of Bigeum Island, of South Korea were assigned to the genus *Streptomyces* (66%) and the remaining were identified as *Nocardiopsis* (18%), on the basis of their morphological, physiological and biochemical properties (Parthasarathi et al., 2012). Isolation of *Nocardiopsis* sp. have also been reported from in shore marine environment and mangrove ecosystem at 8 different locations of Kerala, West Coast of India (Remya and Vijayakumar, 2008).

The isolation of the rarer, non streptomycete actinomycetes produce diverse and unique, unprecedented, sometimes very complicated compounds exhibiting excellent bioactive potency and usually low toxicity (Berdy, 2005; Kurtboke, 2010).

The results of the present study are in agreement with earlier findings which states that *Streptomyces* species are mainly found in shelf and shallow areas when compared to other genera of actinomycetes (Nithya et al., 2012; Thorne and Alder, 2002). Salient finding that relative numbers of *Streptomyces* species decreased with depth in marine sediments suggested that most of the *Streptomyces* species in such sediments originate from terrestrial sources, and have been washed off shore (Bredholdt et al., 2007; Jensen and Fenical, 1991; Weyland, 1981). However, it has been realized that there are several actinomycetes present in the ecosystem which either occur in fewer numbers or grow comparatively slowly or do not produce spores in abundance like the genus *Streptomyces* (Srinivasan et al., 1991).This could be a reason for the difficulty in isolation of rare actinomycete genera other than *Streptomyces* and *Nocardiopsi*s. Schutze and Kothe (2012) indicated that several morphological, physiological and reproductive characteristics of *Streptomyces* (filamentous growth, formation of hyphae and the production of spores) would allow it's species to occupy extreme environments.

Marine actinomycetes exhibited remarkable organic compound degradation capability. The present study highlights the metabolic potential of actinomycetes from marine sediments which is superior to that of high altitude shola soils (Varghese et al., 2012) and Venezualen soils (Taddei et al., 2006). The actinomycetes from marine sediments were ahead of the terrestrial counterparts in decomposition of tyrosine, hypoxanthine, xanthine and urease

activity while more than 50% of actinomycetes from soil samples (Shola soils and Venezuelan soils) and marine sediments decomposed esculin and casein.

The degradation of the substrates, casein, tyrosine and xanthine was variable according to each streptomycete isolate from Venezuelan soils (Taddei et al., 2006) which was observed in the present study also. In this study, the data generated by physiological and biochemical characterization have been used for the segregation of potential isolates with special properties for future work. Actinomycetes from marine habitats have evolved to tide over the harsh environmental conditions and have altered their biochemical and metabolic machinery. Outstanding and diverse physiological traits of microbial population can be attributed to their underlying genetic diversity and also their mechanisms of generating genetic variation (Li et al., 2013).

Out of the 230 isolates screened, only 4% isolates had the ability to produce melanin in the recommended melanin production media. The results correlated with previous findings where streptomycetes from various sources were screened and only less than 10% were found to produce melanin pigments (Devan, 1999; Dhevendaran and Annie, 1999; Mathew, 1995). Only very few actinomycete isolates from various ecological niches exhibited the ability to produce melanin. It was noticed that out of 30 isolates from Egyptian soil, only a single strain had the ability to produce melanin (Shaaban et al., 2013). Nine strains among 180 (5%) *Streptomyces* isolates from soil samples of Gulbarga region produced a diffusible dark brown pigment on both peptone-yeast extract iron agar and synthetic tyrosine agar (Dastager et al., 2006).

Carbohydrate utilization test plays a prominent role in the taxonomic characterization of actinomycetes. Carbohydrate utilization, reported by

Pridham and Gottlieb (1948) for species differentiation of actinomycetes was found to be confusing in the present study due to inconsistent results. The physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physiological conditions (Baskaran et al., 2011). The ability to utilize a wide range of substrates suggests better survival in different environments (Williams et al., 1989).

Actinomycetes have a reputation for marked nutritional versatility which is supported by the results of our analysis. In the present study, more than 75% of the isolates were able to utilize and grow in a variety of carbon sources. Earlier, Proser and Palleroni (1978) during their study on nutritional characterization of actinomycetes, found that actinomycetes utilized variety of sugars for their growth. The difference in carbon utilization may be as a result of availability of the carbon source and adaptation of isolates to different niches in the marine environment. Rhamnose was utilized by almost 99% of the isolates as the sole carbon source and was found to induce heavy growth and sporulation compared to other sugars. Marine actinomycetes grew poorly in the presence of D-xylose, but formed abundant mycelium on the media with D-glucose, L-arabinose, L-rhamnose and D-mannitol, similar observation was reported in an earlier study by Lyons et al. (1969).

Biogranules of various sizes were produced by 82% of the marine actinomycete isolates; majority (61%) produced small size granules. Even though few reports on granulation of fungus and anaerobic granulation is available, granulation property of actinomycetes is least investigated. It was reported that in *Streptomyces coelicolor*, spores germinate to form initial compartmentalized mycelia, which subsequently form spherical pellets that grow in a radial pattern. It was also found that, following a first growth phase, a first death phase is initiated at the center of the pellets and this central core

of dead cell mass grows radially, subsequently, a second growth phase is initiated by the appearance of secondary mycelium (Manteca et al., 2008). It was evident from the present study that concentration of spores in the inoculum influences the granule formation. A high concentration tends to produce a dispersed form of growth whilst a low concentration normally results in pellet formation (Lawton et al., 1989).

In our investigation on marine actinomycetes, all the isolates were screened for antibacterial property using very light inoculum of spores in the basal nutrient broth medium. This was an attempt to select the most potent strain which exhibits remarkable antibacterial activity unlike the production of secondary metabolites in an enriched medium. Approximately 50% of the isolates exhibited antibacterial activity against Gram positive and Gram negative pathogens. Previously it was reported that, out of 82 actinobacterial isolates collected from marine sediments of Bay of Bengal, about 25% had antimicrobial activity (Ellaiah et al., 2002). It is hypothesized that antibiotic production confers a competitive advantage to microbes and thus may contribute significantly to microbial fitness in the soil (Maplestone et al., 1992; Williams et al., 1989), likewise survival advantages in the marine environment. It was evident that size of the inoculum and media influence antibiotic production which is in agreement with previous studies. Earlier it was reported that size of inoculum affects both morphology and tetracycline production in *S. aureofacien*s (Young et al., 1987).

In a study, from the soil samples of Karanjal regions of Sundarbans of Bangladesh about 55 actinomycetes of different genera were isolated and screened for antibacterial activity and 36.36% isolates were active against the test organisms (Arifuzzaman et al., 2010). The antibacterial activity of streptomycete isolates from soil samples of West of Iran was investigated and

only 13.30% isolates showed activity against the test bacteria (Dehnad et al., 2010). Marine actinomycetes are indeed more potent than their terrestrial counterparts to tide over extremes of climatic conditions and hence elaborate wide spectrum bioactive compounds.

According to Sattler et al. (1998), most of the metabolites extracted from actinomycetes inhibit the growth of Gram positive bacteria, but are ineffective against Gram negative bacteria due to alteration of peptidoglycan and its building blocks in the cell wall. In the current study, on screening for antibacterial activity, the marine actinomycetes, showed better antagonistic activity against Gram positive than Gram negative bacteria which agreed with the earlier findings of actinomycetes from other marine environments (Arasu et al., 2008; Nithya et al., 2012; Sacramento et al., 2004; Valli et al., 2012). Actinomycetes screened from various ecological niches have proved *Streptomyces* sp. as the most potent in inhibiting Gram positive and Gram negative bacteria, which was evidenced in the present study also (Adegboye and Babalola, 2013; Rahman et al., 2011). Also, *Streptomyces* sp. exhibited significant antibacterial activity against pathogens i.e., *Bacillus*, *Staphylococcus* and *Vibrio* sp. as reported earlier (Devi et al., 2006; Dhanasekaran et al., 2009; Valli et al., 2012). Several researchers had already reported similar antimicrobial activity of actinomycetes against various human pathogens (Krishnakumari et al., 2006; Thangapandian and Ponmurugan, 2007).

A possible explanation for the positive reaction of different biochemical tests and extracellular enzymes is that the marine derived actinomycetes are metabolically active (Moran et al., 1995) and they are adapted physiologically to grow in sea water and sediments (Jensen and Fenical, 1991). With the advent of whole genome sequencing studies, it has become apparent that as

organisms adapt to the environment, deterioration and loss of many genes that are not required in the environment occurs and there are very few genes that are conserved (Case et al., 2007).

Very few published reports are available regarding the enzyme profile of marine actinomycetes. The results regarding enzymatic activities of actinomycetes are extremely interesting for biocatalytic exploitation (Torres et al., 2008). Screening of the actinomycetes for hydrolytic enzyme production showed that almost all the marine actinomycetes were gelatinase producers, while, previous reports from Bay of Bengal, reported only 116 out of 208 isolates as gelatinase producers (Ramesh and Mathivanan, 2009). Since proteins are one of the main components of sedimentary marine particulate organic matter (POM), proteases are the most abundant extracellular enzymes detected in marine bacteria isolated from the Antarctic and South China sea sediments (Tropeano et al., 2013; Zhou et al., 2009). Enzyme activity of actinomycetes from fresh water systems of Andhra Pradesh revealed 87% as protease producers and cent percent as amylase and lipase producers (Selvam et al., 2011). It is a well established fact that marine *Streptomyces* spp. are degraders of recalcitrant compounds chitin, pectin and lignin. More than 95% of the isolates showed at least one of the extracellular enzymatic activities, and among the 230 isolates tested, 26 strains (11.3%) produced up to 8 extracellular enzymes; majority belonged to *Streptomyces* spp. Tropeano et al. (2012) observed that in marine bacterial strains, when the presence of one extracellular hydrolytic activity was detected, production of other hydrolytic enzymes was frequently associated. With growing awareness on environmental protection, chemical catalysts are being replaced by microbial enzymes in various pharmaceutical, food, textile and agricultural industries. It is revealed that the Bay of Bengal is a potential source for a wide spectrum of

antimicrobial and industrial enzyme producing actinomycetes. Moreover, it can be an imperative resource for bioprospecting novel rare *Streptomyces* spp., which could yield valuable bioactive molecules (Ramesh et al., 2009). Many methods targeting complex phenotypic characteristics of prokaryotes suffer from differing degrees of reproducibility (Moore et al., 2010). It is essential that rigorous standardization for the analysis of phenotypic data is required along with the establishment of comprehensive databases for phenotypic characteristics of actinomycetes (Rossello-Mora, 2011; Tindall et al., 2010).

Even though the highlight of actinomycetes, preferably *Streptomyces* is antibiotic production, much interesting features of actinomycetes are still unexplored due to lack of characterization studies. The study unveils the metabolic potentialities of *Streptomyces* and *Nocardiopsis* from the marine habitat which would be promising as a source of bioactive molecules.

3

Molecular Identification and Phylogenetic Analysis of Marine Actinomycete Isolates

3.1 Introduction

Molecular ecological studies have revealed that deep-sea sediments contained more than 1300 different actinobacterial operational taxonomic units, most of which were predicted to represent novel species, genera and families (Stach and Bull, 2005). It is not surprising, therefore, that members of 50 genera, including 12 novel genera, of actinobacteria have been isolated from marine habitats (Goodfellow and Fiedler, 2010), but less effort has been devoted to molecular characterization and phylogenetic studies of actinobacteria inhabiting marine sediments. A fundamental understanding of their phylogenetic diversity and eco physiology, is essential to assess the ecological significance of this relatively overlooked component of the marine bacterial community (Gontang et al., 2007).

The application of molecular techniques to the analysis of bacterial genomes has contributed considerably to our knowledge of bacterial taxonomy. Occasionally biochemical characteristics of some organisms do not fit into patterns of any known genus and species (Woo et al., 2000). With the

discovery of the polymerase chain reaction (PCR) and DNA sequencing methods, elucidation of closely related taxon with better authenticity has been made effectively in comparison to other conventional methods (Bosshard et al., 2003; Clarridge, 2004; Lane et al., 1985). Besides this, phylogenetic analysis of 16S rRNA gene is an important area of evolutionary study (Michael and Sharon, 2007).

Traditional methods used for the identification of the aerobic filamentous actinomycetes are laborious, time consuming and often require a series of specialized tests (Harvey et al., 2001; Steingrube et al., 1995; Wilson et al., 1998). Therefore rapid molecular methods including polymerase chain reaction (PCR) using genus specific primers and amplified ribosomal DNA restriction analysis (ARDRA), would be extremely useful to discern between the genera and species of large number of environmental actinomycetes which helps in its identification (Khanna et al., 2011).

Vandamme and colleagues (1996), defined polyphasic taxonomy as a process that generated classifications based on a consensus data gathered by the available methods that would be consistent with phylogenetic classification. In their view, ‘phylogenies’ were to be established using 16S rRNA or 23S rRNA gene sequence data. Conventional and molecular taxonomy need to be exclusive but need to complement each other in a process called polyphasic taxonomy (Valente et al., 1999).

Considering the importance of *Streptomyces* and its relatives in terms of both biological behaviour and metabolic products, it becomes essential to understand its evolutionary relationships to other species in the diverse *Actinomycetales* order. A clear appreciation of the phylogenetic relationships and diversity of *Streptomyces* would be of great benefit, as it would not only

allow better taxonomy and identification but it would give insight into the evolution of antibiotic production in this industrially important genus (Embley and Stackebrandt, 1994). The use of phylogenetic and molecular evolutionary approaches has greatly helped the classification methods and differentiation of species (Babalola et al., 2009; Hozzein and Goodfellow, 2011).

Marine environment contains a wide range of distinct *Streptomyces* that are not present in the terrestrial environment (Valan et al., 2012b). Information obtained from a well-resolved phylogeny can be used for the comparison of genome sequences, comparative genome reannotation, and genome visualization. A robust phylogeny is central for ongoing efforts in many groups to reconstruct system-wide metabolic models of *Streptomyces* and related species (Borodina et al., 2005), which are used for systematic strain-engineering in biotechnology (Alam et al., 2010).

Comparison and analysis of nucleotide sequences between strains provides a rapid and accurate method of establishing relatedness. Techniques for carrying out the comparisons include DNA DNA hybridization and PCR based gene sequence analysis.

3.1.1 Molecular detection methods

DNA–DNA hybridization of total chromosomal DNA has been used to determine species identity within the actinomycetes. This is performed by monitoring the reassociation of single-stranded DNAs from different organisms. The degree of relatedness is expressed as percentage homology and the genomic definition of species is considered to encompass strains with ≥70% DNA–DNA relatedness and ≤50°C difference in the melting temperature (ΔT_m) between the homologous and heterologous hybrids formed using standard stepwise denaturation conditions (Wayne et al., 1987). Low-

frequency restriction fragment analysis (LFRFA) is another molecular technique that uses the entire bacterial chromosome to provide taxonomic information. The principle of the method is to digest total chromosomal DNA with restriction endonucleases that cut infrequently. In the case of actinomycetes, rare AT cutters are used. The resulting fragments are examined by Pulsed-Field Gel Electrophoresis (PFGE) to provide a restriction finger print that is indicative of relatedness. Beyazova and Lechevalier (1993) investigated 59 strains from eight species groups within the *Streptomyces* genus by this method and were able to demonstrate its utility for the clustering of some strains. There were however some discrepancies, for example *Streptomyces cyaneus* strains cluster at 90% similarity using physical tests, in contrast to LFRFA results where the same strains group at 58% similarity. Therefore, PFGE'LFRFA reflects the heterogeneity of the *Streptomyces cyaneus* cluster and is useful for finding very closely related strains, but it cannot resolve interspecific relationships; LFRFA can also be misleading if there are large chromosomal amplifications or deletions (Rauland et al., 1995).

Specific genes can be investigated for differences at the nucleotide level using either restriction analysis (Clarke et al., 1993; Fulton et al., 1995) or by monitoring the mobility of the product using specialized gel electrophoresis techniques (Clarke et al., 1993; Hain et al., 1997; Heuer et al., 1997). The potential of RFLP of rRNA for taxonomic differentiation and delineation of the *Streptomyces* genus using strains from the *Streptomyces albidoflavus* cluster (subgroups 1A and 1B) was investigated. Purified rRNA was restricted using a combination of enzymes (*Bg*1I, *Eco*RI, *Pst*I and *Pvu*II) and the patterns obtained varied between species groups and were sufficient for the differentiation of species, thus enabling phenotypically similar strains to be distinguished. Ribosomal restriction analysis was further developed by Fulton

et al. (1995) and *Mse*I fingerprints of rRNA operons were used to group 98 named streptomycete strains of the phenotypic cluster groups A (sub clusters 1–41) and F (sub clusters 55–67) (Williams et al., 1983a), but highly related strains could not be resolved by this method.

RAPD-PCR assays (Williams et al., 1990) use single primers with arbitrary nucleotide sequences to amplify DNA, using a low annealing temperature so that polymorphisms can be detected. RAPD is a rapid method by which actinomycetes can be screened for strain similarity. It requires stringent standardization of the reaction parameters including primer sequence, annealing temperatures, buffer components, concentration and quality of template DNA etc. The arbitrary primer sequence used can affect the usefulness of this technique; unless a highly specific actinomycete primer was used, reducing the effectiveness of the method as a taxonomic tool for species delimitation (Mehling et al., 1995). The use of RAPD PCR for the resolution of interspecific relationships among members of the *Streptomyces albidoflavus* cluster was evaluated and exemplified the variability of the profiles obtained with changes to the concentration of the target DNA (Huddleston, 1995) and the reaction mixture (Mehling et al., 1995). However, the interspecific relationship of *Streptomyces lavendulae* and *Streptomyces virginiae* could not be sufficiently resolved, though it was possible to distinguish between duplicate strains. The use of species specific primer is not applicable to actinomycetes from marine environment, as there are several species and prior knowledge of species is required to design the primer. Hence in the present study ARDRA, was used as a quick and easily accessible approach to group the large number of marine actinomycete isolates, in order to attain an ARDRA profile and select representative isolates for 16S rRNA gene sequence analysis and identification.

3.1.1.1 Amplified ribosomal DNA restriction analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) is a commonly used tool to study microbial diversity that relies on DNA polymorphism (Deng et al., 2008). Clones containing 16S rDNA gene fragments, obtained by applying either universal or genus specific primer sets, are amplified and digested by tetra cutter restriction endonucleases (REs), followed by separation of the resulting fragments on high density agarose gels. The pattern obtained is said to be representative of the species analysed and several restriction enzymes can be used to phylogenetically characterize cultured isolates and 16S genes obtained through cloning from community DNA (Sklarz et al., 2009).

Cook and Meyers (2003) developed a rapid method for identifying filamentous actinomycete genera based on 16S rRNA gene restriction fragment patterns. The patterns were generated by using specific restriction endonucleases to perform *in silico* digestion on the 16S rRNA gene sequences of filamentous actinomycete isolates from soil. Amplified 16S rDNA of soil actinomycetes was restricted with selected endonucleases, *Sau*3AI, *Asn*I, *Kpn*I and *Sph*I and the restriction fragment patterns of the unknown isolates were easily compared to the established patterns. Significantly, the genus *Streptomyces* could be differentiated from all other actinomycete genera by using only four restriction endonucleases.

ARDRA has been shown to be useful in differentiating between bacterial species within a genus and bacterial strains within a species (Gurtler et al., 1991; Kohler et al., 1991). As a 16S rDNA gene based molecular technique, ARDRA has been used for phylogenetic and taxonomic studies in *Clostridium* (Gurtler et al., 1991), *Streptococcus* (Jayaro et al., 1991), *Mycobacterium*

(Vaneechoutte et al., 1993), *Acinetobacter* species (Vaneechoutte et al., 1995), *Brevibacillus* (Logan et al., 2002). Heyndrickx and colleagues (1996) have developed an ARDRA method for identification of strains of the genera *Alcaligenes*, *Bordetella*, *Bacillus* and *Paenibacillus*. Medically important species of aerobic actinomycetes belonging to the genera *Actinomadura*, *Actinoplanes*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces* and *Tsukamurella* have also been identified using ARDRA (Franco-Correa et al., 2010; Harvey et al., 2001; Laurent et al., 1999; Steingrube et al., 1997; Wilson et al., 1998).

ARDRA was reported as a rapid and convenient method in grouping actinobacteria isolated from sponges (Jiang et al., 2010). The taxonomic utility of PCR restriction pattern analysis for rapid identification of clinical isolates of aerobic actinomycetes to genus level using four restriction endonucleases, *Sau3*AI, *Asn*I, *Kpn*I and *Sph*I was evaluated and found cost effective (Muharram et al., 2010).

3.1.2 Nucleic acid sequence comparisons

Zuckerkandl and Pauling (1965) first proposed the use of gene sequences as a molecular clock to decipher phylogenetic relationships. Woese and Fox (1977) introduced the use of rRNA genes for this purpose, which served as the basis for their definition of the three domains of life. DNA based molecular methods have been used for species differentiation and the identification of streptomycetes. The sequence analysis of the genes coding for the ribosomal subunits (16S, 23S, and 5S rRNA) in particular, the 16S rRNA gene has become an important tool in bacterial identification, since it provides information about the phylogenetic placement of species (Brenner et al., 2001; Woese, 1987). The 16S rRNA gene has been widely used for phylogenetic and

diversity studies for several reasons (Weisburg et al., 1991). It consists of conserved and variable regions which allow the development of primers and probes with variable levels of specificity. The conserved regions carry information about phylogenies at the higher taxonomic levels, since they have evolved slowly and are highly similar among the different taxa, whereas the variable regions have undergone more mutations during evolution, and are more useful for classification at the intraspecies level (Woese, 1987). However the 16S rDNA sequence information alone is not sufficient for species identification (Rossello-Mora and Amann, 2001).

Target sites other than rRNA viz., hsp 65 gene has been used for typing environmental and clinical isolates. Even though the method is sensitive, easy and rapid compared to 16S rRNA, it defines a greater number of RFLP genotypes within one species. However, the interspecies genetic diversity of the hsp 65 gene may not reflect phylogenetic relationships as reliably as do rRNA sequences (Dobner et al., 1996; Hughes et al., 1993; Roth et al., 2000).

The comparison of rRNA sequences is a particularly powerful tool in streptomycete taxonomy. *Kitasatosporia* was included in the genus *Streptomyces*, despite having differences in cell wall composition, on the basis of 16S rRNA similarities (Wellington et al., 1992). This was revoked by Zhang et al. (1997) who demonstrated that members of the genus *Kitasatosporia* always formed a stable monophyletic clade away from streptomycetes when sequences from the entire 16S rRNA genes were compared.

Molecular techniques as comparisons of the 16S rRNA sequences have been proven valuable to actinomycetes taxonomy (Zhi et al., 2009). rRNA sequence comparisons have also been useful for answering questions

concerning the horizontal transfer of genes within the genus (Huddleston et al., 1997). These genes are highly conserved within bacteria. Three regions within the 16S rRNA gene have been observed to have enough sequence variation so as to be useful for genus-specific (α and β regions) and species specific (γ regions) probes within the *Streptomyces* genus (Stackebrandt et al., 1992). 23S rRNA, 5S rRNA genes (Mehling et al., 1995) and ribosomal protein sequences have also been used to investigate species relationships within the genus *Streptomyces* (Liao and Dennis, 1994; Ochi, 1995). rRNA sequences used alone can be misleading due to intraspecific variation and intragenomic heterogeneity. Clayton et al. (1995) found a high degree of intra specific variation within the prokaryotic sequence database. Additional genes have been used to examine inter and intraspecific relationships in Frankia (Hirsh et al., 1995). Genes that are conserved between species, such as housekeeping genes (elongation factors and ATPase sub units), are the primary target genes to be studied (Ludwig and Schleifer, 1994). Tryptophan synthase genes was used, in addition to 16S rRNA comparisons to determine the phylogeny of streptomycin producing streptomycetes and provide evidence for the horizontal transfer of antibiotic resistance genes (Huddleston et al., 1997).

Sequence analysis of rRNA genes has been applied to streptomycete taxonomy to investigate relationships at the genus, species and strain level. Relationships obtained differed according to the variable region (α, β and γ) selected for comparison and correspond to nucleotides 982–998, 1102–1122 and 158–203 respectively. Kataoka et al. (1997) conducted a comprehensive study of the γ region from 89 streptomycete type strains that represented several of the major clusters as defined by Williams et al. (1983b). The study verified that these variable regions can be used to resolve inter and intra species relationships within the streptomycetes, despite being too variable for

determining generic relationships. It is also known that microbes with 16S rRNA sequence similarity up to 97% identical should be considered as members of the same genus (Petit et al., 1990). The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus level identification in most cases (>90%) but less so with regard to species (65-83%); about 1-14% of the isolates remaining unidentified (Drancourt et al., 2000; Mignard and Flandrois, 2006; Woo et al., 2003). Hain et al. (1997) also investigated the use of 16S rRNA probes to determine intraspecific relationships within *Streptomyces albidoflavus* and found sequence comparison to be useful for species delimitation, but of no value for strain differentiation.

These methods have now been superseded by multilocus sequence analysis (MLSA) as the use of multiple gene sequences helps to buffer distortions on phylogeny generated by recombination (Laskaris et al., 2012).

Multilocus sequence typing (MLST) is a widely used system for typing microorganisms by sequence analysis of housekeeping genes. The main advantage of MLST in comparison to other typing techniques is the unambiguity and transferability of sequence data (Boers et al., 2012). An alternative to DDH, MLSA scheme is of great biological significance by providing an elaborate taxonomic grouping of streptomycete diversity, and will benefit both ecology and bioprospecting of these ubiquitous microorganisms (Rong and Huang, 2010). MLSA is reliable and has sufficient interspecific discriminatory power for *Streptomyces* strains. The great extent of taxonomic diversity of this category could be a surrogate for chemical diversity (Jensen, 2010; Penn et al., 2009). MLSA scheme of five housekeeping genes (atpD, gyrB, recA, rpoB and trpB) is suggested as a

valuable alternative for creating and maintaining operational protocols for the *Streptomyces* species assignment (Rong and Huang, 2012).

3.1.3 Phylogenetic studies

While early research estimated low numbers and patchy distribution of actinomycetes in marine environment, (Jensen and Fenical, 1991; Mincer et al., 2002) later studies suggested higher abundance and diversity of actinobacteria with numerous novel taxa (Gontang et al., 2007). It was indicated that majority of Gram positive bacteria from tropical marine sediments in the intertidal zone of Republic of Palau belonged to the class actinobacteria (65.6%) as revealed by phylogenetic analysis of representative isolates, based on 16S rRNA gene sequence data. Based on molecular studies, representatives from six families have been recognized in the marine environment. The representative families are Micromonosporaceaea, Nocardiacaeae, Nocardiopsaceae, Pseudonocardiaceae, Streptomycetaceae, and Thermomonosporaceae (Das et al., 2008). The diversity of cultured actinomycete bacteria was compared between near and off shore marine sediments (San Diego and San Francisco) analyzed for 16S rRNA gene sequence diversity and indicated a high level of actinomycete diversity in marine sediments. Of the 8 genera recognized, *Streptomyces* was the dominant followed by M*icromonospora, Actinomadura, Saccharomonospora, Verrucosisspora, Nocardiopsis, Streptomonospora* and *Streptosporangium* (Prieto-Davo et al., 2008).

The streptomycete taxon, largest in class actinobacteria, and the most productive genera, contains more than 500 validly described species, representatives of which have been assigned to lumpy groups based on 16S rDNA sequence data (Bull et al., 2005). It is evident that the genus

Streptomyces remains underspeciated (Manfio et al., 2003; Sembiring et al., 2000) though several validly described species have become subjective synonyms of previously described species (Kumar and Goodfellow, 2010). Taxonomy of *Streptomyces* species needs to be based on a combination of phenotypic and genotypic properties (Kim et al., 2004; Saintpierre-Bonaccio et al., 2004) and even on a multilocus phylogeny (Guo et al., 2008). Tauqeer et al. (2010) determined that single gene analysis can lead to poorly resolved or even misleading streptomycete phylogeny and demonstrated a way of using whole genome analysis to overcome this limitation. They demonstrated that only by a combination of methodologies, it is possible to achieve a fully resolved phylogeny with detailed and well supported inner branches. Assessing the phylogeny of marine actinomycetes provides not only a sound basis for future taxonomic work but also a framework for the rational exploration of their ecology and biotechnological potential (Embley and Stackebrandt, 1994).

The genus *Nocardiopsis* was shown to be phylogenetically coherent and represent a distinct lineage within the order *Actinomycetales* and family Nocardiopsaceae (Rainey et al., 1996). The genus harbors 14 species and one subspecies. Recently, a preliminary investigation on the isolation of actinomycetes from hyper saline soils of Mongolia province, 9 strains among the group of moderate halophilic actinomycetes were phylogenetically detected under the genus *Nocardiopsis* and concluded *Nocardiopsis* as abundant in saline soil (Ara et al., 2013). Members of the genus *Nocardiopsis* have been reported to predominate in saline, alkaline soils (Li et al., 2003), hyper saline soils (Li et al., 2006) and several recognized species have been isolated from such sources (Al-Tai and Ji-Sheng, 1994; Al-Zarban et al., 2002; Chun et al., 2000; Hozzein et al., 2004; Li et al., 2004, 2003). An

actinomycete strain, *Nocardiopsis aegyptia* (SNG49T) representing a novel species within the genus *Nocardiopsis*, was isolated from marine sediment of Abu Qir Bay, on the western seashore of Alexandria, Egypt (Sabry, 2004).

Phenotypic traits are the observable characteristics that result from the expression of genes of an organism, but can largely be modulated by environmental or other conditions (e.g. growth conditions, like temperature, pH-value, etc.) (Moore et al., 2010). Even though they have formed the traditional basis of the characterization of microorganisms, they may comprise simply to recognize, very complex features, and the conditions in which these genes are expressed in an organism.

Major populations of marine actinomycetes reside in ocean sediments and these bacteria display highly evolved marine adaptations (Jensen et al., 2005b). Even though actinomycetes have being routinely isolated from marine environment of Indian peninsula and screened for bioactive compounds, very limited efforts have been directed to their molecular characterization or assessed the species composition in marine sediments. Hence, the objective of the present study was to identify the marine actinomycetes at species level based on 16S rRNA sequencing. For this ARDRA profile was generated and representative isolates were subjected to sequencing taking into consideration their spore morphology and other phenotypic characteristics also.

3.2 Materials and methods

3.2.1 Selection of actinomycete isolates

The marine actinomycete isolates identified up to generic level based on morphological and biochemical characterization were subjected to molecular characterization for species level identity. The isolates were clustered based on morphological, biochemical and physiological characterization by NTSYS clustering and a total of 120 representative isolates were selected for molecular characterization. Genomic DNA was extracted from selected isolates, 16S rDNA amplification was done and the amplicons were subjected to amplified ribosomal DNA restriction analysis (ARDRA). Based on ARDRA profile and the spore mass colour, the isolates (31 nos.) were selected for 16S rDNA sequencing and identified up to species level. In case of ambiguity other phenotypic characteristics were also taken into consideration for species level identification.

3.2.2 Genomic DNA extraction from actinomycete isolates

Total genomic DNA from the selected actinomycete strains was extracted as per standard protocol (Sambrook et al., 1989). Spore suspension of the isolate was suspended in nutrient broth and incubated in an orbital incubator at 30°C, 120 rpm for 48-72 hours to form a pellet of vegetative cells (pre sporulation). The cells were harvested by centrifugation at 15000 g for 10 minutes and then suspended in TEN buffer (100 mM Tris-HCl; 10 mM EDTA; 250 mM NaCl; pH 7.2) having 1% sodium dodecyl sulphate (SDS). Proteinase-K (20 mg/ml) was then added to a final concentration of 100 µg/ml and mixed gently. The suspension of lysed cells was cooled to room temperature and an equal volume of phenol equilibrated with 0.5 M Tris-HCl

(pH 8.0) was added and gently mixed. A third extraction with a 24:1 mixture of chloroform and isoamyl alcohol was carried out and the aqueous phase was collected carefully. DNA dissolved in solution were precipitated after the addition of 0.1 volume of 3.0 M Sodium acetate (pH 7.5) and 0.6 volume of isopropanol or 2 volumes of absolute ethanol. Incubating at -20°C for 12 hours precipitated the DNA. The precipitated DNA was pelleted by centrifugation at 10,000 g for 15 minutes at room temperature. The excess salt was removed by washing the DNA pellets three times in 70% ethanol. The DNA samples were dried under vacuum and dissolved in appropriate volume of TE buffer (10 mM Tris; 1 mM EDTA, pH 7.5) and stored at –20°C. Agarose gel electrophoresis was done to check the purity of DNA.

DNA concentration and purity was assessed spectrophotometrically by comparing absorbance at 260 and 280nm followed by 0.8% agarose gel electrophoresis. Concentration of DNA was found out from the following formula.

$$Conc. of\ DNA\ (\mu g/ml) = OD\ at\ 260\ nm \times 50 \times dilution\ factor$$

Where OD is the Optical Density.

3.2.3 Amplification of 16S rRNA gene

The 16S rRNA gene was amplified using the universal eubacterial primers 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). The primers were used to amplify nearly full length 16S rDNA sequences. PCR was performed in a 25 µl reaction volume containing 1x standard *Taq* buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer and 1U *Taq* DNA polymerase (Fermentas, Inc.). The thermal profile applied

was an initial denaturation of 95°C for 5 minutes, 35 cycles of denaturation (94°C for 20 s), annealing (58°C for 20 s), extension (72°C for 1 minute 30s), and a final extension (72°C for 10 minutes). The PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide (10 µg/ml) to ensure that a fragment of the correct size had been amplified. The gel was visualized on a Gel Documentation System (BIO-RAD).

3.2.4 Amplified ribosomal DNA restriction analysis (ARDRA)

To ascertain the polymorphic groups among the actinomycete isolates from marine sediments, the 16S amplicons of the selected isolates (120 nos.) were subjected to restriction enzyme digestions. The PCR products (0.5 – 1.0 µg) were digested with 3 different tetracutter restriction enzymes viz., *Sau*3A1, (G'ATC) *Hinf*1 (GANT'C) and *Taq*I (T'CGA) in separate reactions. The digestions were performed for 3-4 hours at an incubation temperature of 37°C for *Sau*3A1 and *Hinf*I and at 65°C for *Taq*1 recommended by the manufacturer. The reaction volume consisted of 20 µl containing 8 µl of the PCR product, 2 µl buffer (50 mM NaCl, 10 mM, Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) 8 µl of milliQ and 2 µl (5units) of each restriction enzyme. The restriction fragments were separated by horizontal electrophoresis on 2% agarose gel in 1x TAE buffer for 1 hour at 100 volts. A 100 bp ladder was used as the DNA marker (Fermentas, Inc.). All electrophoresis were carried out with 15x7 cm gels on a mini sub cell unit (10x7cms tray with 16 wells). After electrophoresis the gels were stained with ethidium bromide and photographed under UV transilluminator. Molecular weight determination of PCR products and digests were performed with the Quantity One software of BIO-RAD Geldoc XR unit. Gel images were analysed to construct ARDRA profiles for various actinomycete isolates.

3.2.5 Gene sequence analysis and species identification

ARDRA patterns of three different restriction digestions were analysed and dendrogram was constructed using Primer version 6.0. Based on the size of each ARDRA group, representative isolates, from each unique ARDRA profile were selected for sequencing.

The 16S rDNA amplicons of the segregated (based on ARDRA pattern) actinomycete isolates were purified using Promega PCR clean up system as per the manufacturer's instruction. The PCR products were sequenced using the universal 27F and 1492R primers with an ABI prism model 3700 Big Dye Sequencer (Applied Biosystems, USA) at SciGenom, Kochi, India. The nucleotide sequences obtained were assembled using Gene Tool software and the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at the National Centre for Biotechnology information (NCBI), USA (www.ncbi.nlm.nih.gov).

3.2.6 Nucleotide Sequence Accession Number*s*

The sequences were deposited in the GenBank database using the web based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt).

3.3 Results

3.3.1 Amplification of 16S rRNA gene

16S rRNA gene amplification using a set of universal eubacterial primers: 27F and 1492R yielded a single amplicon of approximately 1500 bp for all the marine actinomycete isolates (Fig. 3.1).

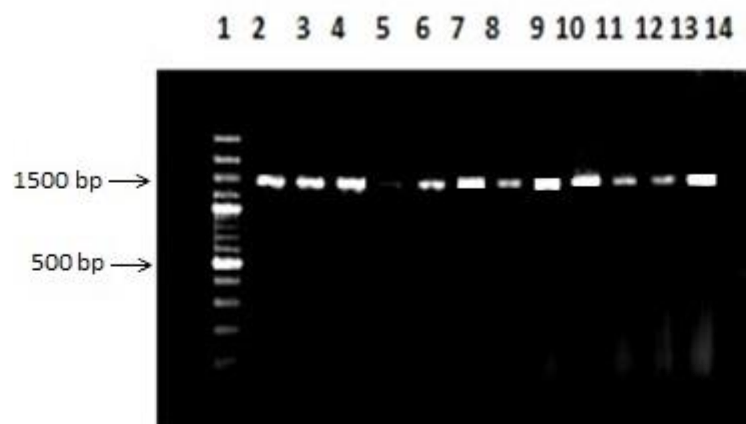


Fig. 3.1 Amplified 16S ribosomal DNA of actinomycete isolates run on 1% agarose gel. Lane 1(100 bp ladder)

3.3.2 ARDRA analysis

The 16S rDNA amplicons of actinomycete isolates subjected to ARDRA using *Sau*3A1, *Hinf*1 and *Taq*1 resulted in patterns. A total of eight polymorphic patterns were obtained for *Sau*3A1 (Fig. 3.2), ten patterns for *Taq*1 (Fig. 3.3) and only six patterns were generated by *Hinf*1 digestions (Fig. 3.4). Fragment smaller than 100 bp could not be reproducibly visualized and were not considered. Faint fragments on the gel which were probably due to incomplete digestion of the DNA were not considered to differentiate between the patterns.

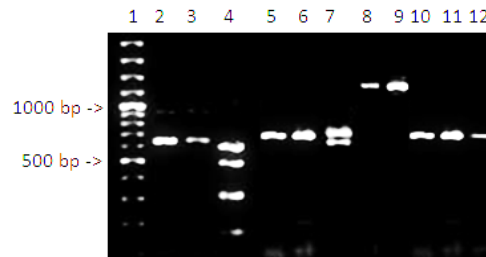


Fig. 3.2 ARDRA patterns of *Sau*3A1 restriction on 16S rDNA amplicons of marine actinomycete isolates

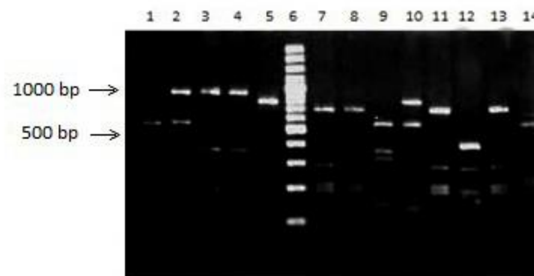


Fig. 3.3 ARDRA patterns of *Taq*1 restriction on 16S rDNA amplicons of marine actinomycete isolates (Lane 6 (100 bp ladder), 1-5 &7-14 samples)

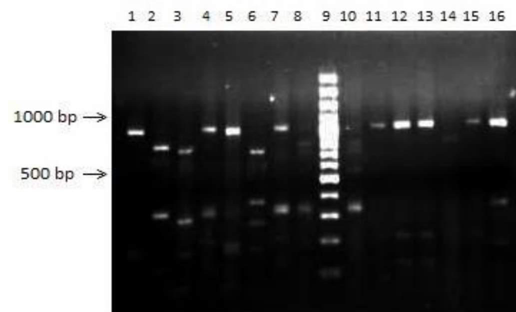


Fig. 3.4 ARDRA patterns of *Hin*f1 restriction on 16S rDNA amplicons of marine actinomycete isolates (Lane 9(100 bp ladder), 1-8 and 11-16 samples)

3.3.3 Gene sequence analysis and species identification

Dendrogram constructed based on ARDRA patterns obtained as a result of restriction digestions of 16S rDNA amplicons of marine actinomycete isolates using *Sau*3A1,(Fig. 3.5) *Hinf*1 (Fig. 3.6) and *Taq*1(Fig. 3.7) are given below. Gene sequencing of representative isolates having dissimilar ARDRA patterns resulted in 23 species representing 2 genera i.e., the dominant genera *Streptomyces* followed by *Nocardiopsis*. Nineteen species could be identified from the genera *Streptomyces* and 4 species from the genus *Nocardiopsis*. ARDRA profile and sequence identity of representative marine actinomycetes isolates based on spore mass colour are given below.

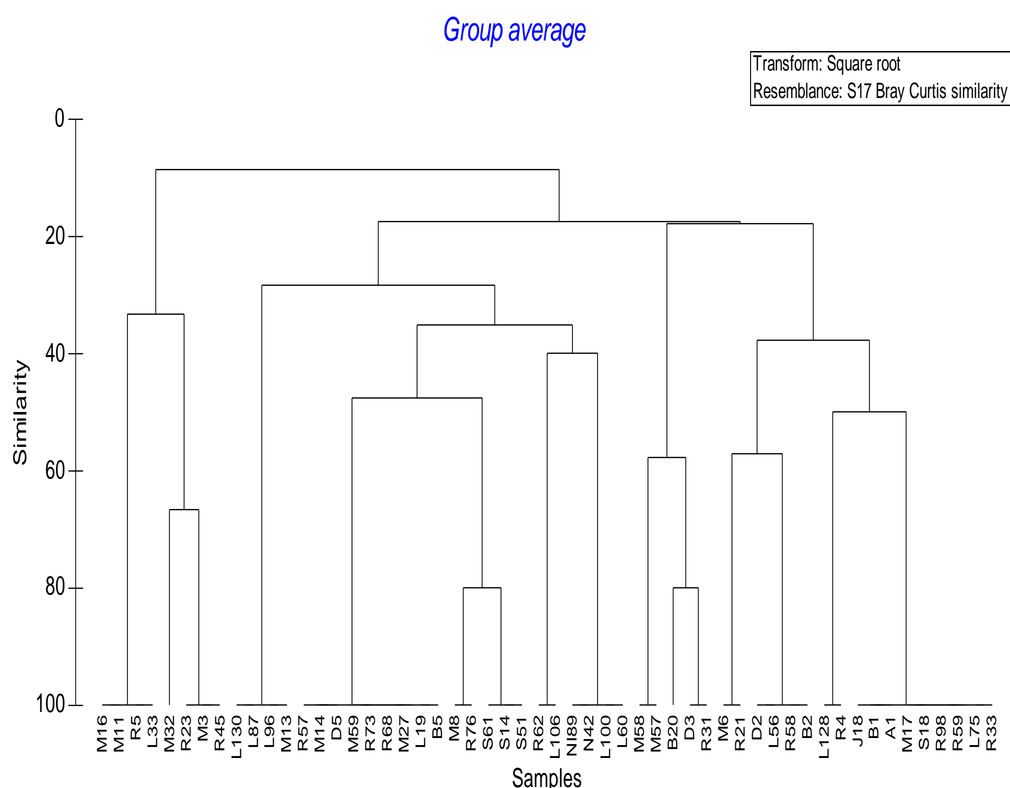


Fig. 3.5 Dendrogram of marine actinomycete isolates constructed based on ARDRA patterns developed by *Sau*3A1

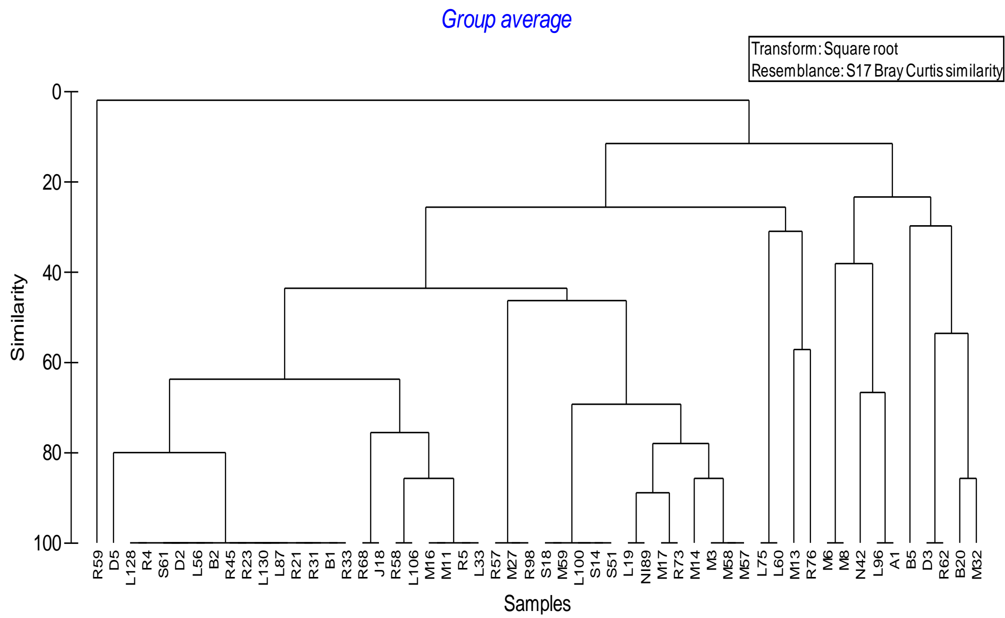


Fig. 3.6 Dendrogram of marine actinomycete isolates constructed based on ARDRA patterns developed by *Hinf*1

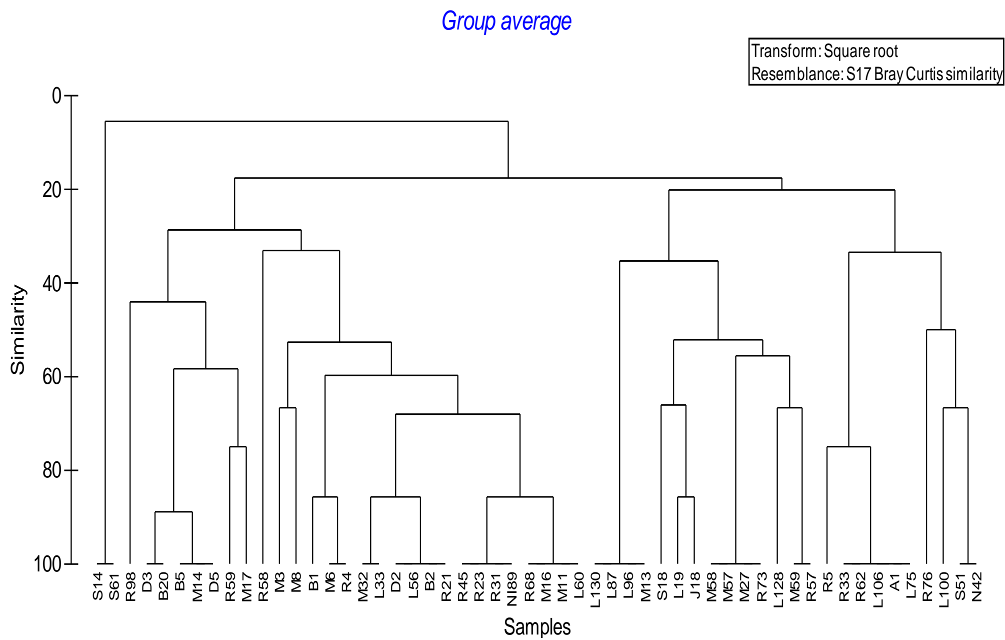


Fig. 3.7 Dendrogram of marine actinomycete isolates constructed based on ARDRA patterns developed by *Taq*1

3.3.3.1 Actinomycete with green spore mass

The ARDRA profile of marine actinomycetes with green spore mass (Fig. 3.8) and the species identity revealed on sequence analysis of representative isolates is given (Table 3.1). The actinomycete isolate no. M14 isolated from the sediments of Bay of Bengal on sequencing and BLAST analysis revealed 98% similarity to *Streptomyces acrimycini* (**FJ486440**) isolated from China. Isolate no. M58 was identified as *Streptomyces thermolineatus* with 100% similarity in BLAST results to *S.thermolineatus* type strain (**AB184618**). Ribotype S14 identified as *N. prasina* with 99% similarity to reference strain *Nocardiopsis alba* subsp.*prasina* **(X97884)** (Table 3.2).

Table 3.1 ARDRA profile of representative actinomycete isolates with green spore mass

Isolate no:	*Invitro* Restriction fragments			Species identity	GenBank Accession
	*Sau*3A1(bp)	*Hinf*1(bp)	*Taq*1(bp)		
M58	650+600+150	800+325+175	550+350+325	*S. thermolineatus*	**KC551929**
M14	650+600+175	850+325+225	400+350+325+150	*S. acrimycini*	**KC570320**
S14	1300+175	800+325	900+325+200	*Nocardiopsis alba* subsp. *prasina*	**KF713523**

Table 3.2 NCBI BLAST results of actinomycetes with green spore mass

Isolate No	Max score	Total score	Query cover	E value	Max ident	Reference Accession no:	BLAST results
M58	883	883	100%	0.0	100%	**AB184618**	*Streptomyces thermolineatus*
M14	1905	1905	100%	0.0	98%	**FJ4846440**	*Streptomyces acrimycini*
S14	2263	2263	100%	0.0	99%	**X97884**	*Nocardiopsis alba* subsp. *prasina*

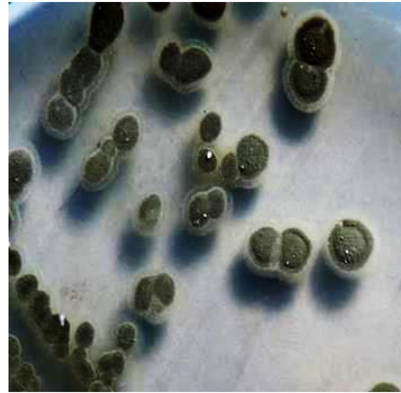


Fig. 3.8 Actinomycete with green spore mass

The phylogenetic tree inferred from multiple sequence alignment (Appendix 3A) depicts the relationship between green spore mass *Streptomyces* and *Nocardiopsis* with other *Streptomyces* sp.(Fig. 3.9).The phylogenetic tree is divided into three clusters, the *Streptomyces* cluster (cluster I and II) consists of the *S. acrimycinii* and *S. viridodiastaticus* cluster and the thermophilic cluster which includes *S.macrosporus*, and *S.thermolineatu*s. Ribotype M14 (GenBank: **KC570320**) is included in the *S. acrimycinii* cluster with a bootstrap support of 98% to the major cluster. Ribotype M58 (GenBank: **KC551929**) comes under the thermophilic cluster, grouped with the minor cluster of *S.thermolineatus* with a bootstrap support of 86% to the main cluster. Cluster III and IV consists of *Nocardiopsis* sp., which includes the ribotype S14 (GenBank: **KF713523**) clustered with *N.alba* subsp *prasina* group (cluster IV) with a bootstrap support of 85% with the main cluster.

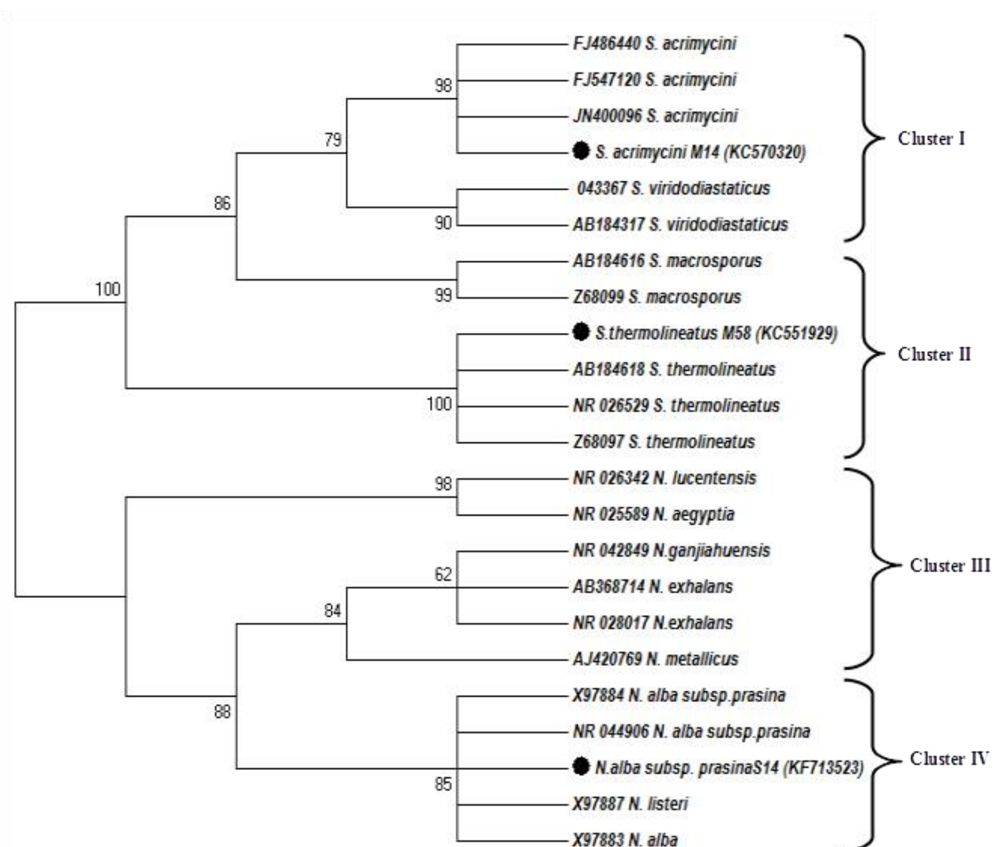


Fig. 3.9 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the green spore mass actinomycetes with other actinomycetes. Values at the node indicate the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

3.3.3.2 Actinomycete with pink red spore mass

The ARDRA profile of representative actinomycetes with pink red spore mass (Fig. 3.10) and the species identity is given (Table 3.3). Isolate no. N42 and N189 on BLAST results revealed 99% identity to reference strain *Nocardiopsis listeri* (**NR026341**). 16S rDNA amplicon of M16 showed 100 % similarity to *Streptomyces rubrolavendulae* (**HQ132776**) from wheat cropping system of fertile regions of Indo-Gangetic Plains (Table 3.4).

Table 3.3 ARDRA profile of representative actinomycete isolates with pink red spore mass

Isolate no:	***In vitro* Restriction Fragments**			**Species identity**	**GenBank Accession**
	***Sau*3A1(bp)**	***Hinf*1(bp)**	***Taq*1(bp)**		
M16	650+600+175	800+300+200+175	600+350+200	*S.rubrolavendulae*	**KC570317**
N189	1300+175	800+325+200+175	900+350+200	*N. listeri*	**KJ158482**
N42	1300+175	800+350+150	850+325+200	*N. listeri*	**KJ158483**

Table 3.4 NCBI BLAST results of actinomycetes with pink red spore mass

Isolate no.	**Max Score**	**Total Score**	**Max Identity**	**E Value**	**Query Coverage**	**Reference Accession No.**	**BLAST results**
M16	2177	2177	100%	0.0	100%	HQ132776	*Streptomyces rubrolavendulae*
M189	1308	1308	100%	0.0	99%	NR026341	*Nocardiopsis listeri*
N42	1423	1423	100%	0.0	99%	NR026341	*Nocardiopsis listeri*

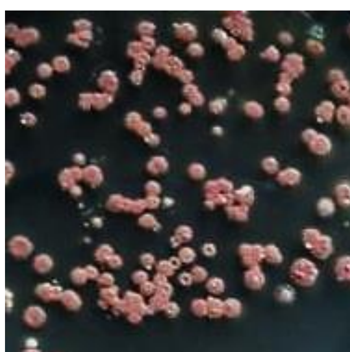


Fig. 3.10 Actinomycete with pink spore mass

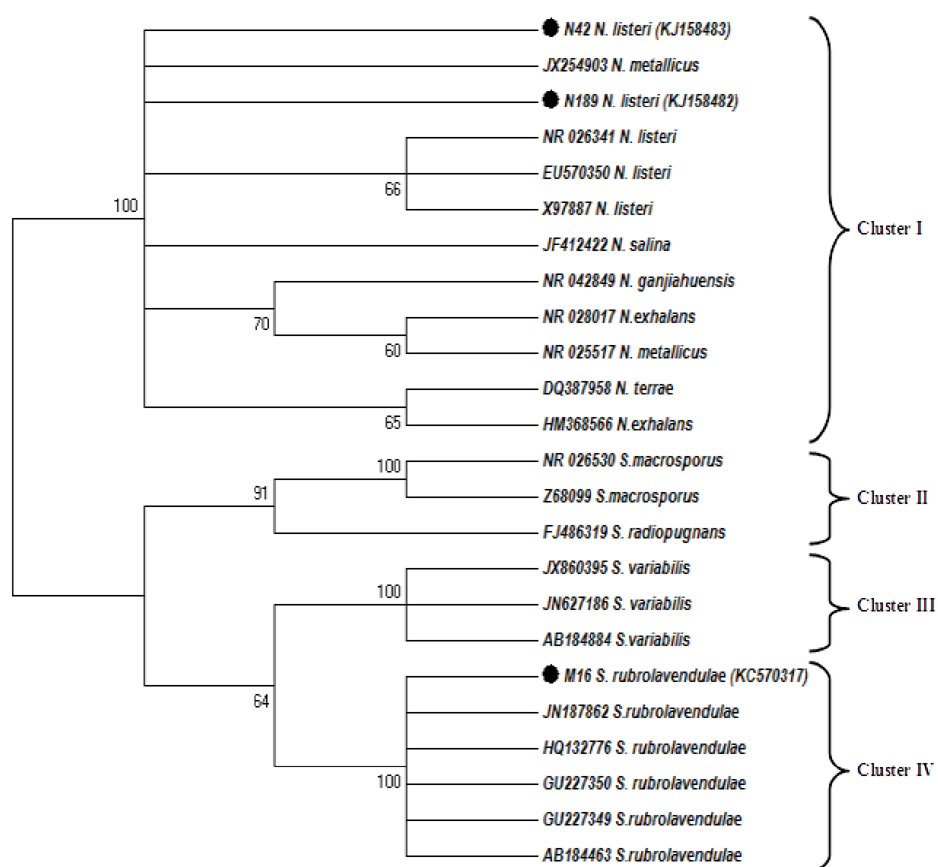


Fig. 3.11 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the pink spore mass actinomycetes with other actinomycetes. Values at the node indicate the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

Phylogenetic tree inferred from multiple sequence alignment (Appendix 3B) of pink red series actinomycetes is shown in Fig. 3.11. The phylogenetic tree is divided into four clusters, cluster I of *Nocardiopsi*s sp. and the other three of *Streptomyces* species. Ribotypes N189 (GenBank: **KJ158482)** and N42 (GenBank: **KJ158483**) are with the *Nocardiposis* cluster and identified as 99% similar to *Nocardiopsis listeri*. Ribotype M16 clustered with the *Streptomyces rubrolavendulae* (GenBank: **KC570317**) cluster of *Streptomyces*

sp. with 100% bootstrap support. Two other clusters represented in the tree are that of *Streptomyces variabilis* and the thermophilic group of *Streptomyces.*

3.3.3.3 Actinomycetes with yellow spore mass

ARDRA profile of representative actinomycetes with yellow spore mass (Fig. 3.12) is shown in Table 3.5. Amplicon of culture No. D2 isolated from sediments of Arabian Sea and isolate no.M3 from Bay of Bengal on BLAST analysis revealed 99% similarity to *Streptomyces chungwhensis* (JX486779), a marine actinomycete isolate from Indian waters. Isolate no. L130 from sediments of Arabian Sea, on BLAST analysis was identified as *Nocardiopsis alkaliphila*, with 99% similarity to its nearest match (NR042798, AY230848), a novel alkaliphilic actinomycete isolated from desert soil in Egypt (Table 3.6).

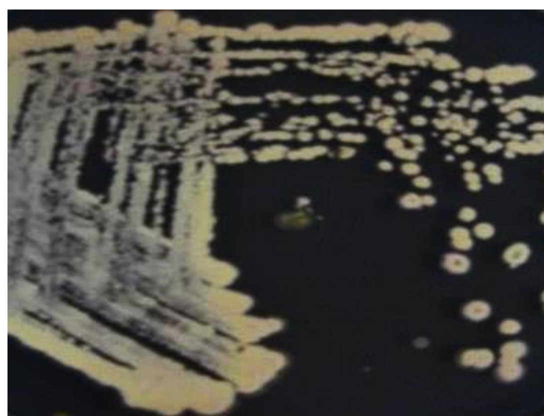


Fig. 3.12 Actinomycete with yellow spore mass

Table 3.5 ARDRA profile of representative actinomycete isolates with yellow spore mass

Isolate no:	*In vitro* Restriction Fragments			Species identity	GenBank Accession
	*Sau*3A1(bp)	*Hinf*1(bp)	*Taq*1(bp)		
D2	650+350+300	1100+325	900+350+175	*S.chungwhensis*	**KF713524**
M3	650+350+300	800+325+175	900+350+175	*S.chungwhensis*	**KC570325**
L130	1350+150	800+225	850+300+150	*Nocardiopsis alkaliphila*	**KF713525**

Table 3.6 NCBI BLAST results of actinomycetes with yellow spore mass

Isolate no	Max score	Total Score	Query Cover	E Value	Max Ident	Reference Accession no.	BLAST results
D2	1967	1967	100%	0.0	99%	**JX486779**	*Streptomyces chungwhensis*
M3	1975	1975	100%	0.0	99%	**JX486779**	*Streptomyces chungwhensis*
L130	1303	1303	100%	0.0	99%	**NR042798**	*Nocardiopsis alkaliphila*

The phylogenetic tree inferred from multiple sequence alignment (Appendix 3C) of yellow spore mass actinomycetes with similar sequences is divided into three clusters (Fig. 3.13). Cluster1 comprise the *Streptomyces rimosus* group, species defined by the major cluster in the numerical classification with a bootstrap support of 92% to the main clade. Ribotype D2 (GenBank: **KF713524**) and ribotype M3 (GenBank: **KC570325**) with 99% similarity to *S. chungwhensis* in BLAST analysis grouped with the same species. Cluster III consists of *Nocardiopsis* sp. L130 (GenBank: **KF713525**) clustered with *N.alkaliphila* with 82% bootstrap support to the main cluster.

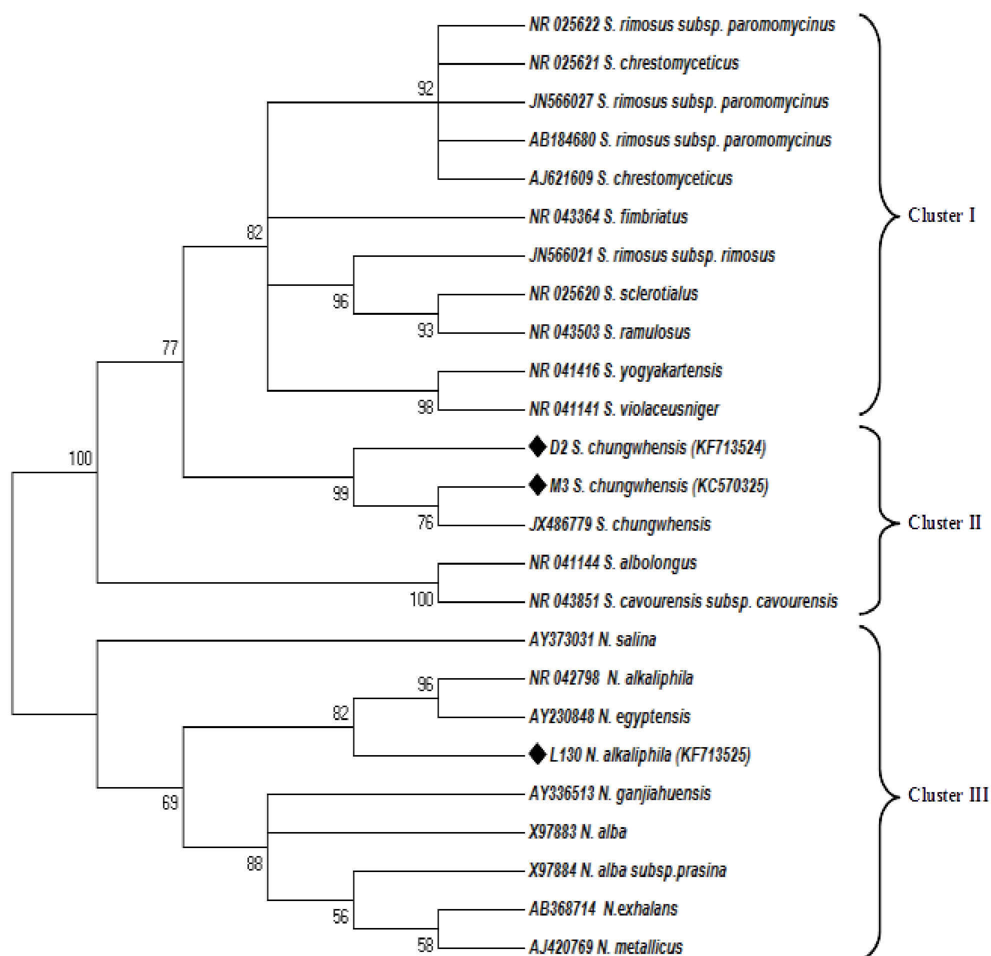


Fig. 3.13 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the yellow spore series actinomycetes with related *Streptomyces* and *Nocardiopsis* sp. Values at the node indicates the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

3.3.3.4 Actinomycetes with grey spore/pigment producing and red substrate mycelium

ARDRA profile of representative grey spore series actinomycetes with red substrate mycelium (Fig. 3.14) and pigment producing property is given (Table 3.7). Isolate M6 and M8, although appeared morphologically similar,

with diffusible pigment production, their amplicons on restriction exhibited different profiles. Both the strains on BLAST analysis showed 99% similarity and 100% query coverage to its nearest match, *Streptomyces bikiniensis* isolated from Pacific and Carribean waters of Costa Rica and also to type strain (**EU741211, AB184602**). Diffusible brown pigment producing strain D3 isolated from Arabian Sea exhibited 99% similarity to various closely related *Streptomyces* species, and was identified based on polyphasic approach as similar to type strain of *Streptomyces griseoaurantiacus* (**AB184676**). Amplicon of isolate B1 and R21 exhibited similar *Hinf*1 restriction profile and slightly different *Sau*3A1 and *Taq*1 profiles and the isolates, on BLAST analysis were identified as 99% similar to *Streptomyces rubrogriseus* (**FJ547131**) isolated from China. Isolate no.B20 was identified based on polyphasic approach as 99% similar to type strain of *Streptomyces diastaticus* subsp. *ardesiacus* **(AB184653)**. Amplicon of B5 with an unique ARDRA profile was identified as 99% similar to type strain of *Streptomyces tendae* (**AB184172**) included in *Streptomyces rochei* cluster (Table 3.8).

Table 3.7 ARDRA profile of representative actinomycete isolates with grey spore mass, pigment producing and red substrate mycelium

Isolate no:	***In vitro* Restriction Fragments**			**Species identity**	**GenBank Accession**
	***Sau*3A1(bp)**	***Hinf*1 (bp)**	***Taq*1(bp)**		
M6	600+550+175	800+300+250+150	900+350+150	*S. bikiniensis*	**KC570315**
M8	625+600+175	800+350+150	900+350+225	*S. bikiniensis*	**JX657681**
D3	700+650+150	825+350+200	400+350+325+150	*S. griseoaurantiacus*	**KJ158466**
B5	650+600+175	800+200+100	400+350+325+150+100	*S. tendae*	**KF713526**
B20	700+650+150	825+350+100	400+350+325+150	*S. diastaticus* subsp.*ardesiacus*	**KJ158467**
B1	650+600	1100+325	900+350+225	*S. rubrogriseus*	**KJ158468**
R21	600+550+175	1100+325	900+350+175	*S. rubrogriseus*	**KJ158469**

Table 3.8 NCBI BLAST results of actinomycete with Grey spore mass and red substrate mycelium

Isolate no:	Total score	Max score	Max identity	E value	Query coverage	Reference Accession no:	BLAST results
D3	2017	2017	100%	0.0	99%	AB184676	*Streptomyces griseoaurantiacus*
M6	1319	1319	100%	0.0	99%	AB184602	*Streptomyces bikiniensis*
M8	1940	1940	100%	0.0	99%	AB184602	*Streptomyces bikiniensis*
BA5	817	817	100%	0.0	99%	AB184172	*Streptomyces tendae*
B20	1953	1953	100%	0.0	99%	AB184653	*Streptomyces diastaticus* subsp. *ardesiacus*
R21	1740	1740	100%	0.0	99%	FJ547131	*Streptomyces rubrogriseus*
B1	1884	1884	100%	0.0	99%	FJ547131	*Streptomyces rubrogriseus*

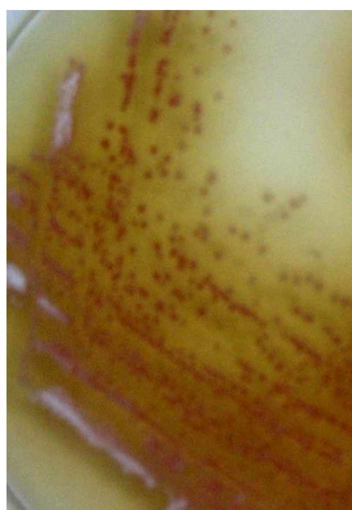


Fig. 3.14 Actinomycete with grey spore and red substrate mycelium

Phylogenetic tree inferred from multiple sequence alignment (Appendix 3D) of grey spore with red substrate and pigment producing actinomycetes with other similar sequences is given (Fig. 3.15).

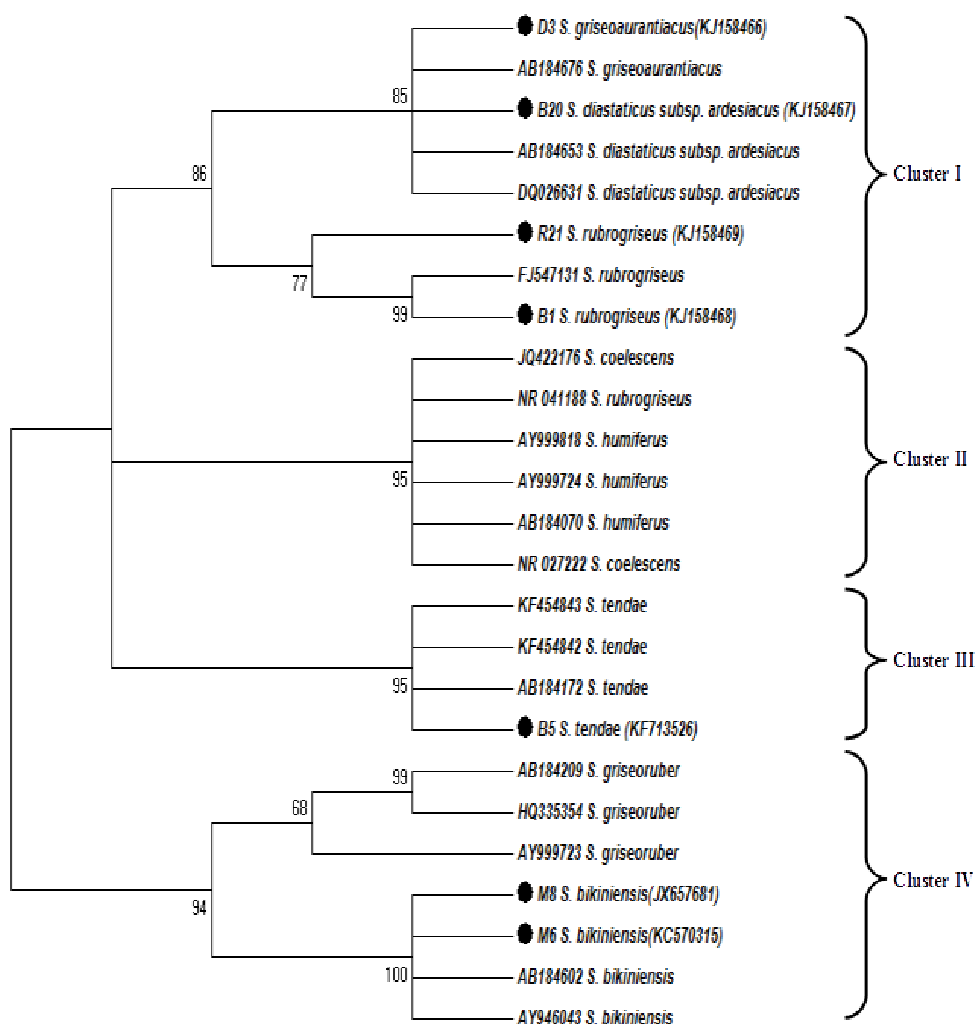


Fig. 3.15 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the grey spore with red substrate and pigment producing actinomycetes with other *Streptomyces* spp. Values at the node indicates the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

The phylogenetic tree is divided into four clusters. Cluster I consists of *S.griseoaurantiacus*, *S. diastaticus* subsp.*ardesiacus* and *S.rubrogriseus* with a boot strap support of 85% to the main group. Ribotype D3 (GenBank: **KJ158466**) and B20 (GenBank: **KJ158467**) falls within cluster I and grouped with *S.griseoaurantiacus* and *S.diastaticus* subsp.*ardesiacus* respectively.

Ribotype B1 (GenBank: **KJ158468)** and R21 (GenBank: **KJ158469)** with 99% sequence similarity to *S. rubrogriseus*, *S.fradiae*, *S.diastaticus* and few other strains, clustered with *S.rubrogriseus* and were identified based on reassessment of phenotypic and biochemical characteristics to complement the molecular analysis. Cluster II consisted of *Streptomyces humiferus*, *S.coelescens* and *S.rubrogriseus.* Ribotype B5 (GenBank: **KF713526**) identified as *S.tendae* heterotypic synonym of *S.rochei* clustered with *S.tendae* of cluster III with a boot strap support of 95% with the main clade. Ribotype M6 (GenBank: **KC570315**) and M8 (GenBank: **JX657681**) clustered with *S.bikiniensis* in cluster 1V with a bootstrap support of 100% to the main group.

3.3.3.5 Actinomycetes with grey spore mass

ARDRA profile of grey spore series actinomycetes (Fig. 3.16) with species identity is given (Table 3.9).

Table 3.9 ARDRA profile of representative actinomycetes with grey spore mass

Isolate no:	*In vitro* Restriction fragments			Species identity	GenBank accession
	*Sau*3A1(bp)	*Hinf*1(bp)	*Taq*1(bp)		
M27	650+600+175	850+325+225	550+350+325	*S. radiopugnans*	**KC570323**
R94	650+600+175	1100+325+100	900+350+150	*S. variabilis*	**KF713527**
B2	650+600	800+325+175	900+350+175	*S. flavomacrosporus*	**KF713528**
R98	600+550	850+325+225	400+375+ 350+300	*S.variabilis*	**KF713529**
R59	600+550	800+300	400+325+375+200	*S.violorubens*	**KF713530**
M17	600+550	800+325+200+175	400+375+350+325	*S.variabilis*	**KC570324**
M13	625+550+175	800+275+300	550+350	*S.radiopugnans*	**KC570319**
M59	650+600+175	800+325	550+375+325	*S.radiopugnans*	**KC570318**

The marine isolates M27 and M59 with slight variation in ARDRA profile were identified as *S. radiopugnans* with 99% similarity to radiation resistant actinomycete *S. radiopugnans* **(DQ912930)** isolated from radiation

polluted soil in China. The amplicon of isolate M13 gave a slightly different pattern for all the three digestions compared to M27 and M59, but the isolate M13 was also identified as 99% similar to *S. radiopugnan*s (**EU841544**) isolated from China. Culture nos. M17 and R98 exhibited similar profile for all the three restriction digestions and was identified as 99% similar to *Streptomyces variabilis* (**JN627186),** isolated from soft coral *Scleronephthya,* while R98 identified as 99% similar to *Streptomyces variabilis* (**JX860395**), an antibiotic producing soil *Streptomyces* from South Africa. Amplicon of isolate R94 with an unique three fragment profile, was also identified as 99% similar to *Streptomyces variabilis* (**JX860395**), an antibiotic producing soil *Streptomyces* from South Africa. Amplicon of R59 was identified as 99% similar to type strain of *Streptomyces violoruben*s (**AB184464**). Amplicon of isolate B2 on BLAST analysis revealed 99% similarity to *S. flavomacrosporu*s (**JX860361),** isolated from rhizospheric soils in Africa (Table 3.10).

Table 3.10 NCBI BLAST results of actinomycetes with grey spore mass

Isolate no	**Max score**	**Total score**	**Max identity**	**E value**	**Query coverage**	**Reference Accession no:**	**BLAST results**
M27	2001	2001	100%	0.0	99%	**DQ912930**	*Streptomyces radiopugnans*
M59	1986	1986	100%	0.0	99%	**DQ912930**	*Streptomyces radiopugnans*
R59	2006	2006	100%	0.0	99%	**AB184464**	*Streptomyces violorubens*
R98	1411	1411	100%	0.0	100%	**JX860395**	*Streptomyces variabilis*
M13	1973	1973	100%	0.0	99%	**EU841544**	*Streptomyces radiopugnans*
B2	1964	1964	100%	0.0	99%	**JX860361**	*Streptomyces flavomacrosporus*
M17	1794	1794	100%	0.0	98%	**JN627186**	*Streptomyces variabilis*
R94	1958	1958	100%	0.0	99%	**JX860395**	*Streptomyces variabilis*

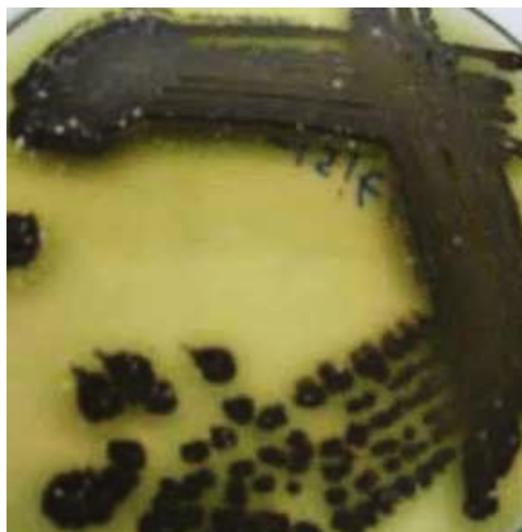


Fig. 3.16 Actinomycetes with grey spore mass

Phylogenetic tree inferred from multiple sequence alignment (Appendix 3E) of grey spore series actinomycetes with closely related GenBank sequences is given in (Fig. 3.17). The grey spore series actinomycetes isolated from marine sediments were identified as *S. variabilis*, heterotypic synonym of *S. rochei* group, *S. violorubens*, *S. flavomacrosporus*, and the thermophilic actinomycetes, *S.radiopugnan*s. The phylogenetic tree is divided into four clusters; Ribotype R94 (GenBank**: KF713527**), R98 (GenBank**: KF713529**) and M17 (GenBank**: KC570324**) identified as *S. variabilis,* clustered with cluster I of S. *variabilis.* Ribotype R59 (GenBank**: KF713530**) is included in the *S. violorubens* cluster with a boot strap support of 63% along with minor cluster of *S. griseoalbulu*s. Ribotype B2 (GenBank**: KF713528**) identified as *S. flavomacrosporus* was included in cluster III along with *S. flavoviridis* with a boot strap support of 62% to the main clade. Ribotype M13 (GenBank**: KC570319**), M59 (GenBank: **KC570318**) and M27 (GenBank: **KC570323**) clustered in the thermophilic clade with 55% boot strap support to the main clade.

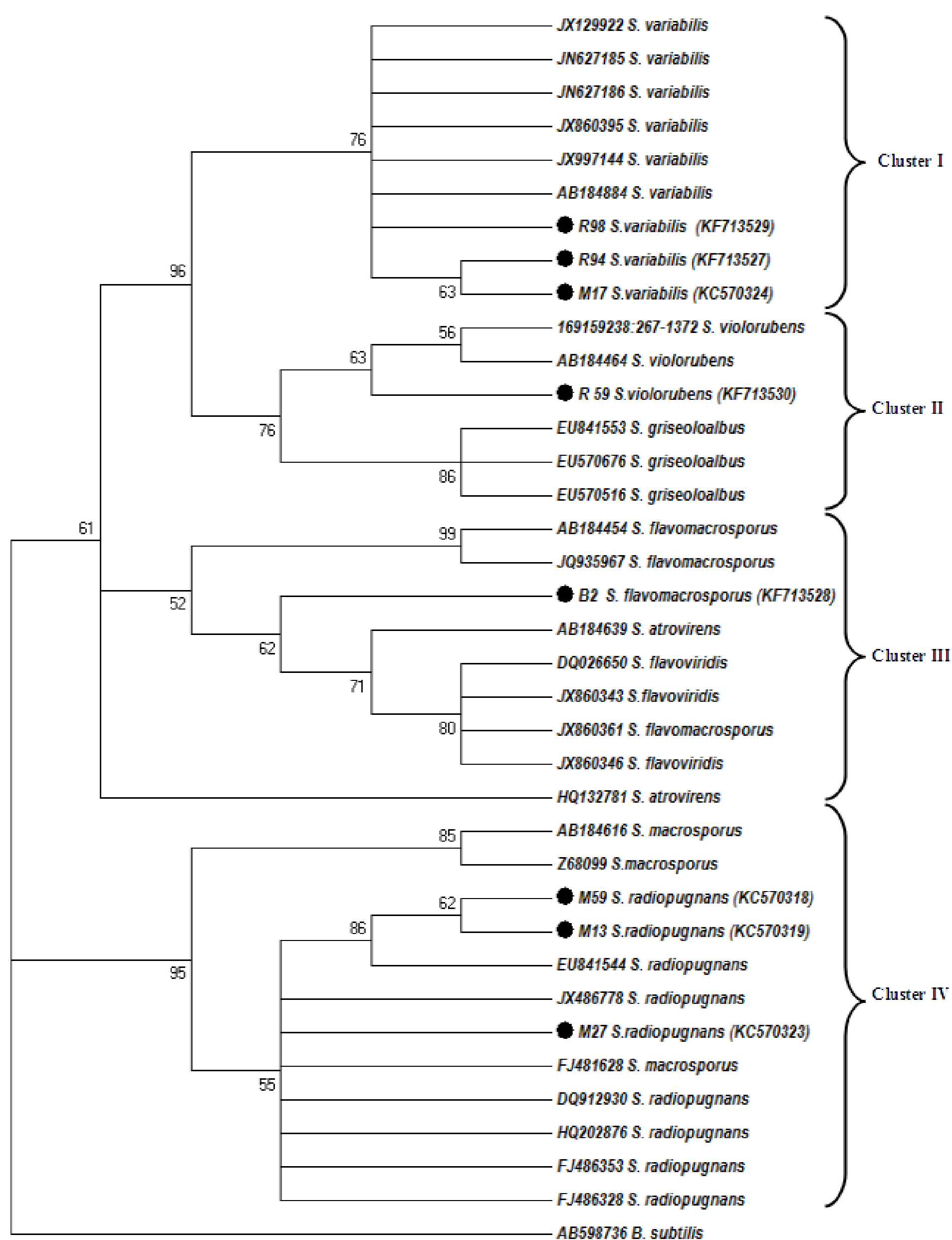


Fig. 3.17 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the grey spore series actinomycetes with other *Streptomyces*. Values at the node indicates the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

3.3.3.6 Actinomycetes with white spore mass

ARDRA profile of actinomycetes with white spore mass (Fig. 3.18 is given (Table 3.11).

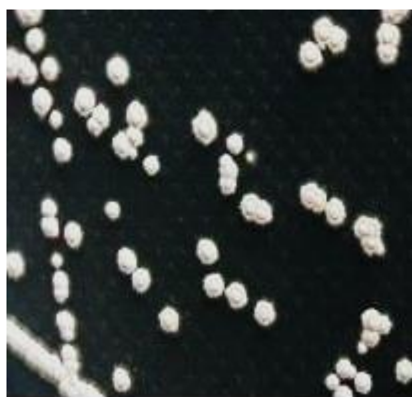


Fig. 3.18 Actinomycete with white spore mass

Actinomycetes with white spore mass (58.26%) was found to be the most abundant form isolated from marine sediments. Amplicon of isolate M31 had 98% sequence identity to *S. radiopugnans* (**HQ202876**), an actinomycete isolated from Kunlun mountains in China. Isolate R33 revealed 99% similarity to *S. champavatii* (**JN652257**), a geosmin producing *Streptomyces* sp. in aquaculture systems. Amplicons of isolate R73 and R57 generated similar fragments for *Sau*3A1 and *Hinf*1 whereas *Taq*1 profile was slightly different, the isolates revealed 100% and 99% identity to reference sequence of *S. exfoliatus* (**NR041229**). Amplicon of L128 revealed 99% identity to reference sequence of *S. luteogriseus* (**NR041128**) included in *Streptomyces cyanaeus* species cluster. The isolate R62 with sclerotia in their substrate mycelium revealed 99% identity to reference strain *Streptomyces minutiscleroticus* (**NR044149**). Amplicon of isolate S18 was identified as 99% similar to reference sequence of *S. albidoflavus* (**NR041095**). Representative isolate L33 could not be assigned to a defined species and was identified as 99% similar to

reference sequence of *Streptomyces* sp. (**NR044582**). Amplicon of isolate B4 revealed 99% similarity to an alkaliphilic salt tolerant *Nocardiopsis alba* isolated from coastal Gujarat, India **(KC798063)** (Table 3.12).

Table 3.11 ARDRA profile of representative actinomycetes with white spore mass

Isolate no:	*In vitro* Restriction fragments			Species Identity	GenBank Accession
	*Sau*3A1	*Hinf*1	*Taq*1		
M31	650+600+225	825+350+150+100	900+350+200	*S. radiopugnans*	**KC570321**
R33	650+600+200	1100+325	550+325+250+225	*S. champavatii*	**KJ158471**
R73	650+600+175	800+325+200+150	550+350+325	*S. exfoliatus*	**KJ158473**
S18	600+175	800+325	550+350+300	*S. albidoflavus*	**KJ158474**
L128	700+600	1100+325	600+550+325	*S. luteogriseus*	**KJ158476**
R57	650+600+175	800+325+200+150	550+375+325	*S. exfoliatus*	**KJ158479**
R62	700+650+175	825+350+200	850+325+250	*S. minutiscleroticus*	**KJ158480**
L33	600+250+225	1100+200+175	900+350+200	*Streptomyces sp.*	**KJ158481**
B4	1300+175	1100+325	850+325+200	*Nocardiopsis alba*	**KF713531**

Table 3.12 NCBI BLAST Results of actinomycetes with white spore mass

Isolate no	Max score	Total Score	Query Cover	E Value	Max Ident	Reference Accession no:	BLAST results
M31	1801	1801	100%	0.0	98%	HQ202876	*Streptomyces radiopugnans*
R33	874	874	100%	0.0	99%	JN652257	*Streptomyces champavatii*
R73	1947	1947	100%	0.0	100%	NR041229	*Streptomyces exfoliatus*
S18	2010	2010	100%	0.0	99%	NR041095	*Streptomyces albidoflavus*
L128	1502	1502	100%	0.0	99%	NR041128	*Streptomyces luteogriseus*
R57	1901	1901	100%	0.0	99%	NR041229	*Streptomyces exfoliatus*
R62	863	863	100%	0.0	99%	NR044149	*Streptomyces minutiscleroticus*
L33	795	795	100%	0.0	99%	NR044582	*Streptomyces* sp.
B4	719	719	100%	0.0	99%	KC798063	*Nocardiopsis alba*

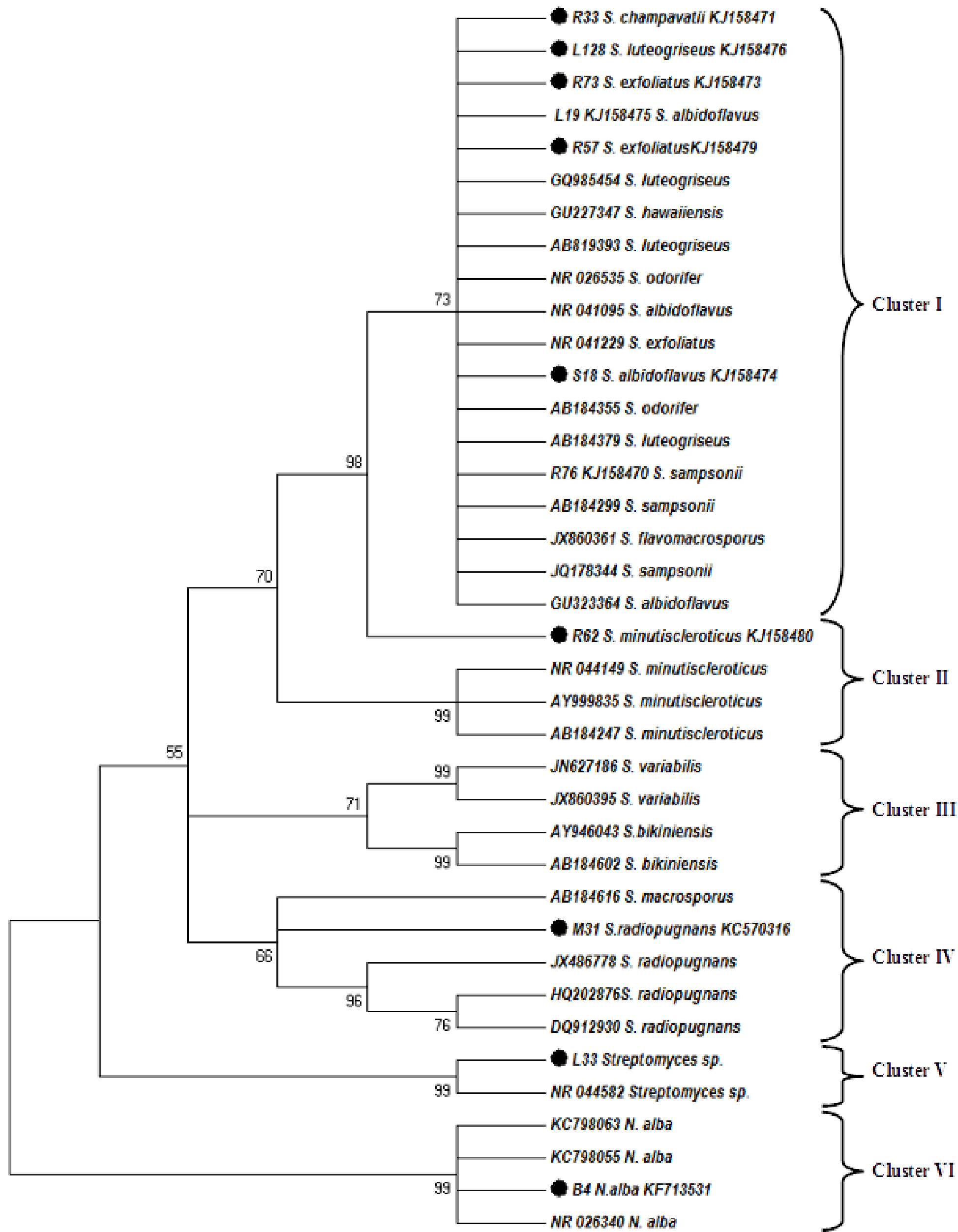


Fig. 3.19 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the white spore series actinomycetes with other actinomycetes. Values at the node indicates the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

The Phylogenetic tree inferred from multiple sequence alignment (Appendix 3F) of white spore series actinomycetes is divided into six clusters (Fig. 3.19). Cluster I consists of *S. albidoflavus* group cluster and closely related off white spore series *Streptomyces* including *S. luteogriseus*, *S. exfoliatus*, *S. champavatii*, *S. sampsonii*, *S. hawaiiensis*, etc. Most of the representative ribotypes M31 (GenBank: **KC570316**) R33 (GenBank: **KJ158471**), R73 (**KJ158473**), S18 (GenBank: **KJ158474**), L128 (**KJ158476**), and R57 (GenBank: **KJ158479**) clustered around the major cluster of *S. albidoflavus* group (cluster1). Ribotype R62 (GenBank: **KJ158480**) clustered with the minor cluster of *S. minutiscleroticus, S. albidoflavus* and *S. variabilis* as closely related neighbours. Ribotype M31 (GenBank: **KC570316**) identified as *S. radiopugnans* clustered with the thermophilic group with 54% bootstrap support to the major cluster. Ribotype L33 identified as *Streptomyces* sp. forms a minor group with bootstrap support of 99% along with major cluster of *Streptomyces* and ribotype B4 (GenBank: **KF713531**) clustered with *Nocardiopsis alba* group with 99% bootstrap support to the major cluster.

3.3.4 Species Composition of Actinomycetes in Arabian Sea and Bay of Bengal

The molecular phylogenetic analysis of marine actinomycetes from sediments of Arabian Sea and Bay of Bengal revealed *Streptomyces* as the dominant genera followed by *Nocardiopsis* sp. *Streptomyce*s genus was represented by over 15 species from the sediments of Arabian Sea and Bay of Bengal.

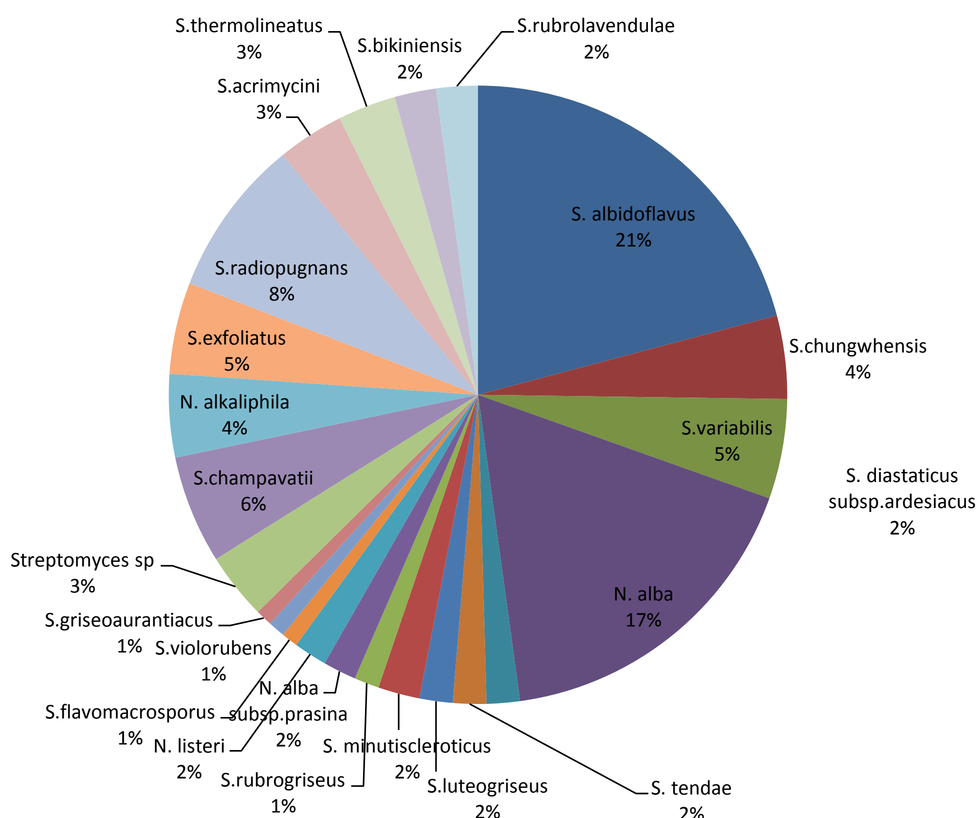


Fig. 3.20 Species composition of marine actinomycete isolates from sediments of Arabian Sea and Bay of Bengal

Of the total 230 marine actinomycete isolates, the most abundant species in marine sediment was identified as *Streptomyces albidoflavus* (20.42%), followed by *Nocardiopsis alba* (17.82%), *Streptomyces radiopugnans* (8.26%), *Streptomyces champavatii* (5.65%), *Streptomyces variabilis* (5.22%), *Streptomyces chungwhensis* (4.34%), *Nocardiopsis alkaliphila* (4.34%), *Streptomyces exfoliatus* (3.4%), *Streptomyces acrimycini* (3.48%), *Streptomyces thermolineatus* (3.04%), *Streptomyces bikiniensis* (2.2%), *Streptomyces rubrolavendulae* (2.2%), *Streptomyces diastaticus* subsp *ardesiacus* (1.73%), *Streptomyces tendae* (1.73%), *S. luteogriseus* (1.73%), *S. minutiscleroticus* (2.17%), *S. exfoliatus* (1.30%), *S. rubrogriseus* (1.30%),

Nocardiopsis alba subsp *prasina* (1.74%), *Nocardiopsis listeri* (1.73%), *S. flavomacrosporus* (0.87%), *S. violorubens*, (0.87%), and *S. griseoaurantiacus* (0.87%), respectively.

Approximately 3.47% actinomycete isolates were not assigned to any species and were recorded as *Streptomyces* sp. (Fig. 3.20). Genus *Nocardiopsis* was represented by *Nocardiopsis alba* (17.82%) as the prominent species followed by *Nocardiopsis alkaliphila* (4.34%), *Nocardiopsis alba* subsp *prasina* (1.74%) and *Nocardiosis listeri* (1.73%) respectively.

Diverse species were represented from sediments of Arabian Sea. Out of the total 164 isolates from Arabian Sea, *Streptomyces albidoflavus* cluster (26.82%) was found to be the most abundant species. *Nocardiopsis alba* represented 19.5% of actinomycete isolates from Arabian Sea and other representative species included, *S. champavatii* (7.92%), *S. exfoliatus* (6.69%), *S. minutiscleroticus* (3.04%), S. *diastaticus* subsp *ardesiacus* (3.65%), *S. tendae* (2.44%), S. *griseoaurantiacus* (1.83%), *S. flavomacrosporus* (1.22%), *S.violorubens* (1.22%), *S. luteogriseus* (2.44%), *S. chungwhensis* (1.82%), *N. alkaliphila* (6.09%), *N. listeri* (2.48%), and *N. prasina* (2.43%) respectively (Fig. 3.21).

Streptomyces radiopugnans (28.78%) was the most abundant *Streptomyces* sp. isolated, among the actinomycete isolates (66) from Bay of Bengal. *Streptomyces thermolineatus*, (10.66%), *S. acrimycini* (12.12%), *S. rubrolavendulae* (7.66%) *S. bikiniensis* (7.57%) were represented only from sediments of Bay of Bengal (Fig. 3.22). Other species included *S. chungwhensis* (10.66%), *S. variabilis* (6.06%), *S. albidoflavus* (4.54%), and *Nocardiopsis alba* (12.12%).

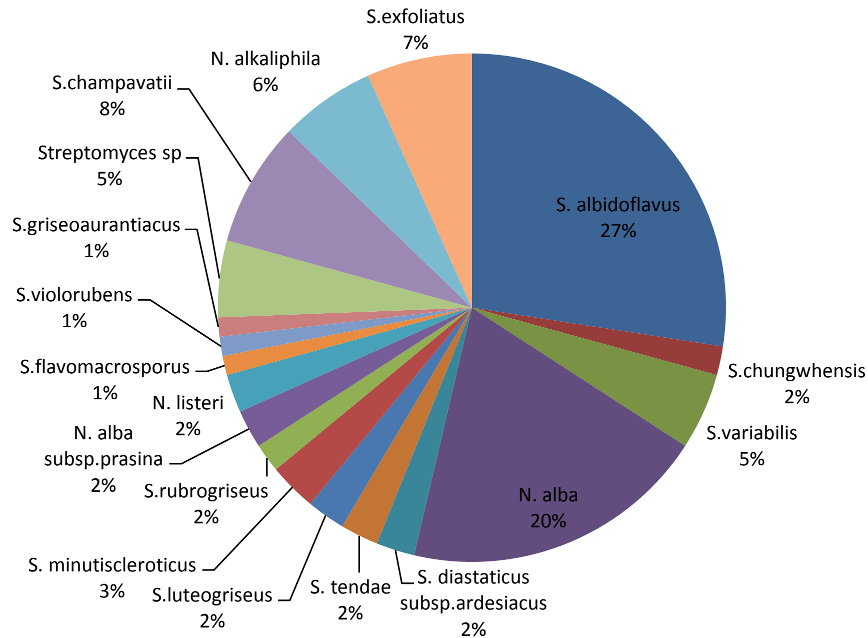


Fig. 3.21 Species composition of marine actinomycete isolates from sediments of Arabian Sea

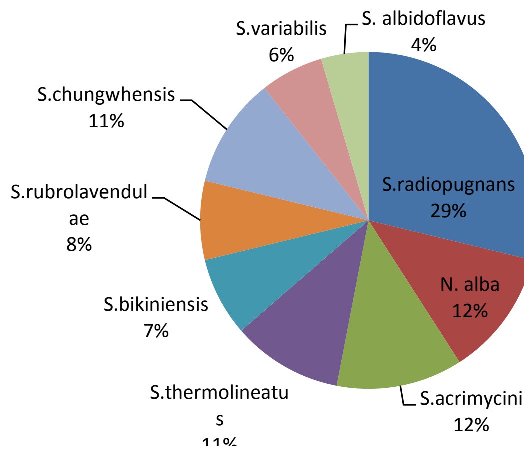


Fig. 3.22 Species composition of marine actinomycete isolates from sediments of Bay of Bengal

3.4 Discussion

Marine environment represents an untapped ecosystem from which rare actinomycetes having potential for producing novel metabolites have been discovered (Arifuzzaman et al., 2010; Solanki et al., 2008; Ward and Bora, 2006). Unique environmental conditions, e.g., high salinity, pH or extreme temperatures may select for proteins with unique sequences and thus biochemical properties. Bacterial functional genes have been retrieved from diverse terrestrial environments. However, similar studies of functional genes from marine ecosystems are extremely rare. Comparison of gene sequences retrieved from similar, but geographically isolated environments could yield insight into the novel functional genes.

Several characteristics of the 16S rRNA gene, such as its essential function, ubiquity, and evolutionary properties, have proposed the same gene as the most commonly used molecular marker in microbial identification. In the present study, phylogenetic diversity of actinomycete isolates from sediments of Arabian Sea and Bay of Bengal were assessed using universal eubacterial primers. The primers were used to amplify the entire 16S rDNA of actinomycetes to obtain maximum discriminatory power. Following PCR amplification, ARDRA was employed to assist in distinguishing among the various marine actinomycete isolates. A restriction enzyme having a recognition site of more than 4 bp would be irrelevant with respect to a gene of approximately 1500 bp such as that coding for the 16S ribosomal subunit (Vaneechoutte et al., 1995). In this study we employed, a combination of *Sau*3A1, *Hinf*1 and *Taq*1 tetracutter restrictase for the ARDRA analysis.

Different combinations of restriction enzymes were tested in the ARDRA assay and found that 3 enzymes yielded similar results in terms of

species identification as did 5 enzymes (Heyndrickx et al., 1996). Even though, reports are available regarding ARDRA analysis of clinical as well as soil actinomycetes, with 16S rRNA and hsp gene, only very few reports are available regarding the molecular characterization or ARDRA patterns of marine actinomycete isolates. Combined with morphological characteristics, 16S rRNA-RFLP could efficiently reduce the number of isolates for sequence analysis.

According to Cook and Meyers (2003), in rapid identification of filamentous actinomycetes, the *Sau*3AI restriction group in which the majority of species of a genus are placed is considered characteristic of that genus. This finding is consistent with those in a previous report by Roth et al. (2003), who compared the full 16S rRNAgene sequences (1,500 bp) of 74 *Nocardia* isolates, and a report by Cloud et al. (2004) who compared the 500 bp 16S rRNA gene sequences of 28 reference *Nocardia* strains. The restriction endonuclease primarily used in the current study was *Sau*3A1. *Sau*3A1 generated a large fragment of 600-700 bp which was most frequently generated, and was considered characteristic of the genus *Streptomyces*. Change in restriction site/fragment size was observed, which might have arose as a result of mutations in the marine environment for better adaptability that created a new *Sau3*A1 recognition site or destroyed a genus characteristic *Sau3*A1 site. This view was suggested by Cook and Meyers (2003) who gave a rapid identification of filamentous actinomycetes to genus level using four restriction enzymes.

*Taq*1 digestions was found to be the most discriminative, with ten polymorphic patterns which helped in the differentiation of species of *Streptomyces* not too closely related up to certain extent, with exceptions. Wu et al. (2006) reported the efficiency of *Taq*I which produced clear ARDRA

patterns, capable of distinguishing species members in each of the 3 genera viz., *Bacillus*, *Paenibacillus* and *Brevibacillus*. *Taq*I is an efficient restriction endonuclease for actinomycete analysis (Jing et al., 2008; Steingrube et al., 1997). In the present study, *Hinf*1 profile was the least discriminative, however it generated certain unique profiles too.

The high variability of 16S rDNA sequences within the actinomycetes genera were evident from the large number of different restriction patterns observed in the present study, as viewed by Vaneechoutte et al. (1995) within the genus *Corynebacterium.* Most of the *Streptomyces* species could be speciated using ARDRA system by comparing either with the theoretical ARDRA pattern or with actual fragment size (Wu et al., 2006), provided their phenotypic characters are assessed.

Considerable variations in the ARDRA patterns between species were evident, although a significant number of common fragments were observed. ARDRA analysis appears to be an accurate and rapid strain identification tool for *Streptomyces*, although relationships based on ARDRA may be valid only for very closely related *Streptomyces* strains or species (Clarke et al., 1993).

In the present study, a preliminary classification of actinomycetes based on colour of spore mass determined six distinct groups, viz., green, pink, yellow, grey, grey with red substrate mycelium and white series. Actinomycetes with green spore mass which accounts to 8.26% of the total, were identified as *Streptomyces thermolineatus*, *Streptomyces acrimycini* from sediments of Bay of Bengal and *Nocardiopsis alba* subsp. *prasina,* with light green spore mass, isolated from sediments of Arabian Sea. The three representative isolates of green spore mass had a different restriction profile. *Sau*3A1 profile was almost similar for the *Streptomyces* sp., whereas

Nocardiopsis sp. had a different profile with the largest fragment size of 1300 bp. *Hinf*1 digestions gave similar profile for all three species with the largest fragment size of 800-850 bp. Significant variation in ARDRA profile was observed for all the three isolates on *Taq*1 digestion. *S.thermolineatus* exhibiting dark green spore mass belongs to the thermo tolerant group of actinomycetes. The *Streptomyces thermolineatus* was associated with a well-defined group formed by the strains of *Streptomyces glaucosporus*, *Streptomyces macrosporus*, *Streptomyces megasporus* and *Streptomyces radiopugnans* (Labeda et al., 2012).

A minor cluster of thermophilic actinomycete, *Streptomyces thermolineatus* was first isolated from sewage compost (Goodfellow et al., 1987) and as endophyte in roots of *Triticum aestivum* (Vanessa and Christopher, 2004). The isolation of unusual dipeptide metabolites from the previously unstudied marine-derived *S. acrimycini* reinforce the importance of studies on marine environment and on secondary metabolism of marine microorganisms (Hernandez et al., 2004). *Nocardiopsis alba* subsp *prasina* represent only 1.73 % isolates from Arabian Sea and Bay of Bengal. Genus *Nocardiopsis*, a widespread group in phylum actinobacteria, has received much attention owing to its ecological versatility and ability to produce a rich array of bioactive metabolites (Li et al., 2013).

Representative isolates with leathery, yellow spore mass, on amplification and ARDRA analysis revealed identity as *Streptomyces chungwhensis* and *Nocardiopsis alkaliphila*. *Streptomyces chungwhensis* was isolated from Arabian Sea and Bay of Bengal and represented 4.34% of total isolates, while *Nocardiopsis alkaliphila* was isolated from sediments of Arabian Sea only, and contributed to 6.09% of the total isolates from Arabian

Sea. L130 revealed 99% similarity to novel sp. *Nocardiopsis alkaliphila* isolated from desert soil in Egypt (Hozzein et al., 2004).

Out of the total 230 isolates, 4% isolates were represented by the most colourful powdery, pink spore mass actinomycetes. Of the total sediment strains, 2.2% were identified as *Streptomyces rubrolavendulae* isolated from Bay of Bengal and 1.73% as *Nocardiopsis listeri* isolated from sediments of Arabian Sea.

The fragmentation profiles of *Hinf*1 and *Taq*1 of the three ribotypes of pink spore mass was shared by other species in the grey and white series. According to data of (Acinas et al., 2004), bacterial genomes display up to 15 rRNA operons, 40% of which have copy numbers of 1–2 with <1% sequence divergence. Sklarz et al. (2009) have also expressed the same view, that fragmentation profiles were not necessarily unique for each sequence in the database, resulting in different species sharing fragmentation profiles.

Comparison of the 16S rDNA sequence data also clearly demonstrate that the genus *Nocardiopsis* is a phylogenetically homogenous and shallow taxon with all the six species falling within the same lineage (Rainey et al., 1996). Differentiation of *Nocardiopsis* species was based on the color of the mycelium and the results of comparative physiological tests (Kroppenstedt, 1992).

In the present study, among the marine actinomycete isolates, grey spore mass series accounting to 20.85% of the total, was found to be second in abundance to white series. The different ribotypes of grey series identified were *Streptomyces bikiniensis* (2.2%), *Streptomyces diastaticus sub*sp.*ardesiacus* (1.73%), *Streptomyces tendae* (1.73%), *S. rubrogriseus* (1.30%), *S. flavomacrosporus* (0.87%), *S. violorubens* (0.87%),

S.griseoaurantiacus (0.87%), *Streptomyces variabilis* (5.27%), and *S. radiopugnans* (8.26%). *S. radiopugnans* was isolated from sediments of Bay of Bengal and found to be the most abundant of the grey series. *Streptomyces variabilis* of the *Streptomyces rochei* cluster was isolated from Arabian Sea and Bay of Bengal. Phylogenetic diversity of actinomycetes from multipond solar saltern, Tuticorin reported *Streptomyces* as the dominant genera with *Streptomyces radiopugnans* representing 18% of the total isolates (Jose et al., 2012).

Few *Streptomyces* sp. with grey spore mass and red substrate mycelium were identified as *Streptomyces tendae*, *S. diastaticus* subsp. *ardesiacus*, *S. griseoaurantiacus* and *Streptomyces rubrogriseu*s. It was observed that restriction profiles were different for same species. Even though, ribotype R21 and B1 were identified as similar to *S. rubrogriseus*, their ARDRA profiles were similar in the case of *Hinf*1, slightly different in the case of *Taq*1 and *Sau*3A1. In the phylogenetic tree, even though all the strains are clustered in the same clade, they were distinguished based on a polyphasic approach. Polyphasic taxonomy is particularly important in the taxonomy of streptomycetes due to strain heterogeneity among the species (Kim et al., 2004).

Differential phenotypic properties notably morphology and pigmentation features highlighted in numerical phenetic studies can be reliably weighted for differentiation of closely related *Streptomyces* species (Kim et al., 1998). Ribotypes M6 and M8 exhibited different fragmentation profiles, but both were identified as *S. bikiniensi*s. Even though the isolates M6 and M8 reveal similar identity, the difference in profile may be attributed to sequence divergence that might arise as result of multiple rRNA operons. The phylogenetic tree of the grey series was consistent with the BLAST results,

species *Streptomyces radiopugnans*, *Streptomyces variabilis*, *Streptomyces violorubens* and *S. flavomacrosporus* formed distinct clades. *S. radiopugnans* distributed in both grey and white series exhibited different ARDRA profile.

Most of the white spore mass *Streptomyces* clustered around the largest cluster of Streptomycetaceae, the *S. albidoflavus* cluster group. The various white species encountered in the present study, *S. albidoflavus, S. sampsonii*, *S. champavatii*, *S. luteogriseus*, *S. radiopugnans* and *Nocardiopsis alba.* Sequence divergence and heterogeneity of strains within species was evident in the case of the common largest species cluster namely, *Streptomyces albidoflavus* cluster. Although some of the white and grey isolates showed 99% similarity with many species, their taxonomic positions were further confirmed by comparing the morphological, physiological and biochemical characteristics with reference strains. Radiotolerant *Streptomyces radiopugnans* was first isolated from a radiation contaminated soil sample collected from Xinjiang Province, north-west China (Mao et al., 2007). Recently, thermotolerant, halotolerant *Streptomyces radiopugnans* (PM0626271) producing novel antibacterial compounds was reported from soil collected from Antarctica (Bhave et al., 2013). In this particular study, *Streptomyces radiopugnans* was identified in both grey and white spore mass series of actinomycetes. In the present study, the BLAST results of the most abundant type *Streptomyces albidoflavus* cluster revealed different species at the same identity level. Many of the observed species are consistent with earlier morphological and numerical taxonomic studies, but it is apparent that insufficient variation is present in the 16S rRNA gene sequence within the species of Streptomycetaceae family to permit bootstrap supported resolution of relationships (Labeda et al., 2012).

In general, great consistency between number of bands and their corresponding molecular weights were observed, when comparing the patterns generated in ARDRA with those obtained from the *in silico* restriction. Inconsistencies could be due to the difference in lengths of some of the GenBank 16S rDNA sequences that do not fully match with the lengths of the sequences of isolates in the present study. This might be due to the difference in primers used for amplification. Absolute length of fragments is not as important as the pattern (Peter et al., 2007). On the other hand, in some isolates the presence of additional faint bands were observed after the digestion, suggesting incomplete digestion of the amplicons.

One fact that has been overlooked is that multiple copies of 16S rRNA gene are often present in a given bacterium. According to Kampfer and Glaeser (2012), several *Streptomyces* species contain up to six ribosomal rRNA gene sets. These intragenomic copies can differ in sequence, leading to identification of multiple ribotypes for a single organism.

Accordingly, the hypotheses used to explain the existence of multiple rRNA operons within a genome are, that the multiple rRNA operons provide a multiplier effect on translation, allowing a bacterium to grow rapidly in response to environmental change (Klappenbach et al., 2000) and the functional differentiation between rRNA operons allows for differential expression of rRNA operons in response to environmental change (Gunderson et al., 1987). Hence, the reliability of molecular characterization alone in resolving *streptomycete* taxonomy is challenging and the importance of polyphasic taxonomic approach plays its role in resolving taxonomic confusion. Genes and genomes can only unfold their potential within a cell, and it is the phenotype that in combination with the natural selection ‘drives’

evolution in a given environment. In this context, the 'polyphasic taxonomic approach' should be revisited again (Kampfer and Glaeser, 2012).

Usually, microbial genomes evolve dynamically by both losing and gaining genes. Genome reduction is considered an evolutionary feature of intracellular pathogenic bacteria, in which gene loss is more likely to occur than gene gain (Li et al., 2013). Differential gene losses help create new species, and the evolutionary loss process has been investigated in many studies. Gene gain is also an important evolutionary force, especially in ecologically versatile species. However, is not yet known how, the highly-adaptable species, such as members of *Nocardiopsis*, maintain the genomic flexibility to survive across such a broad range of ecologies.

Amplified ribosomal DNA restriction analysis followed by sequencing of representative marine actinomycete isolates from sediments of Arabian Sea and Bay of Bengal revealed *Streptomyces* as the dominant genera followed by *Nocardiopsis*. Investigations on phylogenetic diversity analysis of 16S rDNA from representative isolates of actinomycetes from coastal multipond solar saltern in Tuticorin, displayed the dominance of genus *Streptomyces* followed by *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora* and *Nonomuraea* (Jose et al., 2012). The present molecular investigation unveils the species composition of culturable marine actinomycetes in the benthic realm from Indian waters. In the present study, *Streptomyces albidoflavus* was identified as the predominant species from marine sediments, which is in clear agreement with earlier reports that *Streptomyces albidoflavus*, *S. diastaticus*, and *S. lydicus* were found to be common in fresh water habitats, the former predominating in marine sediments (Goodfellow and Haynes, 1984; Goodfellow and Simpson, 1987). Molecular characterization of antagonistic *Streptomyces* sp. from Manakudy estuary, West coast of India, identified eight different species of *Streptomyces*, viz., *S. griseoruber*, *S. calestis*, *S. bikiniensis* etc. (Kannan and Vincent, 2011). The dominance of three clusters

of *Streptomyces* namely, *S. exfoliatus, S. halstedii* and *S. diastaticus* from Indian habitats was reported (Jain et al., 2009). *Streptomyces exfoliatus* and *S. diastaticus* were also isolated in the present study, but both the species were not found dominating, although it represented only 3.4% and 2.6% of the total actinomycete isolates. In Indian peninsula 41 species of marine actinomycetes were reported in which the genus *Streptomyces* was more frequently recorded (Suthindhiran and Kannabiran, 2009).

Diversity of culturable *Streptomycete*s from wheat cropping system of Indo Gangetic plains revealed the presence of *S. variabilis*, *S. acrimycini*, *S. rubrolavendulae*, *S. ambofaciens*, *S. fradiae*, *S. viridodiastaticus* by ARDRA and sequence analysis (Nityanand et al., 2011).

Taxonomic characterization of *Streptomyces* isolates has been a challenge due to the large number of described species, greater than any other microbial genus, resulting from academic and industrial activities (Labeda et al., 2012). It is apparent that the description of *Streptomyces* species needs to be based on a combination of phenotypic and genotypic properties (Kim et al., 2004; Saintpierre-Bonaccio et al., 2004) and even on a multilocus phylogeny (Guo et al., 2008).

The sequences of multicopy rRNA genes are mostly identical or nearly identical, which was evident in the present study. Therefore, several other single copy genes, which are conserved among bacteria, have been suggested and any of them can be considered as an alternative to 16S based phylogeny. Multilocus sequence typing based on single copy housekeeping genes viz; atpD, gyrB, recA, rpoB and trpB is gaining importance in the *Streptomycete* taxonomy (Rong and Huang, 2010). The single major obstacle resides in a fundamental property of protein encoding genes, i.e., the saturation of all third codon positions over a long evolutionary time scale, which makes it more difficult to the design universal primers for housekeeping genes (Case et al.,

2007). Also in the present scenario, it is not economical and feasible to sequence all five house keeping genes, besides the 16S rRNA for resolving the species identity.

As a conclusion to present study, polyphasic taxonomy coupled with ARDRA based on 16S amplicons was found to be reliable and accurate tool for the species identification of actinomycetes. Only few reports are available regarding ARDRA of actinomycete isolates (Cook and Meyers, 2003; Hall, 1999; Muharram et al., 2010). The combination of *Sau*3A1, *Hinf*1 and *Taq*1 used in the present study was not tested previously by researchers, although the enzymes have been used individually with other enzymes. This technique provided reliable results especially with the restriction enzyme *Taq*1 and *Sau*3A1 compared to *Hinf*1 in terms of fragment size and accuracy, but the existing database is insufficient to provide an accurate identification of marine isolates. The restriction profile generated in the study can contribute to database to help tentative identification of *Streptomyces* and *Nocardiopsi*s.

In the present study, resolution between species by ARDRA and sequence analysis was quite difficult in the case of most abundant group of *Streptomyces* with the white and grey spore mass. The presence of multiple copies of the rRNA operon and intra genomic heterogeneity of the 16S genes is considered as another limiting factor for the use of this gene for species identification (Rajendhran and Gunasekaran, 2011). 16S rRNA gene is still a significant phylogenetic marker in *Streptomycete* taxonomy (Rong and Huang, 2012). Hence, in this regard polyphasic approach and 16S rRNA sequencing have played its role in resolving the species identity of various *Streptomyces* and *Nocardiopsis* species in the current study. The present study has contributed to unveiling the species composition of *Streptomyces* and *Nocardiopsis* distributed in the sediments of Arabian Sea and Bay of Bengal.

4

Antimicrobial Property of Marine Actinomycetes with Special Reference to Antivibrio Activity

4.1 Introduction

The discovery of Penicillin from fungus *Penicillium notatum* and actinomycin from soil *Streptomyces antibioticus* heralded the era of antimicrobials and since then began the extensive screening of microorganisms for antibiotics. Antibiotic drugs were initially used to combat severe pathogenic illnesses, but indiscriminate use of antibiotics and self-medication has led to the phenomenon of drug resistance among microorganisms. Hence, the constant need for research and development of new antibiotics with novel active principles to combat resistant pathogens. Different offensive strategies are known to be adopted by bacteria for competitive elimination of related species or even subpopulations of the same species.

Marine microbes are particularly attractive because they produce highly potent bioactive compounds to be effective in the marine environment, due to the diluting effect of sea water. Actinomycetes comprise 10% of the total bacteria colonizing marine aggregates (Ward and Bora, 2006). Bioactive

compounds from marine actinomycetes possess distinct chemical structures that may form the basis for synthesis of new drugs that could be used to combat resistant pathogens.

Penaeid shrimp culture has become an important economic activity in many developing countries, particularly in Asia, where shrimp farming represents a substantial source of revenue. However, the shrimp farming industry is constantly under threat due to the outbreak of infectious diseases and environmental problems. Bacteria are among the groups of microorganisms causing serious losses in shrimp culture throughout the world. Vibriosis is one of the most common and significant factor of aquaculture loss in tropical countries. Hence newer and more effective strategy is required for the control of outbreaks caused by *Vibrio* spp. Despite the recognition of actinomycetes as promising candidates of novel antibiotics, less attention has been given in the exploration of antagonistic activity of *Streptomyces* towards *Vibrio* spp. in aquaculture.

4.1.1 Vibriosis in aquaculture

Vibriosis is a bacterial disease caused by Gram negative, motile, facultative anaerobic bacteria of the family Vibrionaceae. *Vibrio* species are the prominent microorganisms in the marine environment and usually constitute the majority in the normal microflora of farmed and wild penaeid shrimp. They become opportunistic pathogens when the natural defense mechanisms are suppressed (Lightner, 1993). The major species causing vibriosis in shrimp are *V. harveyi*, *V. alginolyticus*, *V. anguillarum* and *V. parahaemolyticus* (Goarant et al., 1999).

Members of the genus *Vibrio*, including *V. parahaemolyticus* and *V. harveyi*, have been described as the main pathogenic species in shrimps and

are responsible for most of the larval deaths. These pathogens cause serious infections, decreased production both in the hatchery and grow out ponds, reduced feed conversion and growth rates in surviving individuals, thus having a negative impact on the overall financial status of the business.

4.1.1.1 Strategies for shrimp disease prevention and control

Several strategies to control vibriosis in shrimp hatcheries have been proposed. Use of antibiotics to control the infectious agents has led to problems of drug resistance and resulted in trade restrictions in export markets. Vaccination or immunostimulation of shrimp is another widely accepted technology that promotes the immune response but they generally cannot be used as a universal disease control measure in aquaculture because efficiency of response on subsequent encounters is limited and they are too labour intensive.

Probiotics are another means of effective and environment friendly approaches of improving shrimp health and disease control. The mode of action of the probiotics is rarely investigated, but possibilities include competitive exclusion, that is, the probiotics actively inhibit the colonisation of potential pathogens in the digestive tract by production of bactericidal substances, competition for nutrients and space, and modulation of the immune system. The stimulation of host immunity and exclusion of pathogens may provide greater nonspecific disease protection as a result of both immunity enhancement and competitive exclusion (Rengpipat et al., 2000). There is accumulating evidence that the prophylactic use of beneficial bacteria is effective at inhibiting a wide range of fish pathogens. Increased concern about antibiotic resistant microorganisms (Amabile et al., 1995) has led to suggestions of alternative disease prevention methods, including the use of

nonpathogenic bacteria as probiotic biocontrol agents (Austin et al., 1995; Moriarty, 1997).

4.1.1.2 Competitive Exclusion

Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis in intensive larviculture systems (Garriques and Arevalo, 1995). Competitive exclusion is one of the ecological processes that can be manipulated to modify the species composition of a soil or water body or other microbial environment. Small changes in factors that affect growth or mortality rates will lead to changes in species dominance. Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. The competitive exclusion mechanism (competition for nutrients, space or oxygen)is based on the substitution of pathogen by beneficial population (Fuller, 1989; Gatesoupe, 1999; Moriarty, 1998). It is possible to reduce the adherence of pathogenic strains in the host animal and consequently reduce the risk of disease. Specific inhibition of *V. harveyi* by *Pseudomona*s *aeruginosa* has been reported (Torrento and Torres, 1996). Many bacterial isolates, which are common members of the non pathogenic microflora of fish and shellfish culture systems, have been shown to inhibit fish and prawn pathogens *in vitro*. This has been demonstrated for *Planococcus* (Austin and Billaud, 1990), lactic acid bacteria (Gatesoupe, 1994), *Vibrio* (Austin et al., 1995), *Carnobacterium* (Robertson et al., 2000), *Bacillus* (Rengpipat et al., 2000), *Pseudomona*s (Chythanya et al., 2002), *Micrococcus* (Jayaprakash et al., 2005) and *Streptomyces* (Das et al., 2010).

The antibacterial effect may be due to the production of antibiotics (Williams and Vickers, 1986), bacteriocins (Vandenbergh, 1993), hydrogen peroxide, or alteration of pH by producing organic acids (Sugita et al., 1997). Researchers claimed that certain strains of bacteria associated with *Artemia* and prawn culture systems have the ability to control pathogens by means of competitive exclusion or by the production of inhibitory compounds (Verschuere et al., 2000).

Hence, in recent years, there has been a considerable interest in isolating beneficial microorganisms from sea water, sediments and gastro intestinal tracts that are capable of producing antimicrobial substances that can inhibit pathogens *in vitro* as well as to avoid sudden changes in the microflora of the ecosystem (Banerjee et al., 2007; Rengpipat et al., 1998).

4.1.2 Antivibrio activity of marine actinomycetes

Actinomycetes isolated from marine samples collected from various stations along the Tuticorin coast were found to be inhibitory to bacterial pathogens of fishes (Patil et al., 2001). Marine actinomycetes have been proven to be antagonistic to *Vibrio* spp. pathogenic to shrimps. The use of marine *Streptomyces* sp. antagonistic to shrimp pathogenic *Vibrio* spp. was recommended as probiotic strains due to their ability to degrade macromolecules, production of antimicrobial substances, and heat resistant spores (You et al., 2005) Due to their potential to breakdown complex organic molecules such as starch and protein, actinomycetes could play an important role in the food webs of the marine environment. The activity of marine actinomycete as a potential organism against biofilms produced by *Vibrio* spp. was reported and recommended the use of actinomycetes to prevent the disease caused by *Vibrio* spp (You et al., 2007). Actinomycetes isolated from

marine environment of little Andamans identified as *Streptomyces xantholiticus*, *S. aureofasciclus*, *S. galtieri*, *S. vastus*, *S. galbus*, and *S. rimosus* exhibited varying degrees of antagonistic activity to *Vibrio* spp. (Sahu et al., 2007). Marine sponge associated *Streptomyces* strains represent a promising source of antibacterial agents against fish and shellfish pathogens especially *V. harveyi* and *V. parahaemolyticus* (Dharmaraj, 2010). *Streptomyces citricolor* isolated from coastal soil of Parangipetti showed the best level of antibacterial effect against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Vibrio cholerae* (Sathiyaseelan and Stella, 2011). Antagonistic marine *Streptomyces* sp. LCJ94 isolated from Bay of Bengal with inhibitory activity to *V. harveyi*, *V. alginolyticus* and *V. vulnificus* can act as biocontrol agents in controlling the diseases in shrimps caused by *Vibrio* spp. (Mohanraj and Sekar, 2013).

4.1.3 Application of Aerobic Granulation Technology

The applications of cell immobilisation technology have been widely recognised and developed since many years ago. Immobilised cell systems are extensively used in the production of enzymes (e.g. enzymes digesting cellulose, protein and chitin), antibiotics, alcohols, complex polysaccharides, surfactants and food additives. Microbial communities in the form of biofilms, or biogranules have been also employed in environmental bioremediation technologies e.g., wastewater treatment for removal of chemical oxygen demand (COD), nitrogen, phosphorus, heavy metals, hydrocarbons and cyanide and in biosensors (Venugopalan et al., 2005). Studies also demonstrated that aerobic granules can be used for phosphate and ammonia removal (Dulekgurgen et al., 2003; Tsuneda et al., 2003). With their compact structure and high degradation efficiency, aerobic granules show excellent ability in degradation of toxic compounds, such as phenol (Jiang et al., 2002).

Earlier studies have reported the diverse morphological forms of actinomycetes in submerged cultures (Lawton et al., 1989). Actinomycetes are unique among prokaryotes due to their filamentous nature, in shake cultures, the filaments entangle and aggregate to form bead like structures known as biogranules due to secretion of extracellular polymeric substances and cell surface hydrophobicity.

Actinomycete biomass of biogranule can be quantified by the estimation of ATP. The ubiquitous distribution of ATP in living cells, the rapid loss from dead cells, the fairly constant concentrations in microorganisms (Karl and Holm-Hansen, 1978), and the ease of extraction and measurement have fostered the use of ATP as an indicator of active microbial biomass (McCarthy, 1991). A typical method for ATP detection involves the chemical or enzymatic extraction of ATP from bacterial cells, followed by the measurement of light emission derived when the dissolved ATP reacts with the Luciferin Luciferase complex (Sakakibara et al., 1997; Webster et al.,1985). ATP measurement can provide a better estimate of the total active biomass than heterotrophic plate counts, where only a small fraction (<1%) of total cells are quantified ((Maki et al., 1986; Siebel et al., 2008).

Hence, a preliminary attempt was made to ascertain the efficacy of application of *Streptomyces* sp. in the form of biogranules to eliminate pathogenic *Vibrio* spp. which can find wide application in the field of bioremediation in aquaculture.

4.2 Materials and Methods

4.2.1 Selection of marine actinomycetes for exclusion experiments

Marine actinomycete culture *Streptomyces* sp.(M56) exhibiting broad spectrum antibacterial activity against aquaculture pathogens with significant biogranulation property was selected for *in vitro* competitive exclusion experiments.

4.2.2 Characterization of potent antibacterial strain *Streptomyces* sp. M56

4.2.2.1 Morphological, Biochemical and Physiological characterization

Morphological ,biochemical and physiological characterization of the strain was done as given in section 2.2.3, 2.2.4 and 2.2.7 respectively.

The sensitivity of the culture to various antibiotics penicillin G (10 i.u), tobramycin (10 μg), gentamicin (10 μg), streptomycin (25 μg), vancomycin (30 μg), neomycin (30 μg), cephaloridine (30 μg), rifampicin (30 μg) was done according to Kirby Bauer disc diffusion assay.

4.2.3 Phylogenetic analysis

The sequences retrieved on BLAST analysis of M56 were multiple aligned using the programme Clustal W (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the actinomycete genera retrieved from GenBank. Phylogenetic tree was inferred by the Neighbor joining (NJ) method (Saitou and Nei, 1987), with MEGA 5.0 package (Tamura et al., 2011). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

4.2.4 Carbon utilization profile

The carbon utilization/nutritional profile of potential antibacterial strain were done using BIOLOG SFP2™ microplate*. BIOLOG SFP2™ plates are 96 well plates that have 95 carbon sources and a water control. The first well A-1 (water control) is the reference well for growth comparison. The isolate was inoculated in to nutrient agar plates prepared with sea water until good sporulation. The spores were scraped off from the media and suspended in 15 ml sterile distilled water with 0.01% triton to reduce surface hydrophobicity of spores. The optical density (OD) of spore suspension was adjusted to 0.2 and 100 µl spore suspension was inoculated into each of the 96 wells. The microplate was incubated at 28°C for 7 days and the OD was read at 590 nm in a microplate reader (TECAN Infinite Tm, Austria). The utilization profile was scored as positive and negative by comparing absorbances with the reference well A-1.

4.2.5 Application potential of biogranule in aquaculture

4.2.5.1 *In vitro exclusion of Vibrio* spp. by actinomycete biogranule (Co-culture experiment)

The efficacy of actinomycete biogranules in exclusion of vibrios in aquaculture was tested using co-culture experiments. Co-culture experiments with actinomycete isolate (M56) and aquaculture pathogens belonging to *Vibrio* spp. viz., *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Vibrio fluvialis* were carried out following the method of Gram et al. (1999) with slight modifications. The vibrios were pre cultured separately in 100 ml flasks at 28°C on a shaker at 120 rpm overnight. The actinomycete granules were produced in nutrient broth medium by inoculating spore suspension into the medium, incubated at 28°C on a shaker at 150 rpm for 3-5

days. The medium sized granules were harvested, washed in sterile seawater and 15 granules (approximately 0.5 g wet weight) was inoculated into fresh nutrient broth (1/10 strength), with 1.5% NaCl to support the viability of *Vibrio* spp. and actinomycetes. The optical density of overnight culture of different *Vibrio* spp. was noted, and the OD was adjusted as to inoculate approximately an initial cell density of 10^5 cfu/ml of vibrios separately in the experiment set up of nutrient broth (1/10) with actinomycete granules (Fig. 4.1). Respective *Vibrio* controls were set up in nutrient broth (1/10) with 10^5 cfu/ml of *Vibrio* sp. without actinomycete granules. All the experiments were set up in triplicates.

Flasks were incubated at 28°C on a rotary shaker at 120 rpm and samples (1 ml) were withdrawn at 24 hour intervals for determination of *Vibrio* count. The samples were serially diluted 10 fold and 0.1 ml aliquots were spread plated on Thiosulphate Citrate Bile salt Sucrose agar (TCBS) (Hi media) in triplicates. The plates were incubated at 28°C for 24 hours and colonies formed on TCBS agar plates were counted and expressed as log10 cfu/ml of *Vibrio* in the co-culture.

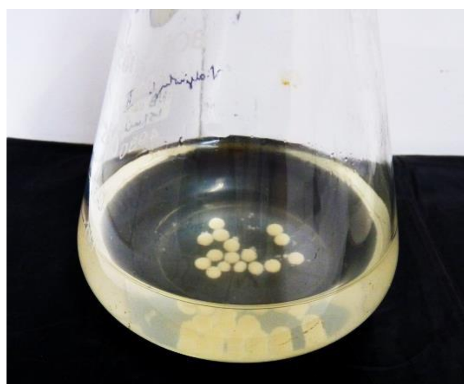


Fig. 4.1 Co-culture of actinomycete biogranule (M56) with *Vibrio* species

4.2.5.2 Biomass estimation of biogranule

Estimation of ATP

To estimate the biomass of biogranule, ATP estimation was carried out (Parsons et al., 1984). ATP extraction was done using sterile, boiling Tris-HCl buffer (0.02 M, pH 7.8). Five ml of buffer was brought to boiling point in a test tube, covered by a watch glass and actinomycete granule (1-2 Nos.) approximately 4-5 mm diameter was added to boiling Tris-buffer. The temperature of the buffer was maintained as not to be < 96°C after granule was added. After 5 minutes, the beaker was removed to a bath of crushed ice and centrifuged at 1,500 g for 5 minutes. The supernatant thus obtained was then decanted into a graduated test tube and held at -20°C until analysis. The ATP concentration was determined by the luciferin-luciferase reaction, using an ATP bioluminescent assay kit (Sigma chemicals, USA). Bioluminescence ATP assays were performed using a Luminometer (Turner). ATP (Sigma Chemicals, USA) at a concentration of 10-50 ng/ml was used as the standard. The factor value was calculated from ATP standards and biomass (in terms of ATP) per granule was estimated (Karl and Holm Hansen,1978).

$$ATP\ Concenrtation\ per\ wet\ weight\ of\ biogranule(fg/g) = Relative\ Light\ \ Units\ (RLU) \times\ Factor\ Value \times correctionfactor \times Dilution\ Factor/wet\ weight$$

4.2.6 Statistical analysis

The experimental data was analyzed by means of one way analysis of variance (ANOVA) and Duncan’s multiple comparisons of the means. Significance level for the analysis was set to $p < 0.05$. Statistical analysis was carried out using the software SPSS 19.0.

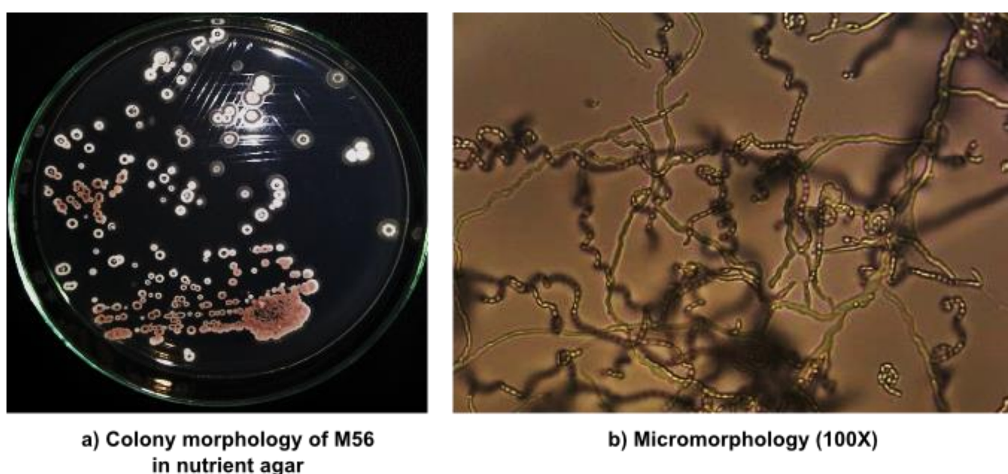
4.3 Results

4.3.1 Characterization of the potent antibacterial strain M56

Morphological characteristics of the strain M56 are given in Fig. 4.2. Microscopic observation of the coverslip culture of M56 revealed the sporophores as spirals. The strain M56 exhibited good growth on starch casein agar, yeast extract malt extract agar, glycerol asparagine agar and nutrient agar, with pink red aerial and light pink substrate mycelium. No diffusible pigments were produced in any of the media. Isolate M56 degraded esculin, urea and casein, resistant to lysozyme, and reduced nitrates. Melanin was not produced in tyrosine agar and peptone yeast extract iron agar. The culture M56 utilized sugars viz., glucose, lactose, galactose, arabinose, rhamnose, xylose, trehalose, sorbitol, mannitol and inositol but acid was produced only from glucose, trehalose and xylose. It was resistant to antibiotics penicillin, streptomycin, neomycin and cephaloridine, and sensitive to gentamicin, tobramycin, vancomycin, and rifampicin. Regarding the hydrolytic enzyme activity, the strain (M56) exhibited protease, amylase, lipase, DNase and phosphatase, while it did not show chitinase, pectinase, ligninase, cellulase and aryl sulphatase activity.

Based on the morphological, biochemical, and physiological characterization and 16S rDNA sequencing, the strain was identified as *Streptomyces rubrolavendulae.* The 1132 bp sequence of 16S rDNA was submitted to GenBank database under the accession number **KJ403746.**

Phylogenetic tree constructed to study the taxonomic position of antagonistic *Streptomyces* sp. M56 with that of 16S rDNA sequences of various actinomycetes revealed that M56 was closely related to type strains of *Streptomyces rubrolavendulae* with 99% similarity on blast analysis (Fig. 4.3). A comparison of the morphological, physiological and biochemical characteristics of the strain with the description of the Bergey's manual of systematic bacteriology, (Williams et al., 1989) revealed that the selected strain had the characteristics consistent with that of *Streptomyces rubrolavendulae*.



a) Colony morphology of M56 in nutrient agar **b) Micromorphology (100X)**

Fig. 4.2 Morphology of *S. rubrolavendulae* (M56)

Table 4.1 Morphological and biochemical characteristics of *Streptomyces rubrolavendulae* (M56) with broad spectrum antibacterial activity

Characteristic		Result
Morphological	Spore chain morphology	Spirals
	Spore mass colour	Pink red
	Aerial mycelium	Pink red
	Substrate mycelium	Off white
	Diffusible pigment production	-
Biochemical reactions	Melanin production (Peptone yeast extract iron agar)	-
	Melanine Production (Tyrosine agar)	-
	Esculin decomposition	+
	Casein decomposition	+
	Tyrosine decomposition	-
	Hypoxanthine decomposition	-
	Xanthine decomposition	-
	Lysozyme resistance (0.05%)	+
	Nitrate reduction	+
	H_2S production	-
	Urea Hydrolysis	+
	Citrate utilization	-
Acid Production from Carbohydrates	Lactose	-
	Mannitol	-
	Glucose	+
	Arabinose	-
	Rhamnose	-
	Galactose	-
	Inositol	-
	Trehalose	+
	Sorbitol	-
	Xylose	+
Resistance to Antibiotics	Penicillin G (10 i.u)	+
	Tobramycin (10 µg)	-
	Rifampicin (30 µg)	-
	Cephaloridine (30 µg)	+
	Streptomycin (25 µg)	+
	Neomycin (30 µg)	_
	Gentamicin (10 µg)	_
	Vancomycin (30 µg)	_
Hydrolytic Enzyme Activity	Protease	+
	Amylase	+
	Lipase	+
	DNase	+
	Phosphatase	+
	Pectinase	-
	Ligninase	-
	Cellulase	-
	Aryl sulphatase	-
	Chitinase	-

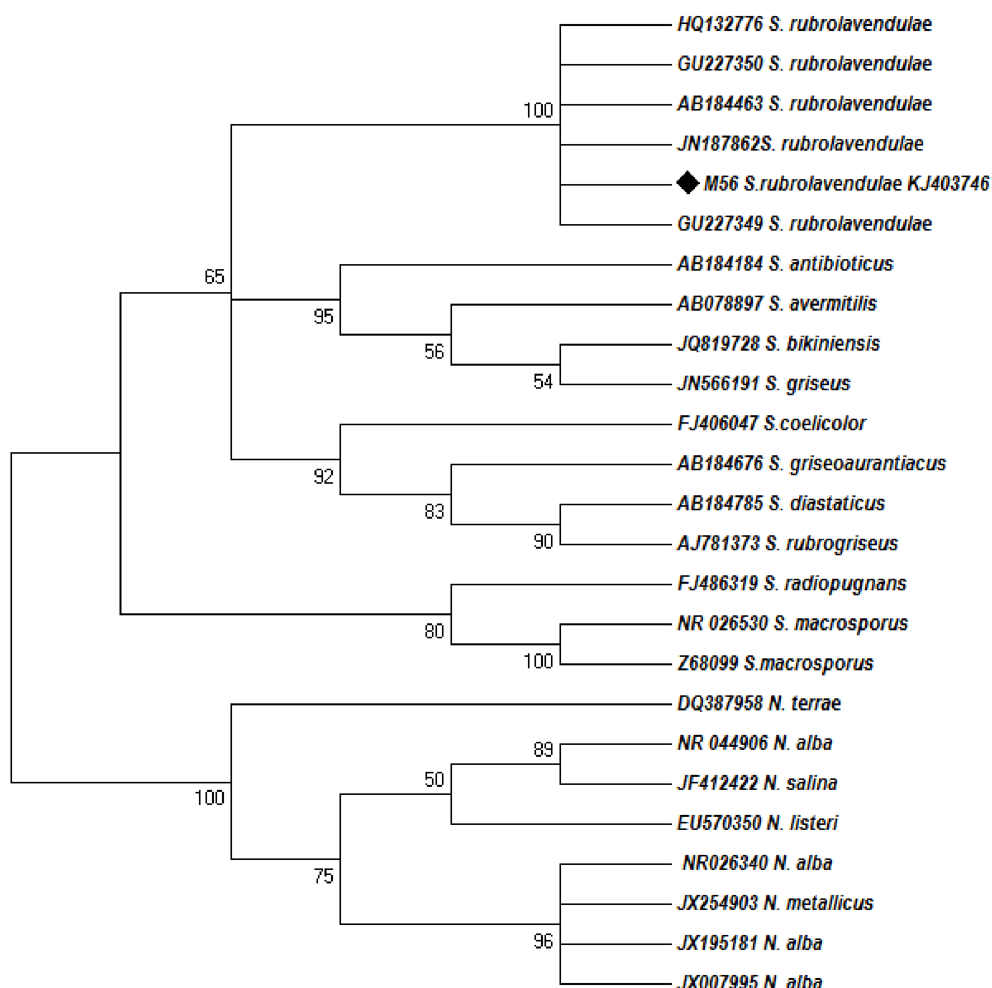


Fig. 4.3 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between *S. rubrolavendulae* (M56) and other related species. Values at the node indicate the percentage with which the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequence

4.3.1.1 Carbon utilization profile

The strain (M56) utilized 44 of the total 95 carbon sources tested in BIOLOG SFP2 microplate.

SF-P2 MicroPlate™

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-⊛-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl-D-Glucose	C12 α-Methyl-D-Glucoside
D1 ⊛-Methyl-D-Glucoside	D2 ζ-Methyl-D-Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvic Acid Methyl Ester	F7 Succinic Acid Mono-Methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl-L Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α–Glycerol Phosphate

Water	Sugars	Polysorbate	Amide	Glycoside	Sugar Alcohol	Carboxylic Acid	Ester	Amino Acid	Nucleoside

Table 4.2 Carbon utilization profile of *S. rubrolavendulae* (M56) (Acc.no: KJ403746) using BIOLOG SFP2 microplate

Carbon Source	Result	Carbon Source	Result	Carbon Source	Result
α- Cyclodextrin	-	L-Arabinose	+	α- D-Lactose	+
β-Cyclodextrin	+	D-Arabitol	-	Lactulose	-
Dextrin	+	Arbutin	-	Maltose	+
Glycogen	+	D- Cellobiose	-	Maltotriose	-
Inulin	-	D- Fructose	+	D-Mannitol	+
Mannan	-	L-fucose	-	D-Mannose	-
Tween 40	+	D-Galactose	+	D-Melezitose	-
Tween80	+	D-Galacturonic acid	-	D-Melibiose	-
N-Acetyl-D-Glucosamine	+	Gentiobiose	-	α-Methyl-D-Galactoside	-
N-acetyl-β-D-Mannosamine	+	D- Gluconic acid	+	β-Methyl-D-Galactoside	+
Amygdalin	-	α-D- Glucose	+	3-Methyl-D- Glucose	-
D-Tagatose		m-Inositol	+	α-Methyl-D -Glucoside	-
D-Trehalose	+	L-Alaninamide	+	Adenosine	+
Turanose	+	D-Alanine	-	2-Deoxy Adenosine	+
Xylitol	-	L-Alanine	+	Inosine	-
D-xylose	-	L-Alanyl glycine	-	Thymidine	-
Acetic acid	-	L- Asparagine	+	Uridine	-
α-Hydroxybutyric acid	-	L-Glutamic acid	+	Adenosine-5 Monophosphate	-
B- Hydroxybutyricacid	-	Glycyl-L-Glutamic acid	-	Thymidine-5-Monophosphate	-
ϒ-Hydroxybutyricacid	-	L-Pyroglutamic acid	-	Uridine-5-Monophosphate	+
p-Hydroxyphenyl aceticacid	-	L-Serine	+	D-Fructose-6-Phosphate	+
α-Ketoglutaricacid	+	Putrescine	+	D-Glucose-1- Phosphate	-
α-Ketovalericacid	+	2,3-Butanediol	-	D-Glucose-6 -phosphate	+
B-methylD glucoside	-	Glycerol	+	D-L-α-glycerol Phosphate	+
α-Methyl D mannoside	-	Lactamide	-	Succinic acid mono methyl ester	-
Palatinose	+	D-Lacticacid methyl ester	+	Propionic acid	+
D-piscose	-	L-lacticacid	-	Pyruvic acid	-
D-raffinose	+	D-Malic acid	-	Succinamic acid	-
L-Rhamnose	+	L-Malic acid	+	Succinic acid	-
D-ribose	+	Pyruvic acid methyl ester	+	Sedoheptulosan	-
Salicin	-	N-Acetyl- L-Glutamic acid	+	Stachyose	-
Sorbitol	+	Sucrose	-		

The various sugars utilized by M56 included glucose, rhamnose, arabinose, D-trehalose, turanose, fructose, galactose, raffinose, palatinose, ribose, glycogen, dextrin, α-D lactose, maltose, mannitol, sorbitol, inositol, adenosine, deoxy adenosine, D-fructose-6-phosphate, D glucose-6-phosphate, β-cyclodextrin, N-acetyl-D-glucosamine, N-acetyl-D mannosamine, D-L-α-glycerol phosphate, uridine-5-monophosphate, amino acids such as L-glutamic acid, L-alanine, L-serine, L-asparagine, putrescine, carboxylic acids such as α-ketoglutaric acid, α-keto valeric acid, D-gluconic acid, propionic acid, L-malic acid and L alaninamide, D-lactic acid methyl ester, pyruvic acid methyl ester and polymers tween 40, and tween 80 (Table 4.2).

4.3.1.2 Biomass estimation

Biomass of the actinomycete granule, was estimated in terms of ATP. ATP concentration of *Streptomyces rubrolavendulae* M56 was found to be 72x 10^{6} fg/g wet weight of the granule. (Table 4.3)

Table 4.3 Biomass of actinomycete granule by ATP measurement

Actinomycete granule	Wet weight of the granule(g)	Relative light units (RLU)	*Amount of ATP /g wet wt. of the granule (fg/g)
Streptomyces rubrolavendulae M56	0.03	3834 ±1.34	72 x 10^{6}

*ATP concentration per wet weight of biogranule = Relative light Units (RLU) x factor value x dilution factor xcorrection factor/wet weight

Factor value (calculated from standards) = 4.5x10^{-6}

4.3.2 *In vitro* exclusion of *Vibrio* spp. by actinomycete biogranule

In co-culture experiments with *Streptomyces rubrolavendulae* (M56) and *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. fluvialis*, a gradual decline in viable *Vibrio* count with time was observed and became undetectable on the third day for all co-cultures.

Cell numbers of vibrios treated with *Streptomyces* (M56) biogranule at 24, 48 and 72 hours were significantly lower than the respective control at 48 and 72 hours ($p < 0.05$). Biogranules of strain M56 showed a competitive exclusion effect on *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis* and *V. harveyi* and reduced the viable counts of this bacterium from 10^5 to 10^1 cfu/ml after 48 hours of incubation (Appendix 2). The viable count of *Vibrio parahaemolyticus* treated with M56 granule showed a decrease from 10^5cfu/ml to 10^2 during time interval of 24 hours, and to 10^1 cfu/ml within 48 hours and no detectable count was observed after 72 hours (Fig. 4.4). On treatment of *Vibrio harveyi* with M56 granule, the viable count showed a steady state with slight increase in cell number after 24 hours and a drastic reduction in cell count was observed after a period of 48 hours from 10^5 cfu to 10^1 cfu/ml and was undetectable by 72 hours (Fig. 4.5). The viable counts of *Vibrio alginolyticus* and *Vibrio fluvialis* showed a gradual decline from 10^5 to 10^4 cfu /ml after 24 hours to a count of 10^2 in the case of *alginolyticus* after 48 hours (Fig 4.6) while *V. fluvialis* count decreased to 10^1 cfu/ml after 48 hours and both the strains were undetectable after 72 hours (Fig. 4.7). *Streptomyces rubrolavendulae* (M56) biogranule could significantly exclude the pathogenic *Vibrio* spp. with respect to untreated controls.

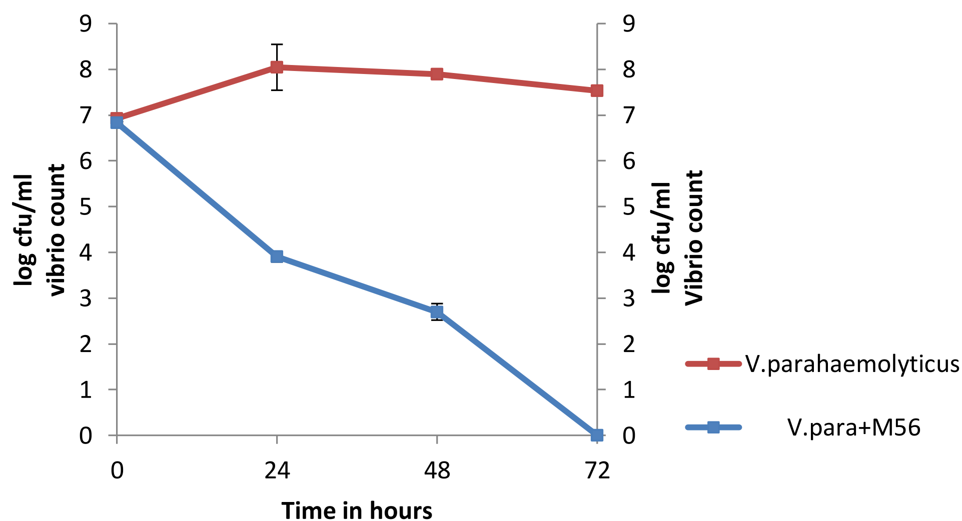


Fig. 4.4 Inhibition of *Vibrio parahaemolyticus* by actinomycete biogranule (M56)

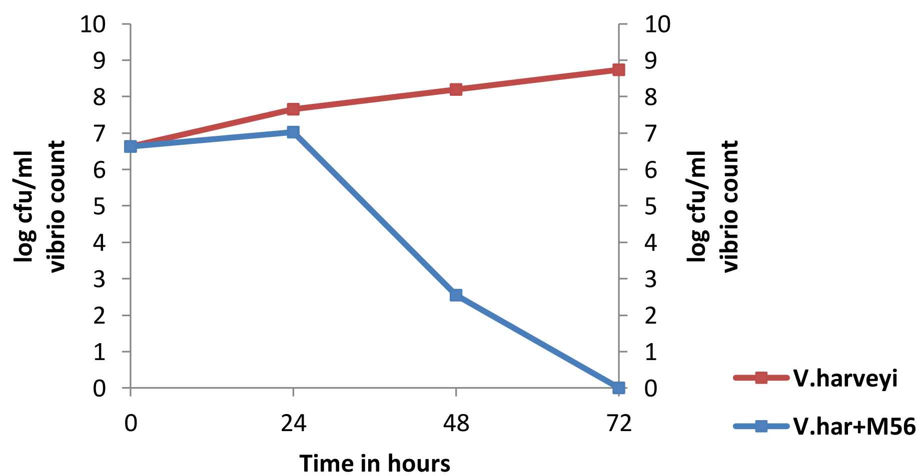


Fig. 4.5 Inhibition of *Vibrio harveyi* by actinomycete biogranule (M56)

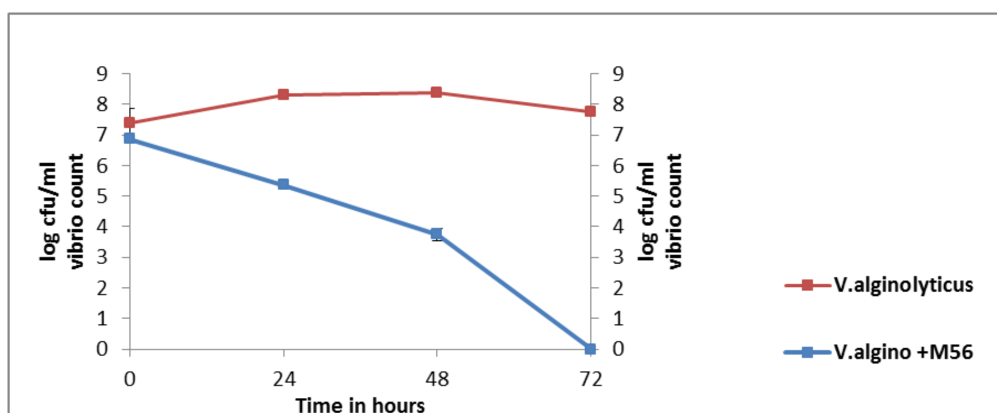


Fig. 4.6 Inhibition of *Vibrio alginolyticus* by actinomycete biogranule (M56)

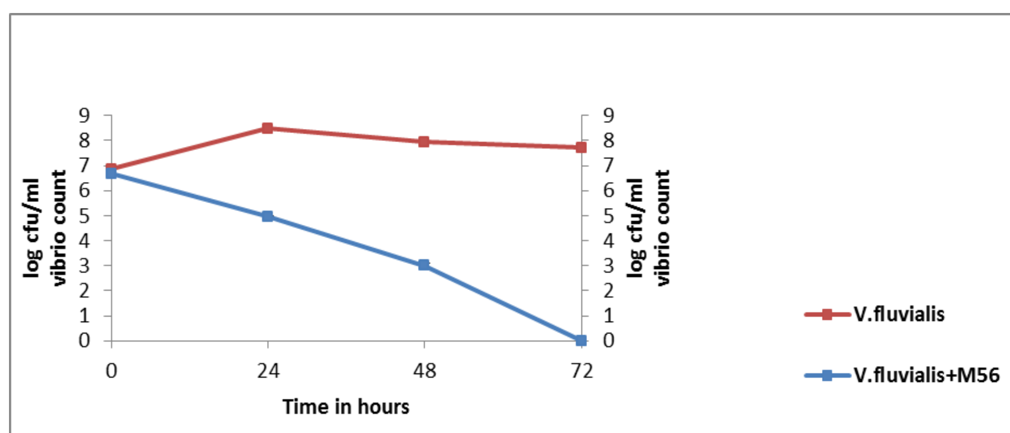


Fig. 4.7 Inhibition of *Vibrio fluvialis* by actinomycete biogranule (M56)

4.4 Discussion

Screening for novel antimicrobial agents is a continuous process to match unending demand for bioactive substances to curtail the phenomenon of drug resistance. Actinomycetes have been intensively screened in several underexplored environments and extreme habitats in various parts of the world

during the last few years. Yet there are only few reports regarding the bioactive metabolites produced by actinomycete isolates from sediments of Arabian Sea and Bay of Bengal. Actinomycetes produce bioactive compounds as a response to growth limiting stress conditions such as nutrient limitation and high cell densities (Bibb, 2005; Demain and Fang, 1995).

In the present investigation, strain M56, isolated from Bay of Bengal, which exhibited broad spectrum antagonistic activity against the *Vibrio* spp. and produced medium sized biogranules were selected for *in vitro* exclusion of pathogenic vibrios. Several studies have shown that secondary metabolite production is correlated with changes observed in actinomycete pellet morphology (Singh and Mahendra, 2013). Morphology and avermectin production by *Streptomyces avermitilis*, were shown to be influenced by factors such as the nitrogen source, dissolved oxygen level and inoculum volume (Yin et al., 2008).

The strain M56 was characterized and identified to species level, as the identification of potential isolates is vital since this provides informative insight about the organism, novelty of bioactive compounds produced etc. (Adegboye and Babalola, 2012). The strain was identified as 99% similar to *Streptomyces rubrolavendulae* based on polyphasic approach including 16S rDNA sequence analysis. Only very few reports are available on the occurrence and distribution of antagonistic *Streptomyces* in the marine environment. Many novel drugs have been developed from *Streptomyces* spp. including *S. griseus*, *S. hygroscopicus*, *S. coelicolor*, *S. avermitilis*, *S. rochei*, *S. plicatus*, *S. fungicidicus*, *S. flaveus*, and *S. globisporus*; belonging to different classes of antibiotics such as aminoglycosides, ansamycins, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines (Baltz, 2007; Murray, 2011).

The use of beneficial bacteria (probiotics) to exclude pathogens by competitive processes is being used as a better remedy than administering antibiotics for the control of aquaculture pathogens (Havenaar et al., 1992).

The new mode of application of *Streptomyces* (M56) in the form of biogranules could effectively eliminate all of the four pathogenic *Vibrio* spp. The biomass of *Streptomyces* biogranule (M56) was calculated based on ATP estimation as approximately 72 x 10^6 fg/g wet weight of biogranule. Greeg (1991) reported that ATP estimation of cell numbers by bioluminescence may agree closely with colony counts on agar plates for gram-positive bacteria. According to Stanley (1989), a bacterium contains approximately $10^{(-15)}$ g (1fg) ATP per cell. Hence it can be estimated that a M56 biogranule contains approximately 7.2 x 10^7 cells/g wet weight of biogranule. According to Liu and Tay (2002) a self-immobilized granule contains millions of organisms per gram of biomass. The biogranules of *Streptomyces rubrolavendulae* (M56) at an initial biomass of 72 x 10^6 fg/g could significantly eliminate the *Vibrio* spp. within 48 hours of co-culture. The results of the present study are in concordance with the suggestions put forth by previous researchers that the antagonist must be present at significantly higher levels than the pathogen and the degree of inhibition increased with the level of the antagonist (Banerjee et al., 2007; Goulden et al., 2012; Jayaprakash et al., 2005). A similar trend was observed in the co-culture experiments with *Pseudomona*s MCCB103 and *V.harveyi* where a cell density greater than 10^5 cfu/ml and 10^6 cfu/ml could significantly eliminate the pathogen (Pai, 2006). In the co-culture experiment study with actinomycetes and *V.harveyi* (at a cell density of 10^8 cfu/ml and 10^3 cfu/ml respectively), a gradual decline in *Vibrio harveyi* count was observed for all co-cultures and became undetectable on the third day of incubation (Lakshmi, 2008). Representative isolates of *Vibrio alginolyticus* VaM11 and

Vibrio parahaemolyticus VpM1, when tested for competitive exclusion using the marine *Bacillus* sp., showed decreased viable counts from 10^8 to 10^2 cfu/ ml (Banerjee et al., 2007).

In the present experiments, a reduction in viable counts of pathogenic vibrios clearly indicated competitive exclusion by the selected marine *Streptomyces* (M56) biogranule. There was no apparent reverse inhibition of selected actinomycete strain (M56) by the pathogenic vibrios. These observations supported the previous works (Dalisay et al., 1997; Nogami and Maeda, 1992) in which niche competition between beneficial bacteria and pathogens were noticed. Action of marine bacteria appears to be significant in protecting the host shrimp against pathogenic bacteria (Banerjee et al., 2007). *Streptomyces rubrolavendulae* (M56) produced five different enzymes, protease, amylase, lipase, DNase, and phosphatase which could also be an antagonistic factor, inhibiting growth of the vibrios. Similar observation was reported for *Bacillus* spp. which produced enzymes including protease, amylase, lipase and gelatinase inhibiting growth of the pathogenic bacteria (Banerjee et al., 2007). Antagonism is a widespread trait implicated in the competitiveness and ecological success of many marine bacteria and is thus considered an important attribute of aquaculture probionts (Gram et al., 2010; Long and Azam, 2001). The ability to degrade organic nutrients, production of antimicrobial substances, and heat resistant spores promotes the use of marine *Streptomyces rubrolavendulae* (M56) antagonistic to shrimp pathogenic *Vibrio* spp. as putative probiotic as earlier recommended by You et al. (2005).

To conclude, the present investigation suggests that *Streptomyces rubrolavendulae* (M56) antagonistic to *Vibrio* spp. can be used as a promising alternative to antibiotics. It can be used to control luminescent and other vibrios in marine larviculture systems.

5

Characterization and Evaluation of Bioactivity of Melanin from Marine Actinomycetes

5.1 Introduction

Colours are vital to the sensing of the environment and have evolved in higher living organisms to guide their interactions with others. Many bacterial species, including those inhabiting the vast marine environment, produce a wide variety of pigments that are important to cellular physiology and survival (Soliev et al., 2011). Colours often provide an easy way of identifying certain microbes, as they are often used in naming of the species. Microbial pigmentation comes in a variety of hues, ranging from bright coloured carotenoids, prodigiosin, quinones, violaceins, and phenazines to the dark pigment melanins. Evolutionary selective pressures act behind the acquisition of microbial pigments that promotes survival. Actinomycetes, one among the most colourful microbes are characterized by the production of various pigments on natural and synthetic media. Pigment production, an important feature in describing the actinomycetes has aroused considerable interest for many years. Some of the bioactive pigmented compounds investigated from marine actinomycetes include prodigiosin, carotenoids, quinones and

phenazines. They also synthesize and excrete dark pigments melanin or melanoid which are considered to be useful criterion for taxonomical studies (Arai and Mikami, 1972; Dastager et al., 2006). Although melanin production of *Streptomyces* was considered a taxonomic criterion, characterization of the melanin pigments from actinomycetes has been the least investigated.

Natural melanins are dark brown or black pigments with high molecular weight formed by oxidative polymerization of phenolic or indolic compounds in plants, animals and microorganisms (Langfelder et al., 2003). Generally, melanins are negatively charged, hydrophobic; insoluble in aqueous or organic solvents, resistant to concentrated acid and susceptible to bleaching by oxidizing agents (Butler and Day, 1998; Jacobson, 2000; Nosanchuk and Casadevall, 1997; Prota, 1992).

Melanin from natural sources possess a broad spectrum of biological activities including photoprotection (Menter and Willis, 1997), antioxidation (Hung et al., 2002), free radical scavenging (Rózanowska et al., 1999) and immunomodulatory effects (Sava et al., 2001).

A great variety of functions have been ascribed to melanins. Melanins can serve as energy transducers, bind diverse drugs and chemicals and affect cellular integrity (Hill, 1992). Melanin is also used for camouflage and sexual display. The ink of the cuttlefish *Sepia officinalis* consists of melanin particles. The coloration in black and red hair arises from different types of melanin and melanins are responsible for feather coloration in certain birds. Melanins in melanocytes of skin provide protection against sunlight and almost certainly contribute to the resistance of melanoma to therapeutic radiation (Hill, 1992).

Production of melanin is one of the most universal, but at the same time enigmatic adaptations of living organisms to the variable conditions of the

Earth (Plonka and Grabacka, 2006). The presence of various kinds of melanins in representatives of almost every large taxon suggests an evolutionary importance of melanogenesis. The basic functions of melanins are still a matter of controversy and speculation, even though their adaptive importance has been proved. Melanogenesis has probably evolved parallely in various groups of free living organisms to provide protection from environmental stress conditions (Plonka and Grabacka, 2006). It was postulated that most pigments evolved initially as a mechanism to combat environmental reactive oxygen species (ROS), but later on, these compounds were adapted to serve divergent functions (Liu and Nizet, 2009). Literature is available regarding the biology and implications of melanin in higher animals and humans. But very little is known regarding melanogenesis in microbes, characteristics of microbial melanin or its function.

5.1.1 Melanins in Actinomycetes

The ability of free living microbes to produce melanin is likely to be associated with survival advantage in the environment (Nosanchuk and Casadevall, 2003). *Aeromonas salmonicida*, *Azotobacter*, *Mycobacterium*, *Micrococcus*, *Bacillus*, *Legionella*, *Streptomyces*, *Rhizobium*, *Vibrio*, *Proteus*, *Azospirillum*, *Pseudomonas aeruginosa*, *Hypomonas*, *Burkholderia cepacia*, *E. coli*, *Bordetella pertussis*, *Campylobacter jejuni*, and *Yersinia pestis* are some of the bacteria known to produce melanin(Geng et al., 2010; Tarangini and Mishra, 2013). Apart from terrestrial microorganisms, explorations of melanin production by marine microorganisms are inadequate in literature.

The most illustrative example of melanin producing marine bacteria is the actinomycetes. This is particularly the case for the genus *Streptomyces*, from which most compounds with known biological activity have been

isolated (Fenical and Jensen, 1993). All *Streptomyces* strains are reported to use tyrosinases in the synthesis of melanin pigments (Claus and Decker, 2006). Diverse species of *Streptomyces* produce melanin and the production of the pigment is used in the taxonomy of the genus (Lindholm et al., 1997). In particular, melanin production is well described for *Streptomyces antibioticus* (Bernan et al., 1985; Katz et al., 1983) and *Streptomyces glaucescens* (Huber et al., 1985). *Streptomyces galbus* laccase production is induced at 42°C, which suggests that melanin formation is a protective response to adverse environmental conditions (Kuznetsov et al., 1984).

Diffusible dark pigment producing actinomycete strains were isolated from sediments of Vellar estuary, Tamil Nadu using starch casein agar. The dark pigment was identified as melanin by Fourier transform infra-red spectroscopy and suspected the pigment as eumelanin type (Vasanthabharathi et al., 2011). They reported sugarcane waste as the cheapest source for melanin production. The effective carbon source for melanin production by *Streptomyces* spp. was starch followed by glycerol and fructose (Dastager et al., 2006; Vasanthabharathi et al., 2011).

The *Streptomyces* sp. isolated from soil samples were the most producer of a diffusible dark brown pigment (melanin) on both peptone yeast extract and tyrosine liquid medium (Amal et al., 2011). Quadri and Agsar (2012) isolated eight thermoalkaliphilic *Streptomyces* sp. from the soil samples and reported that alkaline pH (9.0), higher temperature (45°C) and 3.0% concentration of sodium chloride are the important physiological attributes for the better production of melanin.

5.1.2 Enzymes of microbial melanogenesis

Microbes generally synthesize melanin via various phenoloxidases such as tyrosinases, laccases, or catecholases and the polyketide synthase pathway (Wheeler and Bell, 1988). Tyrosinases catalyze the oxidation of monophenols to o-dihydroxyphenols and further to o-quinones, and have a putative common ancestor with oxygen binding and oxygen transporting proteins (Decker and Terwilliger, 2000). They originate from primitive metalloproteins, which bind dioxygen through the prosthetic group that contains a co-ordinated transient metal cation.

Laccases are also metalloproteins containing one to four copper atoms in their active site. Not related to tyrosinases, they belong to the family of blue copper containing oxidases, together with ascorbate oxidase and ceruloplasmin. Both types of these melanogenic enzymes can be found in Eukaryota and Prokaryota, which confirms their ancient evolutionary origin (Valderrama et al., 2003).

Polyketide synthases (PKS) produce Dihydroxynaphthalene (DHN) melanins, and belong to an old family of multidomain proteins related to the animal fatty acid synthases (Kroken et al., 2003). Numerous microorganisms employ these enzymes to produce pigments, antibiotics, toxins and other products of intermediate metabolism (Hutchinson, 2003; Snyder et al., 2003). Other melanogenic enzymes include p-Hydroxyphenylpyruvate oxidase/hydroxylase (HPPD), for pyomelanin production from homogentisic acid and 4-Hydroxyphenylacetic acid hydroxylase (HPA) acting on phenylacetic acid (Gibello et al., 1995; Kotob et al., 1995).

5.1.3 Types of melanin

Melanins are polymers of phenolic compounds classified into three main types: 1) Eumelanins 2) Pheomelanins 3) Allomelanins.

Eumelanins (black or brown) are present in human hair and skin and are produced by some bacteria and fungi. These are produced by oxidation of tyrosine or phenylalanine to o-dihydroxyphenylalanine (DOPA) and dopaquinone by phenol oxidases which further undergoes cyclization to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (del Marmol and Beermann, 1996; Langfelder et al., 2003).

Pheomelanins (yellow-red) are present in red human hair. These are initially synthesized just like eumelanins, but DOPA undergoes cysteinylation, directly or by the mediation of glutathione. Pheomelanins contain sulphur, by virtue of the presence of cysteine. The end product of this reaction, cysteinyl DOPA, further polymerizes into various derivatives of benzothiazines (Kobayashi et al., 1995; Nappi and Ottaviani, 2000).

Allomelanins are the least studied and the most heterogeneous group of polymers and are found in many fungi and plants. Fungi generally produce the DHN melanin (Babitskaya et al., 2000) , which is produced through oxidation or polymerization of di- (DHN) or tetra-hydroxynaphthalene.

5.1.4 Chemical characterization

Remarkably little is known about the chemical structure of melanins, despite their abundance in the global biomass. This is due to the inability of current biochemical and biophysical techniques to provide a definitive chemical structure, because these complex polymers are amorphous, insoluble, and not amenable to either solution or crystallographic structural studies

(Nosanchuk and Casadevall, 2006). Characterization of melanin is based on qualitative tests, UV-Visible spectroscopy, High performance liquid chromatography, Infra-red spectroscopy, Electron spin resonance signal and Nuclear magnetic resonance spectroscopy.

Previous studies have shown that melanin pigments extracted from various sources including plants (Sava et al., 2001; Wang et al., 2006), animals (Hong and Simon, 2006) and microorganisms (Koroleva et al., 2007) exhibit some common physical and chemical properties (Yong et al., 2009).

5.1.5 Functions of melanin

5.1.5.1 Melanin as electron acceptors

Melanin is a polymer able to donate or accept an electron, making it possible to produce energy in processes analogical to oxidative phosphorylation, but under anaerobic conditions, it can act as a final electron acceptor with insoluble compounds of iron (Menter et al., 1998). Melanin interact with free radicals *via* the simple one electron transfer processes (Rozanowska et al., 1999). The facultatively anaerobic marine bacterium *Shewanella alga* produces pyomelanin and reduces it simultaneously with the oxidation of gaseous hydrogen (Turick et al., 2002). In the mineralized marine deposits the availability of such soluble compounds is limited; therefore production of melanin is an important evolutionary adaptation. The soil aerobic bacterium *Azotobacter chroococcum* employs melanogenesis to enhance utilization of oxygen and to maintain reducing conditions necessary for binding atmospheric nitrogen. The presence of iron and copper ions in the medium significantly increases the rate of *Azotobacter* melanization (Shivprasad and Page, 1989; Sutton and Winterbourn, 1989).

5.1.5.2 Panenvironmental function

Melanins confer resistance to UV light by absorbing a broad range of the electromagnetic spectrum and preventing photo induced damage (Hill, 1992). The most important aspect of this function is lowering the vulnerability of the soil microecosystems to UV irradiation, but also maintaining the proper ion balance in the soil. Increased melanin production is associated with the greater resistance of pigmented fungi to radiation (Zhdanova et al., 1973). The most noteworthy function of melanins is to act as a photoprotectant against radiation, especially in the UV region of the spectrum, scavenge reactive oxygen species (ROS), and consequently reduce UV damage *in vivo* (Hill, 1992; Menter et al., 1998; Riley, 1997). Melanin produced by *Streptomyces lividans* was observed to protect the mosquitocidal activity of the endotoxin prepared from *B. thuringiensis* against 254 nm radiation (Liu et al., 1993). Melanin produced by newly isolated *Aeromonas media* strain from East Lake, China has the potential to be used as a general photoprotective agent for bioinsecticides (Wan et al., 2007). Melanin has been used commercially in photoprotective creams (Joshua and Arturo, 2003) and is generally thought to be environment friendly.

5.1.5.3 Melanin as a factor of virulence

The ability of pathogenic bacteria to produce melanin seems to originate from the evolutionary achievements of free living bacteria. In some genera containing both free living and parasitic strains (e.g. *Vibrio* spp.), there are both pyomelanogenetic and eu/pheomelanogenetic pathways, sometimes simultaneously active in one organism. Free living strains of *Vibrio cholerae* are usually amelanotic or they produce pyomelanin (Kotob et al., 1995). *Azospirillum chroococcum* melanin has been associated with protection

against toxic oxygen compounds (Shivprasad and Page, 1989) and iron binding by melanin in *Azospirillum salinestris* may protect against damage from hydrogen peroxide (Page and Shivprasad, 1995). These observations suggest that melanin contributes to virulence by protecting melanized bacterial cells in tissue against the oxidative burst of activated host effector cells.

5.1.6 Microbial melanin as antioxidants

Reactive oxygen species plays an important role in the development of degenerative diseases. Hence the focus of pharma industries is being directed to the search for natural antioxidants. Though pigments from natural sources like plants, were primarily used as a colouring agent in various industries, during the past decade, researchers have focused the usage of pigments as antioxidants in pharmaceutical and food industries. In this context pigment producing microbes are gaining importance.

Antioxidants can be defined as “any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell and Gutteridge, 1989). Antioxidants play an important role in inhibiting and scavenging reactive oxygen species, thus providing protection to humans against various infectious and degenerative diseases. Some degradation processes of aerobic living organisms are mediated by reactive oxygen species, such as superoxide anion radical, hydrogen peroxide radical and hydroxyl radical. Wang et al. (2008) reported that a significant decrease in singlet oxygen production was observed in the presence of eumelanin extracted from pig retinal pigment epithelium cells. Modern research is now directed towards ecofriendly natural antioxidants from plants and microorganisms with minimal side effects which can serve as safe therapeutics.

Proteus mirabilis, a Gram negative bacterial agent of human urinary tract infections, produces a melanin pigment that can act as a free radical trap (Agodi et al., 1996). A melanin pigment isolated from an epidemic strain of *Burkholderia cepacia* also possesses antioxidant properties that can attenuate macrophage superoxide production (Zughaier et al., 1999).

Few reports are available regarding the antioxidant and cytotoxic potential of secondary metabolites of *Streptomyces* isolated from marine sediments along the Indian coast. The cytotoxic activity of ethyl acetate extract of secondary metabolite of *Streptomyces* from marine sediments of Bay of Bengal was evaluated using HeLa cell lines by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a tetrazole (MTT) assay, hemolytic activity on mouse erythrocytes and the antifungal activity was evaluated by MTT cytotoxic assay against *Aspergillus niger*, *Aspergillus fumigatus* and *Candida albicans*. The cytotoxicity of secondary metabolite was found to be concentration dependent and 24% of isolates showed significant cytotoxic activity (Suthindhiran and Kannabiran, 2010). Similarly antioxidant potential of *Streptomyces* sp. VITSTK7 isolated from marine sediments collected at the Puducherry coast of Bay of Bengal assessed by DPPH• (1,1-Diphenyl-2-picrylhydrazyl) scavenging activity, Fe^{+3} reducing assay, metal chelating assay and DNA inhibition protection assay indicated that the ethyl acetate extract of the isolate possess free radical scavenging activity (Thenmozhi and Kannabiran, 2012).

The melanin pigment was produced by *Streptomyces girseorubiginosus* (desert soil, Rajasthan) and the purified pigment exhibited good DPPH scavenging activity which can lead to the development of a pharmaceutically valuable antioxidant (Radhakrishnan and Balagurunathan, 2011).

Cytotoxicity is considered an important index for evaluating safety of anti-oxidants, antimicrobials and management of chemicals prior to their administration in biological system. For its accomplishment, cell lines could be used which out rightly eliminated animal experimentation, as part of the bioassays (Jose et al., 2010), and is considered as *in vitro* models for toxicity studies (Allen et al., 2005). Such an approach could forecast their *in vivo* effects as well as assisting their optimization for field level application. Cytotoxicity assays have been developed which use different parameters associated with cell death and proliferation. Moreover, the most convenient, modern assays have been optimized for the use of micro titre plates to allow many samples to be analysed rapidly and simultaneously (Weyermann et al., 2005).

In the present study, an attempt was made to extract and characterize the melanin from marine actinomycetes. As the natural melanins are considered to exhibit photoprotective and antioxidant properties, evaluation of the antioxidant potential by *in vitro* free radical scavenging and phototoxicity assays were carried out. In order to ascertain the biocompatibility and nontoxicity of extracted melanin, standard cytotoxicity and antioxidant assays were performed, which can help contribute melanin to the pool of natural antioxidants free of adverse effects.

5.2 Materials and Methods

5.2.1 Selection of melanogenic actinomycete

Out of the positive melanogenic marine actinomycetes from marine sediments of Arabian Sea and Bay of Bengal (Refer section 2.2.4.7 and 2.3.3),

the culture M8 (*Streptomyces* sp.) which exhibited high positive melanin production ability on peptone yeast extract iron agar and weak reaction in tyrosine agar was selected for further extraction and characterization studies of melanin.

5.2.2 Melanin Formation test

The melanin formation test was done to confirm whether the diffusible black /brown pigment formed in peptone yeast extract iron agar and synthetic tyrosine agar is melanin (not melanoid pigments). Mineral basal media supplemented with carbon and nitrogen sources were used for the purpose and the effect of carbon and nitrogen sources on melanin formation was tested using L-DOPA as substrate (Arai and Mikami, 1972).

5.2.2.1 Effect of carbon source on melanin production

The selected culture M8 which had the ability to produce melanin on melanin production media was tested for the effect of carbon source on melanin formation.

The effect of carbon source on melanin production was done using the basal medium ($NaNO_3$ 2.0 g; K_2HPO_4 1.0 g; $MgSO_4.7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4.7H_2O$ 0.01 g; in 1000 ml distilled water pH7.2) supplemented with 1% carbon sources viz., glycerol, starch, dextrin, lactose, sucrose, fructose, and glucose as the sole carbon source. The carbon sources were prepared in 10% solutions, sterilized using bacteriological filter and added to the basal medium to give final concentration of 1%. Spore suspension (100 μl) of the actinomycete culture M8 was inoculated into 10 ml basal media supplemented with the respective carbon sources and incubation was done at 28°C for 9 days. Estimation of melanin production was done using the culture filtrate.

5.2.2.2 Effect of nitrogen source on melanin production

The culture M8 which had the ability to produce melanin on melanin production media was tested for the effect of nitrogen source on melanin formation. The effect of various nitrogen sources was studied with the same basal medium supplemented with L-asparagine, L-arginine, L-citrulline, L-histidine, glycine, L-proline, sodium glutamate, ornithine and addition of 1% glycerol as carbon source. All nitrogen sources were prepared in 10% solution, sterilized using bacteriological filter, and added to the basal medium to give a final concentration of 1%. Spore suspension (100 µl) of actinomycete culture was inoculated into 10 ml basal media supplemented with the respective nitrogen sources. Incubation was carried out at 28°C for 7 days. Estimation of melanin production was done using the culture filtrate.

5.2.2.3 Melanin estimation

Culture supernatant (2 ml) was mixed with 2 ml of 0.1 M phosphate buffer (pH 5.9) and 1 ml (0.4%) L-DOPA as substrate and incubated at 37°C for 1-2 hours. Red brown coloration due to dopachrome formation was read spectrophotometrically at 480 nm (Scribner et al., 1973).

5.2.3 Characterization of Melanogenic strain (M8)

5.2.3.1 Morphological and Cultural Characterization

Refer section 2.2.3

5.2.3.2 Biochemical and Physiological Characterization

Refer section 2.2.4

Screening of the culture (M8) for various hydrolytic enzymes viz; protease, amylase, lipase, pectinase, ligninase, DNase, phosphatase, cellulase, chitinase, arylsulphatase was done following the methods (Holding and Collee, 1971) with slight modifications (refer section 2.2.7). The sensitivity of the culture to various antibiotics penicillin G (10 i.u), tobramycin (10 μg), gentamicin (10 μg), streptomycin (25 μg), vancomycin (30 μg), neomycin (30 μg), cephaloridine (30 μg), rifampicin (30 μg) was done according to Kirby Bauer disc diffusion assay.

5.2.3.3 Molecular Characterization

Refer section 3.2.2 and 3.2.3

The 16S rDNA amplicons of (M56) were sequenced using the universal 27F and 1492R primers with an ABI prism model 3700 Big Dye Sequencer (Applied Biosystems, USA) at SciGenom, Kochi, India.

5.2.3.4 Phylogenetic analysis and GenBank Accession

The nucleotide sequences obtained were assembled using Gene Tool software and the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI) USA (www.ncbi.nlm.nih.gov). The sequences were multiple aligned using the programme ClustalW (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the actinomycete genera retrieved from GenBank. Phylogenetic tree was inferred by the Neighbor-joining (NJ) method (Saitou and Nei, 1987), with MEGA 5.0 package (Tamura et al., 2011). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

The sequences were deposited in the GenBank database using the web based data submission tool, BankIt. (http://www.ncbi.nlm.nih.gov/BankIt).

5.2.4 Carbon utilization profile of melanogenic strain M8

The carbon utilization/nutritional profile of melanogenic strain M8 were done using BIOLOG SFP2^{TM} microplate* as per the manufacturer's protocol. (Refer section 4.2.4)

5.2.5 Extraction of melanin

Spore suspension (1 ml) of the melanogenic strain (M8) was inoculated into 500 ml peptone yeast extract iron broth and incubated in a rotary shaker at 150 rpm for 7 days at 28°C. Extraction of melanin from the culture medium was done according to the protocol (Gadd, 1982) with slight modifications. Black diffusible pigment produced in the culture broth by the strain M8 was recovered by centrifugation at 8000 rpm for 10 minutes. The alkaline pigmented supernatant was further acidified to pH 2.0 with concentrated HCl to precipitate melanin. Melanin was pelletised by centrifugation at 10,000 rpm for 10 minutes. Melanin pellets were washed with deionized water thrice and centrifuged at 10,000 rpm for 10 minutes to obtain the purified pigment. Purified pigment designated as M8 melanin was lyophilized and stored at -20°C until further use.

5.2.6 Physicochemical characterization of extracted melanin

5.2.6.1 Solubility

The solubility of melanin was determined according to Wang et al. (2006). Crude melanin (0.1 g) was added to various solvents (10 ml) i.e.,

water, aqueous acid, organic solvents (chloroform, ethanol, methanol, acetic acid, petroleum ether, hexane, acetone) and dilute alkali (sodium hydroxide, aqueous ammonia), stirred for 1 hour and filtered. The absorbances of solutions were recorded at 400 nm in a Hitachi U 2001 double beam spectrophotometer to measure the solubility of melanin.

5.2.6.2 UV-VIS spectra of melanin

Crude melanin (0.3 mg) was dissolved in 1 ml alkaline distilled water (pH 10.0) prepared by adding 0.1 ml aqueous ammonia (25%) to 10 ml distilled water. Solution was scanned with UV-VIS Spectrophotometer (Hitachi U 2001) at wavelengths 230-600 nm and analyzed.

5.2.6.3 Nuclear magnetic resonance (NMR) spectra of melanin

Proton one dimensional (1D) NMR of M8 melanin and Sepia melanin standard (Sigma), was obtained at Eastman Chemical Company, USA. Sepia melanin standard (Sigma) was dissolved in 1M NaOD in D_2O at 80°C on a shaker. M8 was dissolved in 1M NaOD in D_2O at room temperature. All solutions were centrifuged, and then transferred to 5 mm NMR tubes. Proton 1D NMR spectra were collected on a Bruker Avance III 600 NMR spectrometer using a CH cryoprobe operating at 298°K (25°C). Spectral data were collected and processed as follows: 16 (M8) and 128 (Sepia Melanin) scans, 32,768 points, 25 ppm sweep width centered at 5.0 ppm, exponential apodization with 5.0 (M8) and 2.0 (Sepia melanin) Hz broadening, and 15 sec pulse delay. Spectra were processed using the JEOL DELTA software package (version 4.3.6) on a MacBook Pro (operating system version 10.5.8).

5.2.6.4 Fourier Transform Infra Red Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying the functional groups and interpretation of structure of unknown compounds. FTIR spectrum of M8 melanin was recorded at 4,000- 400 cm^{-1} using Spectrum 100 Perkin Elmer FTIR spectrophotometer (USA) at the Inter University Centre for Marine Biotechnology, CUSAT. The spectra of M8 melanin was compared with that of Sepia and synthetic melanin standards.

5.2.6.5 Scanning Electron Microscope – Energy Dispersive Spectrometer analysis (SEM EDS)

Surface topography and qualitative elemental analysis on the surface of melanin (M8) was performed with SEM-EDS combination. Different elements and surface topographies emit different quantity of electrons, due to which the contrast in a SEM micrograph is obtained. SEM observations and elemental analysis were performed on gold coated samples that had been previously air-dried on glass slides. An analytical SEM (JEOL JSM-6390LV) equipped with EDS (JEOL JED – 2300) was used for the analysis, performed at Sophisticated Analytical Instruments Facility, STIC, CUSAT. The SEM EDS of M8 melanin was compared with synthetic and Sepia melanin standards.

5.2.6.6 Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

The atomic spectrum emitted by melanin (M8) is used to determine its elemental composition by ICP-AES analysis. The wavelength at which emission occurs identifies the element, while the intensity of the emitted radiation quantifies its concentration. Synthetic melanin was used as standard.

The analysis was carried out in an ICP-AES Thermo Electron IRIS INTREPID II XSP DUO at Sophisticated Analytical Instruments Facility, STIC, CUSAT.

5.2.6.7 Elemental analysis of M8 melanin (CHNS analysis)

The percentage of elements carbon, hydrogen, nitrogen and sulphur in melanin (M8) sample was analyzed over a wide range of sample matrices and concentrations with Elementar Vario EL III Operation at Sophisticated Analytical Instruments Facility, STIC, CUSAT. Synthetic melanin was used for comparison.

5.2.7 Assay for antioxidant activity

The antioxidant activity of extracted crude melanin (M8) was assayed based on two approaches; a peroxy radical inhibition assay in that the extent of the scavenging by hydrogen or electron donation of a preformed free radical is a marker of antioxidant activity. Secondly, a decolorization technique in which the stable free radical generated is decolourized on reduction with putative antioxidants.

The extracted melanin (M8) was tested for antioxidant activity using the following inhibition and free radical scavenging assays:

- Lipid peroxidation inhibition assay
- ABTS (2,2-azinobis3-ethyl benzothiazoline-6-sulfonicacid) radical scavenging activity
- DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging activity

5.2.7.1 Lipid peroxidation inhibition assay

A modified thiobarbituric acid reactive substances (TBARS) assay (Dasgupta and De, 2004) was used to measure the lipid peroxide formed using egg yolk homogenate as lipid rich media. Egg homogenate (0.5 ml of 10% v/v) and M8 melanin of different concentrations (200–2000 μg/ml) were added to a test tube and made up to 1 ml with distilled water; 0.05 ml of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and the mixture was incubated at 37°C for 30 minutes. Then 1.5 ml of 20% trichloro acetic acid and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate was added to quench the reaction. The resulting mixture was vortexed and then heated at 95°C for 60 minutes. After cooling, 5.0 ml of butanol was added to each tube and the mixture centrifuged at 3000 rpm for 10 minutes. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation (%) by the melanin was calculated.

$$\mathrm{I}\,\% = \left(1 - \frac{E}{C}\right) \times 100$$

Where,
***C** is the absorbance of the fully oxidized control and*
***E** is the absorbance in the presence of melanin.*

Butylated hydroxy toluene (BHT) was used as positive control.

5.2.7.2 Assay of 2, 2-azinobis 3-ethylbenzothiazoline-6-sulfonicacid (ABTS) radical scavenging activity

The ABTS radical scavenging activity of crude melanin (M8) was measured as described by Miller and Rice Evans (1997). ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution (8 mM) with 3 mM

potassium persulphate and the mixture was allowed to stand in dark at room temperature for 12-16 hours for the completion of free radical generation. The (ABT$S^{\cdot+}$) solution was diluted with phosphate buffer (pH 7.4) in order to obtain an absorbance of 0.8±0.01 at 734 nm. Melanin solution 10 µl of different concentrations (100 - 1000 µg/ml) were mixed with 240 µl of diluted (ABT$S^{\cdot +}$) solution and incubated at 30°C. The absorbance at 734 nm was read after 10 minutes in a microplate reader (TECAN Infinite). Trolox (1 mM) was used as standard. The ability of M8 melanin to scavenge ABTS radicals was calculated using the following equation:

$$I\ \% = \frac{(Ac - As)}{Ac} \times 100$$

Where,

***Ac** is the Absorbance of fully oxidized control(ABTS)*

***As** is the Absorbance of ABTS treated with different concentrations of melanin*

5.2.7.3 Assay of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging activity

Free radical scavenging activity of M8 melanin was determined according to the procedure described by Chen et al. (2008). The 0.2 mM solution of DPPH in 95% ethanol was prepared freshly before UV measurements. Melanin (1 ml) of different concentrations (100–1000 µg ml^{-1}), was thoroughly mixed with 2 ml of freshly prepared DPPH solution (0.1 mM in 95% ethanol) and 2 ml of 95% ethanol. The mixture was shaken vigorously and left to stand for 30 minutes in the dark, and then the absorbance was measured at 517 nm against a blank. The ability to scavenge the DPPH radical was calculated using the following equation:

$$I\% = \left[1 - \frac{Ai - Aj}{Ac}\right] \times 100\%$$

Where,

Ac *is the absorbance of DPPH solution without sample (2 ml DPPH + 3 ml 95% ethanol),*

Ai *is the absorbance of the test sample mixed with DPPH solution (1 ml sample + 2 ml DPPH + 2 ml 95% ethanol) and*

Aj *is the absorbance of the sample without DPPH solution (1 ml of sample + 4 ml 95% ethanol).*

The effective concentration of an antioxidant needed to decrease the initial DPPH/ABTS concentration by 50% (EC_{50}) is a parameter widely used to measure antioxidant activity (Nie et al., 2007).

5.2.8 Biocompatibility and cytotoxicity assays of melanin in human HEp-2 cancer cells

For biocompatibility studies, HEp-2 (Human larynx epithelial cells) cell lines were used. Biocompatibility and non toxicity of melanin (M8) was evaluated by sequential cytotoxicity assays viz., mitochondrial dehydrogenase activity by reduction of XTT (2, 3-Bis [2- methoxy 4 nitro-5-sulfophenyl]-2H-tetrazolium-5 carboxanilide), lysozomal activity by neutral red uptake (NRU) assay, and the assay for protein synthesis using sulforhodamine B dyes (SRB).

5.2.8.1 2, 3-Bis [2-methoxy 4 nitro-5-sulfophenyl]2H tetrazolium 5carboxanilide] XTTassay

For the above sequential assays, ~1×10^6 HEp-2 cells were inoculated into each well of a 96 well tissue culture plate containing MEM (minimal essential medium) supplemented with 10% fetal bovine serum (FBS) and incubated for 12 hours at 37°C. After incubation, the cells were copiously washed with phosphate buffered saline (PBS), and the medium was exchanged

with MEM containing different concentrations of melanin. The cells were incubated for 24 hours at 37°C, and after 24 hour incubation the wells were observed under Inverted phase contrast microscope (Leica, Germany) and sequential cytotoxicity assays were performed following manufacturer's instruction (Cytotox-PAN I, Xenometrix, Germany). Briefly, melanin treated cells were incubated with 50 µl pre-warmed XTT at 37 ºC for 4 hours, mixed the formazan formed in each well and absorbance was measured as a function of reduction of XTT to soluble formazan by healthy cells at 480 nm in a microplate reader (TECAN Infinite) with a reference wave length at 690 nm, and percentage of viable cells were calculated.

$$Percentage\ of\ viable\ cells = 100 - \left(\frac{Ac}{Ao} \times 100\right)$$

Where,

***Ac** is the average absorbance of cells at a particular concentration of melanin, and **Ao** is the average absorbance of control cells without melanin.*

5.2.8.2 Neutral red uptake (NRU) assay

For neutral red uptake assay, XTT solution from each well was discarded and washed with 300 µl of wash solution. Added 200 µl neutral red labeling solution diluted (1:200) with growth medium and incubated for 3 hours at 37ºC. Discarded the labeling solution and added 100 µl fixing solution to each well and discarded after 1 minute, added 200 µl solubilization solution to each well and incubated for 15 minutes at room temperature (RT). After incubation, mixed gently using a multichannel pipette and the absorbance were measured at 540 nm in a micro plate reader with a reference wavelength at 690 nm and percentage of viable cells was calculated.

$$Percentage\ of\ viable\ cells = 100 - \left(\frac{Ac}{Ao} \times 100\right)$$

Where,

***Ac** is the average absorbance of accumulated neutral red in cells at a particular concentration of melanin, and **Ao** is the average absorbance of accumulated neutral red in control cells without melanin.*

5.2.8.3 Sulforhodamine B (SRB) assay

For SRB assay, the solubilization solution from NRU test procedure was removed, with the cells remaining attached to the bottom of the wells. Washed with 300 µl wash solution and added 250 µl fixing solution, incubated the plate for 1 hour at 4ºC. Washed the cells and added 50 µl labeling solution, incubated for 15 minutes at room temperature. Washed 2 times with 400 µl rinsing solution and air dried the cells. Dissolved the air dried cells with 200 µl solubilization solution, incubated for 1 hour at room temperature and the absorbance was read at 540 nm with a reference wavelength at 690 nm and percentage of viable cells at each concentration of melanin were calculated (Vichai and Kirtikara, 2006).

$$Percentage\ of\ viable\ cells = 100 - \left(\frac{Ac}{Ao} \times 100\right)$$

Where,

***Ac** is the average absorbance of cells at a particular concentration of melanin and **Ao** is the average absorbance of control cells without melanin.*

5.2.8.4 Superoxide anion (Nitroblue tetrazolium reduction) assay

To assess the intracellular superoxide anion levels induced by melanin, HEp-2 cell lines were incubated with varying concentrations of M8 melanin.

The intracellular generation of superoxide anions were determined using standard nitroblue tetrazolium (NBT) reduction assay (Song and Hsieh, 1994). NBT solution (2 mg ml^{-1}) in 0.05 mol l^{-1} Tris HCl buffer (pH 7.6) was added to the melanin exposed cell lines and incubated for 1 hour at 10°C. The samples were then centrifuged at 5000 rpm for 5 minutes, discarded the supernatant and quenched the reaction by adding 500 µl absolute methanol. Following incubation for 10 minutes, the insoluble formazan residue was fixed by rinsing thrice with 500 µl of 50% methanol. The formazan residue was solubilized with 120 µl of 2 M KOH and 140 µl dimethyl sulphoxide (DMSO) and absorbance was read at 620 nm in a microplate reader (TECAN Infinite).

5.2.8.5 Estimation of Superoxide dismutase activity

Superoxide dismutase activity of melanin exposed cell lines was estimated (Kakkar et al., 1984). Assay mixture contained 240 µl sodium pyrophosphate buffer (pH 8.3), 20 µl phenazine methosulphonate, 80 µl nitro blue tetrazolium dye, 60 µl NADH, 240 µl distilled water and 60 µl of melanin treated HEp-2 cell lines. Tubes were incubated at 30°C for 2.5 minutes and reaction was stopped by the addition of 200 µl glacial acetic acid. Reaction mixture shaken vigorously with 800 µl n-butanol and absorbance of chromogen was measured at 560 nm.

5.2.9 Phototoxicity assay of M8 melanin

HEp-2 cells were prepared in 96 well microplate in minimal essential medium and treated with 50, 100, 200 µg ml^{-1} of melanin (M8). The cells were exposed to UV radiation for 10 minutes in an ice chest. Cells were washed copiously with phosphate buffered saline and reactive oxygen species (ROS) were analyzed by nitroblue tetrazolium reduction assay (Song and Hsieh, 1994) (Refer section (5.2.8.4). Control cells (HEp-2) without melanin

under UV exposure and without UV exposure were also maintained. Synthetic melanin (50 μg ml^{-1}) was used as positive control.

5.2.10 Statistical analysis

The data of all experiments were recorded as mean ± SD and were analyzed with SPSS (version 17.0 for Windows, SPSS Inc.). The IC_{50} was calculated using probit analysis of SPSS.

5.3 Results

5.3.1 Effect of carbon source on melanin production

The diffusible pigment formed in complex media was confirmed as melanin by melanin formation test using various carbon sources and L-DOPA as substrate. Results revealed starch as the most effective carbon source for melanin production of melanogenic strain (M8) followed by dextrin, glycerol, sucrose, and glucose as given in Fig. 5.1.

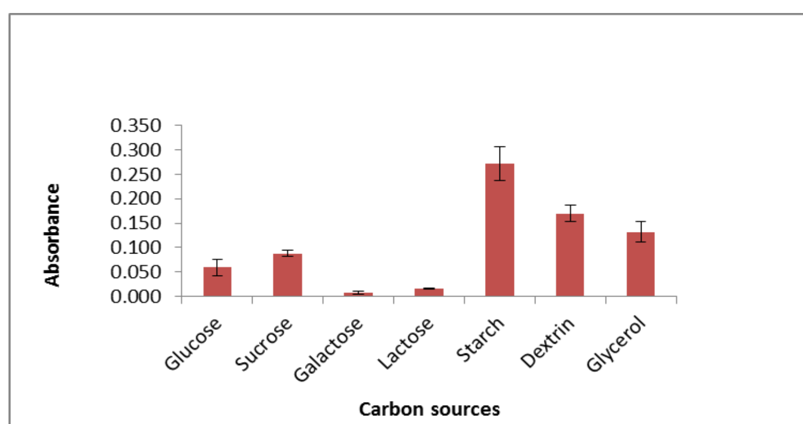


Fig. 5.1 Effect of different carbon sources on melanin production by M8

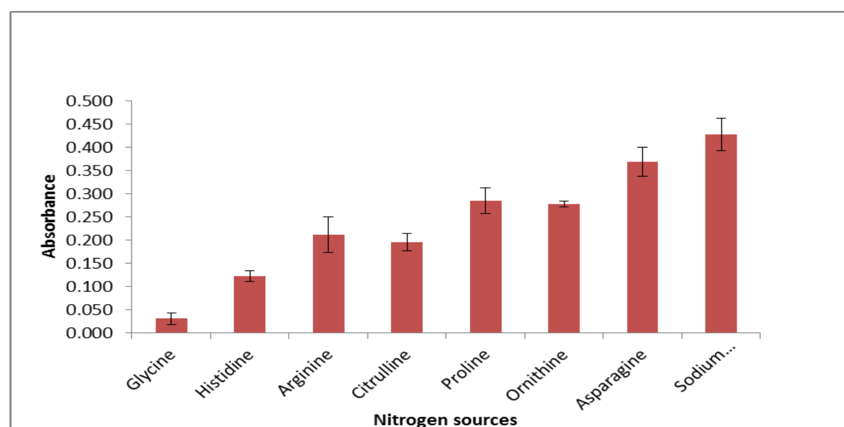


Fig. 5.2 Effect of different nitrogen sources on melanin production by M8

5.3.2 Effect of nitrogen source on melanin production

The diffusible pigment formed in complex media was confirmed as melanin by melanin formation test using various nitrogen sources and L-DOPA as substrate. The comparative efficiency of various nitrogen sources for the production of melanin by the melanogenic strain (M8) is shown in (Fig. 5.2). Sodium glutamate was the most effective nitrogen source, followed by asparagine, proline, ornithine, arginine, citrulline, histidine and glycine.

5.3.3 Characterization of melanogenic culture (M8)

5.3.3.1 Morphological and Cultural characterization

The actinomycete isolate M8 exhibited good growth on starch casein agar, glycerol asparagine agar, yeast extract malt extract agar and nutrient agar, with greyish white to grey aerial mycelium and off white substrate mycelium. Diffusible brown pigment was produced only in nutrient agar (Fig. 5.3).

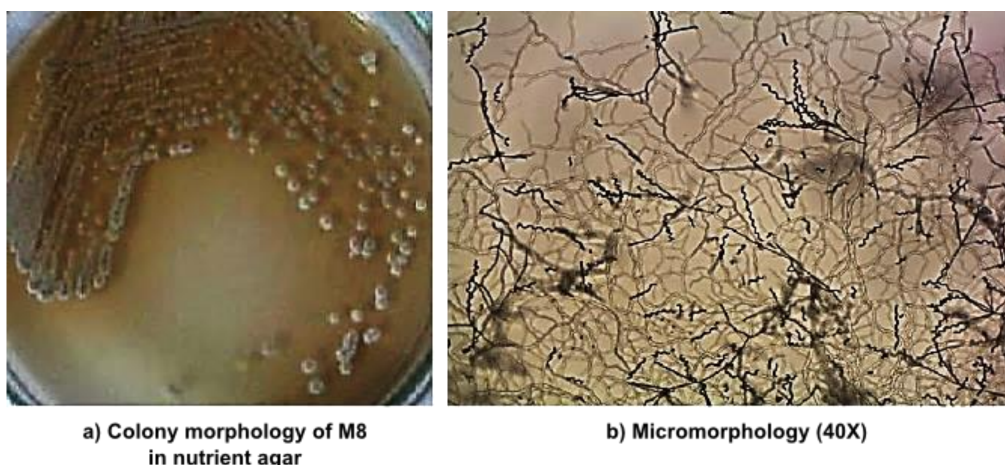


Fig. 5.3 Morphology of melanogenic culture (M8)

Microscopic observation of the coverslip culture of the strain revealed the sporophores as rectiflexibiles. The culture degraded esculin, casein, tyrosine, hypoxanthine and urea. It exhibited lysozyme resistance, nitrate reduction and hydrogen sulphide production. It was unable to utilize sodium citrate and xanthine was not degraded. It utilized almost all the carbon sources, but acid was produced only in media supplemented with glucose, mannitol, galactose and xylose. There was no acid production in media supplemented with lactose, arabinose, rhamnose, inositol, trehalose and sorbitol. The strain was resistant to penicillin G (10 i.u) and cephaloridine (30 µg). Hydrolytic enzyme activity exhibited by the strain included protease, amylase, lipase, DNase and phosphatase and there was no chitinase, ligninase, aryl sulphatase, pectinase and cellulase activity (Table 5.1).

Table 5.1 Morphological, biochemical and physiological characteristics of melanogenic culture (M8)

Characteristic		Result
Morphology	Spore chain morphology	Rectiflexibiles
	Spore mass colour	Grey
	Aerial mycelium	Grey
	Substrate mycelium	Offwhite
	Diffusible pigment production	Brown
Biochemical reactions	Melanin production (Peptone yeast extract iron agar)	+
	Melanin production (Tyrosine agar)	+w
	Esculin decomposition	+
	Casein decomposition	+
	Tyrosine decomposition	+
	Hypoxanthine decomposition	+
	Xanthine decomposition	-
	Lysozyme resistance (0.05%)	+
	Nitrate reduction	+
	H_2S production	+
	Urea Hydrolysis	+
	Citrate utilization	+
Acid production from carbohydrates	Lactose	-
	Mannitol	+
	Glucose	+
	Arabinose	-
	Rhamnose	-
	Galactose	+
	Inositol	-
	Trehalose	-
	Sorbitol	-
	Xylose	+
Resistance to antibiotics	Penicillin G (10 i.u)	+
	Tobramycin (10 µg)	-
	Rifampicin (30 µg)	-
	Cephaloridine (30 µg)	+
	Streptomycin (25 µg)	-
	Neomycin (30 µg)	_
	Gentamicin (10 µg)	_
	Vancomycin (30 µg)	_
Hydrolytic enzyme activity	Protease	+
	Amylase	+
	Lipase	+
	DNase	+
	Phosphatase	+
	Pectinase	-
	Ligninase	-
	Cellulase	-
	Aryl sulphatase	-
	Chitinase	-

5.3.3.2 Molecular phylogenetic analysis

The 16S rRNA gene sequence of the culture (M8) revealed 99% similarity with type strains of *Streptomyces bikiniensis* (**AB184602, AY946043**) on blast analysis. The strain also showed 99% similarity to other melanin producing strains. Hence a comparison of the cultural characteristics and morphological criteria of the strain with the description of the Bergey's manual of systematic bacteriology (1989) revealed that the selected strain had the characteristics similar to that of *Streptomyces bikiniensis*.

16S rRNA gene sequence analysis was carried out to elucidate the taxonomic position of melanogenic strain with type strains of other melanogenic *Streptomyces* spp. The tree is divided into four clusters, M8 clustered with *Streptomyces bikiniensis, Streptomyces violaceorectus*, *S.hirsutus* with a boot strap support of 87%. (Fig. 5.4).

5.3.3.3 Sequence Accession Number

The 1083 bp sequence of 16S rDNA determined was submitted to Genbank database under the accession number **JX657681.**

5.3.3.4 Carbon utilization profile of melanogenic strain M8

The strain utilized 70 out of the 95 carbon sources tested. The carbon sources utilized included the various sugars viz., β-cyclodextrin, dextrin, glycogen, mannan, N-acetyl-D-glucosamine, amygdalin, tagatose, trehalose, turanose, xylose, palatinose, piscose, raffinose, ribose, arabinose, cellobiose, fructose, galactose, gentiobiose, α-D-glucose, α-D-lactose, maltotriose, mannose, melezitose, melibiose, 3-methyl-D-glucose, α-D-glucose-1 phosphate, 2-deoxy adenosine, inosine, sugar alcohols such as xylitol, glycerol, arabitol, sorbitol, mannitol, D-L-α-glycerol phosphate, and

carboxylic acids viz., acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ- hydroxy butyric acid, α- ketoglutaric acid, p-hydroxyphenylaceticacid, D-galacturonic acid, D-gluconic acid, succinamic acid, succinic acid, propionic acid, pyruvic acid, also amino acids such as D-alanine, L- alanine, L-alanyl glycine, L- asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, glycosides such as arbutin, nucleosides viz; thymidine, uridine, thymidine-5-monophosphate, uridine-5-monophosphate, and the ester utilized was succinic acid mono-methyl ester (Table 5.2).

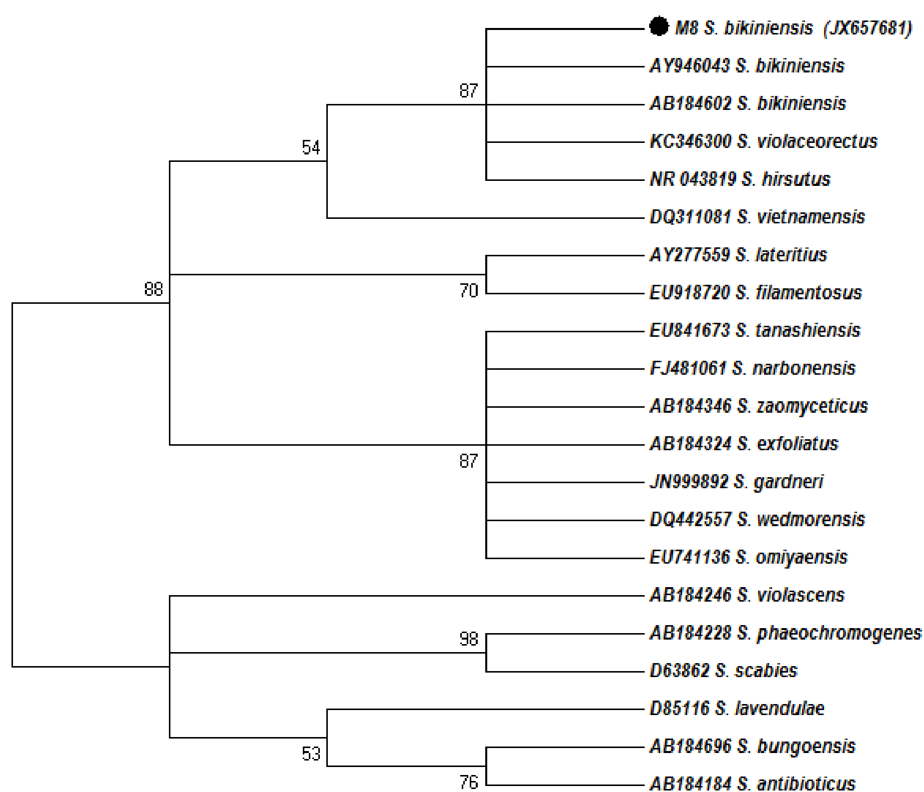


Fig. 5.4 Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequences showing the phylogenetic relationship of melanogenic culture M8 with related melanogenic Streptomyces spp. Bootstrap values are expressed as percentages of 1000 replications. Bootstrap values >50% are shown at branch points

Table 5.2 Carbon utilization profile of S. bikiniensis (M8) (Acc.no: JX657681)

Carbon source	Result	Carbon source	Result	Carbon source	Result
α-Cyclodextrin	-	L-Arabinose	+	α-D-Lactose	+
β-Cyclodextrin	+	D-Arabitol	+	Lactulose	-
Dextrin	+	Arbutin	+	Maltose	-
Glycogen	+	D-Cellobiose	+	Maltotriose	+
Inulin	-	D- Fructose	+	D-Mannitol	+
Mannan	+	L-Fucose	-	D-Mannose	+
Tween 40	+	D- Galactose	+	D-Melezitose	+
Tween 80	+	D-Galacturonic acid	+	D-Melibiose	+
N-Acetyl-D- Glucosamine	+	Gentiobiose	+	α-Methyl-D-galactoside	-
N-Acetyl-β-D-Mannosamine	-	D- Gluconic acid	+	β-Methyl-Dgalactoside	+
Amygdalin	+	α-D-Glucose	+	3-Methyl-D-glucose	+
D-Tagatose	+	m-Inositol	-	α-Methyl-D-Glucoside	-
D-Trehalose	+	L-Alaninamide	+	Adenosine	-
Turanose	+	D-Alanine	+	2-Deoxy Adenosine	+
Xylitol	+	L-Alanine	+	Inosine	+
D-Xylose	+	L-Alanyl glycine	+	Thymidine	+
Acetic acid	+	L- Asparagine	+	Uridine	+
α-Hydroxybutyricacid	+	L-glutamic acid	+	Adenosine- 5- monophosphate	-
β- Hydroxybutyricacid	+	Glycyl-L-glutamic acid	+	Thymidine-5-monophosphate	+
γ- -Hydroxybutyricacid	+	L-Pyroglutamic acid	+	Uridine-5-monophosphate	+
p-Hydroxyphenylaceticacid	+	L-Serine	+	D-Fructose- 6-Phosphate	-
α-Ketoglutaricacid	+	Putrescine	-	α-D-glucose-1-phosphate	+
α-ketovalericacid	-	2,3-Butanediol	-	D- Glucose- 6- phosphate	-
β-Methyl-D-Glucoside	-	Glycerol	+	D-L-α-Glycerolphosphate	+
α-Methyl-D-Mannoside	-	Lactamide	+	Succinic acid mono methyl ester	+
Palatinose	+	D-Lactic acid Methyl ester	+	Propionic acid	+
D-Piscose	+	L-Lactic acid	+	Pyruvic acid	+
D-Raffinose	+	D-Malic acid	-	Succinamic acid	+
L-Rhamnose	-	L-Malic acid	+	Succinic acid	+
D-Ribose	+	Pyruvic acid methyl ester	-	Sedoheptulosan	-
Salicin	-	N-acetyl-L-glutamic acid	+	Stachyose	-
Sorbitol	+	Sucrose	-		

5.3.4 Extraction of melanin

The yield of melanin per litre of peptone yeast extract iron broth was 0.166 g dry wt/L from 2.52 g dry weight of biomass (Fig. 5.5).

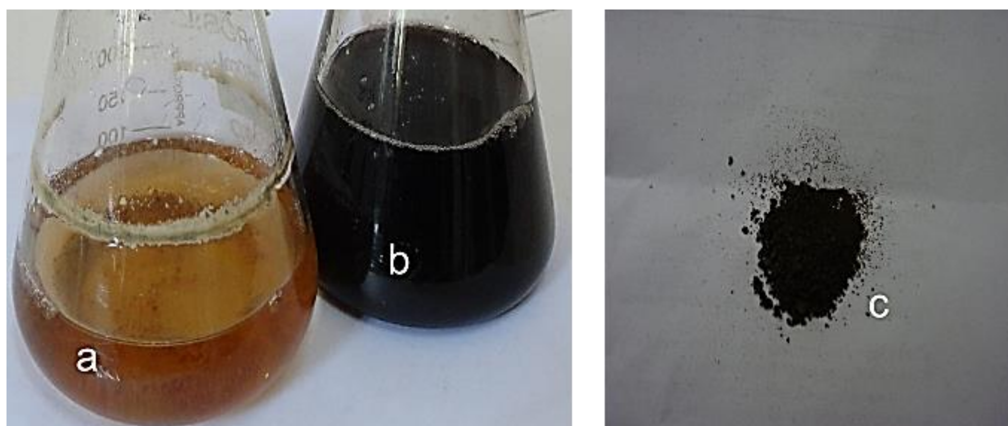


Fig. 5.5 Melanin production in peptone yeast extract iron broth (a) Medium inoculated with negative control (b) Streptomyces bikiniensis (M8) culture broth showing melanin production (c) Extracted M8 melanin (lyophilized)

5.3.5 Physicochemical characterization of extracted melanin

5.3.5.1 The solubility of melanin

The solubility experiments indicated that the melanin was insoluble either in water, aqueous acids or most organic solvents (e.g. methanol, ethyl acetate, ethanol, chloroform, ether, petroleum ether, hexane, acetone or acetic acid). It was partially soluble in 1 M NaOH, and dissolved only in aqueous ammonia (25%) solution and displayed a brown black colour. It was precipitated in acidic aqueous solution (0.1 M HCl), below pH 3. Therefore, the solubility of melanin is very similar to typical melanin.

5.3.5.2 UV-VIS Spectra of melanin

The UV and visible spectra of the melanin samples are given in (Fig. 5.6). Wavelength scan of M8 melanin exhibited an absorbance in the UV region with an absorption peak at 230 nm. The spectra of the M8 melanin did not show any different peaks in the 230-600 nm regions. The absorption was highest in the UV region of 230 nm, but decreased towards the visible region, which is the characteristic property of melanin.

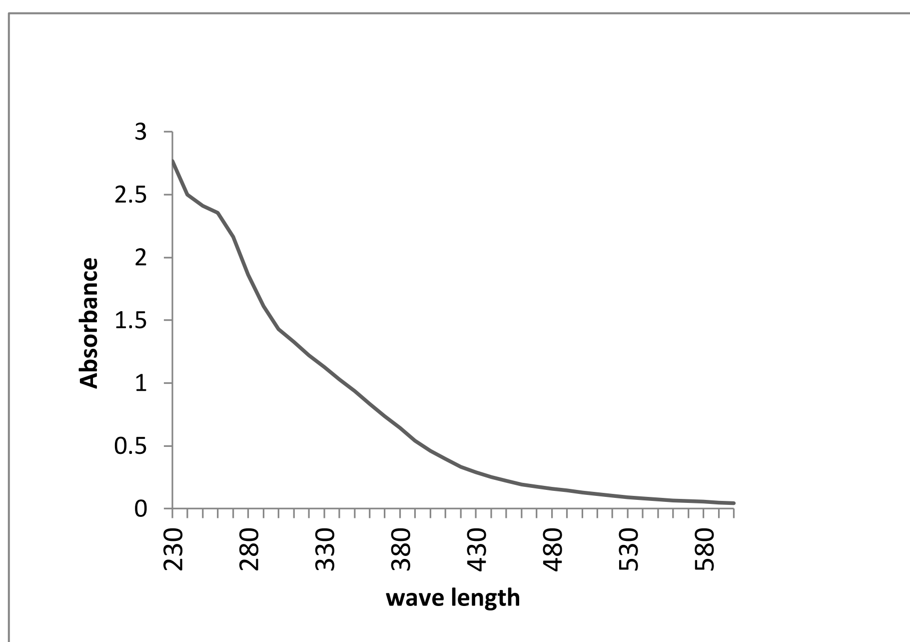


Fig. 5.6 UV-VIS Spectra of M8 melanin

5.3.5.3 NMR spectra of Melanin

Proton NMR spectra of Sepia melanin (A), and M8 melanin (B) are presented in Fig. 5.7. The broad resonances in M8 melanin at 7.60, 7.35, 7.00, and 6.60 ppm were also observed as minor components in the Sepia melanin

(Sigma). Sharp, well-defined aromatic resonances observed in the Sepia melanin were not observed in M8 melanin. NMR spectra for M8 melanin resembled spectra for human hair melanin except for the increased level of N and/or O attachment, less residual protein, and a lower level of methyl functionality relative to residual protein content. M8 melanin contains roughly equal levels of aromatic content relative to N and/or O attached functionality, about 40% and 37%, respectively.

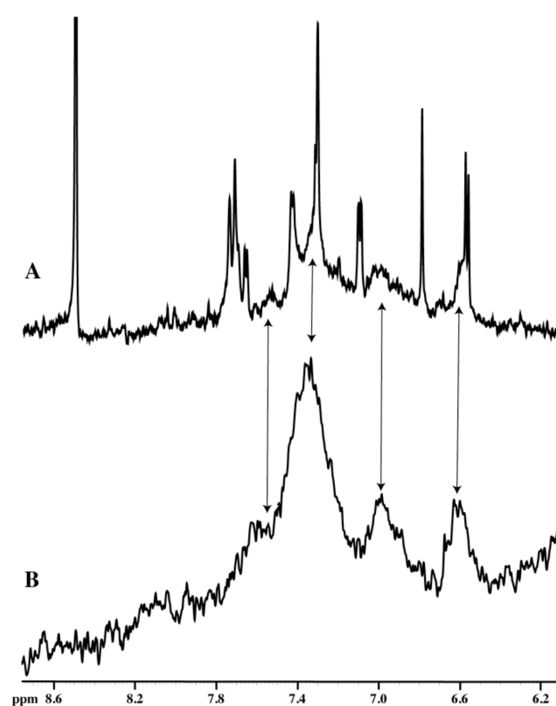


Fig. 5.7 Comparison of the aromatic proton spectral regions of Sepia melanin (A) and M8 melanin (B) plotted between 6.2 and 8.7 ppm. Vertical double arrowed lines indicate the presence of broad aromatic resonances found in M8 melanin also present as minor components in Sepia melanin

5.3.5.4 FTIR spectra

Fourier transform infra red (FTIR) spectra (Fig. 5.8) showed the presence of peaks at 3277.63 (OH or NH stretching), 1633.76 (C = C stretching or C=O stretching), 1537.8 (NH bending) and 1446 cm^{-1} (CH_2-CH_3 bending) characteristic of melanin pigment. Phenolic COH stretching at 1233.9 cm^{-1} relates to phenolic compounds. On the basis of the above results it was concluded that the pigment was eumelanin.

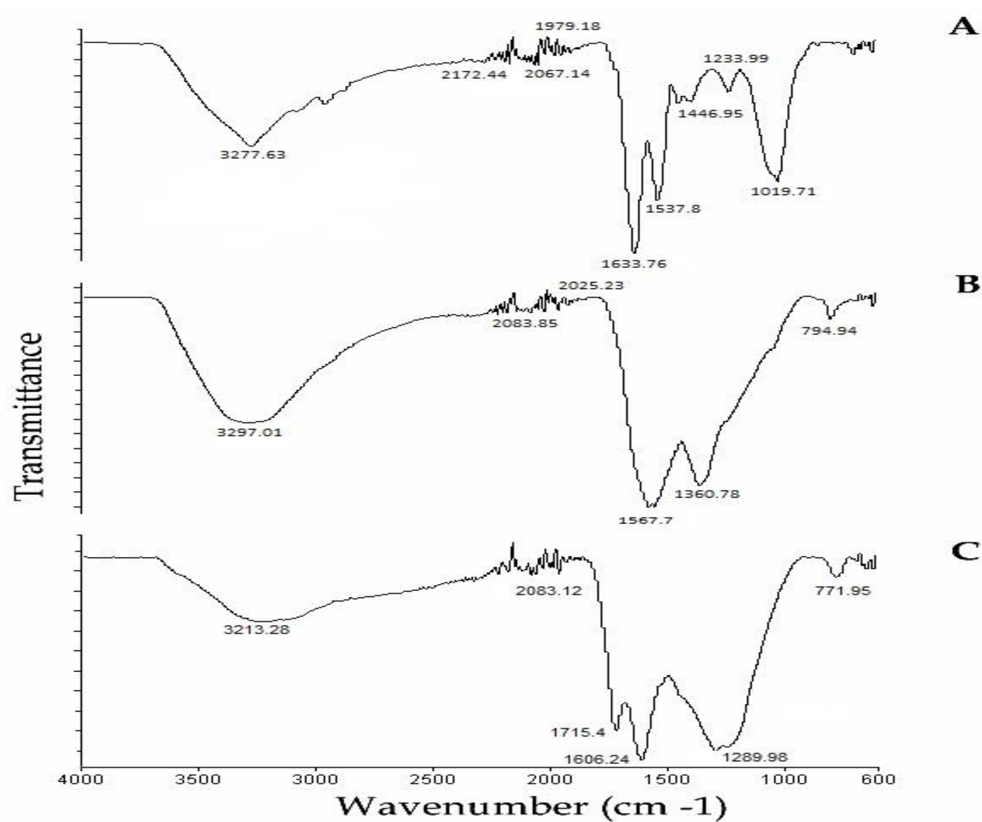


Fig. 5.8 FTIR Spectra analysis A) M8 melanin B) Sepia melanin (Sigma) C) Synthetic melanin

5.3.5.5 SEM -EDS analysis

The structure and elemental composition of the melanin was determined by scanning electron microscope-energy dispersive spectrometer (SEM-EDS) analysis. In the present study, the M8 melanin synthesized by *S. bikiniensis* in peptone yeast extract iron broth appeared to be amorphous by SEM imaging, similar to synthetic melanin, while natural Sepia melanin had a defined spherical structure (Fig. 5.9). The surface elemental composition of M8 melanin consists of 6 elements, carbon as the most abundant followed by moderate amounts of oxygen and iron, small amounts of phosphorous followed by sulphur and a trace of aluminum is also detected. Synthetic melanin consists of only 3 elements, large amounts of carbon, moderate amounts of oxygen and trace of chlorine, where as Sepia melanin consists of 7 elements, carbon being the most abundant. Unlike synthetic and M8 melanin, Sepia melanin has chlorine as the second abundant element next to carbon, followed by moderate amounts of sodium, oxygen, small amounts of calcium and magnesium and trace of sulphur (Fig. 5.10, Table 5.3).

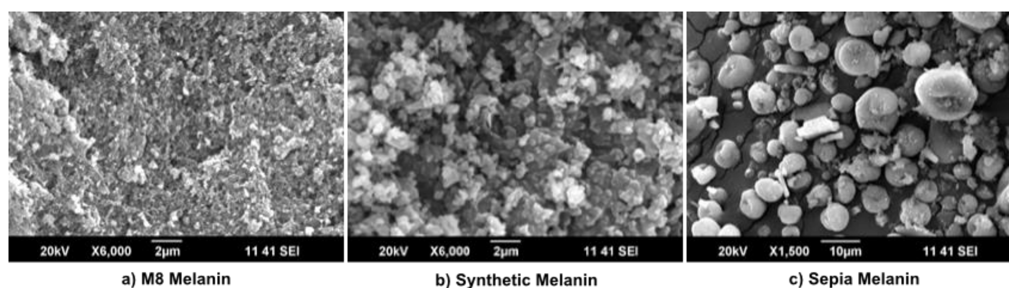


Fig. 5.9 Scanning electron microscope imaging of melanin samples

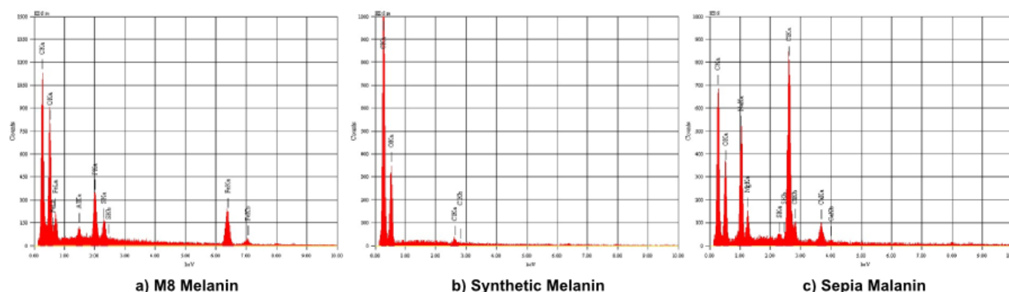


Fig. 5.10 Energy dispersive spectra of melanin samples

Table 5.3 Elemental composition of melanin samples by SEM EDX

Element	(KeV)	M8 melanin		Synthetic melanin		Sepia melanin	
		Mass %	Atom %	Mass %	Atom %	Mass %	Atom %
C K	0.277	55.87	73.21	89.35	92.1	46.92	66.06
O K	0.525	16.82	16.54	9.85	7.62	9.56	10.11
Al K	1.486	0.89	0.52	-	-	-	-
P K	2.013	7.16	3.64	-	-	-	-
S K	2.307	3.19	1.57	-	-	0.48	0.25
Fe K	6.398	16.06	4.53	-	-	-	-
Na K	1.041	-	-	-	-	10.41	7.66
Mg K	1.253	-	-	-	-	2.38	1.66
Cl K	2.621	-	-	0.8	0.28	27.36	13.05
Ca K	3.690	-	-	-	-	2.90	1.22
Total		**99.99**	**100.01**	**100.00**	**100.00**	**100.01**	**100.01**

5.3.5.6 ICP -AES

Melanin is associated with many metal ions bound to various functional groups. The various metals estimated were Mg (II), Ca (II), Na (I), K (I) and Fe (III). The most abundant element is iron in M8 melanin, synthetic melanin and Sepia melanin (data based on literature) but a higher percentage is detected in M8 melanin compared to synthetic melanin. Calcium is second in abundance to iron followed by sodium, potassium and magnesium. (Table 5.4). According to the proposed molecular structures of melanin, the pigment

contains phenolic hydroxyl (OH), carboxyl (COOH) and amine (NH) groups as potential functional binding groups for metal ions.

Table 5.4 ICP AES analysis of melanin samples

Sl. No.	Sample Name	Ca3179	Fe2395	K7664	Mg2802	Na5895	Unit
1	Actinomycete melanin	0.356	9.462	0.053	0.046	0.069	%
2	Synthetic melanin	0.226	0.612	0.024	0.032	0.035	%
	Detection Limit	0.01	0.01	0.01	0.01	0.01	ppm

5.3.5.7 CHNS analysis

Carbon, hydrogen, nitrogen and sulphur analysis of the lyophilized melanin (M8) samples were performed (Table 5.5). Compared with synthetic and Sepia melanins, M8 melanin had a higher percentage of nitrogen, sulphur and hydrogen content. The carbon content was almost similar to Sepia melanin, but synthetic melanin had higher carbon content. The C: N and C: H ratios of M8 melanin were smaller compared to synthetic and eumelanins.

Table 5.5 CHNS Analysis of melanin samples

Sample No.	Sample Name	N%	C%	S%	H%
1	M8 melanin	8.28	31.15	1.31	6.26
2	Synthetic melanin	6.18	49.77	-	3.39
3	Sepia melanin (Sigma)	5.87	32.61	0.33	3.11

5.3.6 Antioxidant assays

5.3.6.1 Lipid peroxidation inhibition assay

Egg yolk lipids undergo rapid non enzymatic peroxidation when incubated with ferrous sulphate with subsequent formation of malondialdehyde (MDA) and other aldehydes that form pink chromogen with thiobarbituric acid at an absorbance of 532 nm. The effect of M8 melanin on

non enzymatic peroxidation is shown (Fig. 5.11). The inhibition effects of M8 melanin on lipid peroxidation increased with corresponding increase in concentration as shown (Table 5.6). At a concentration of 25 μg ml^{-1}, the inhibition was 22.67 ± 4.72%, and for an increase in concentration of melanin from 50 - 150 μg ml^{-1} inhibition of lipid peroxidation increased as 37.90 ± 2.47%, to 65.29 ± 2.15%. At a concentration of 5.5 μg ml^{-1}, positive control butylated hydroxyl toluene (BHT) exhibited 70.84 ± 0.11% inhibition on lipid peroxidation. The inhibitory concentration (IC_{50}) of extracted crude M8 melanin on lipid peroxidation was calculated as 73.70 ± 12.6 μg ml^{-1}.

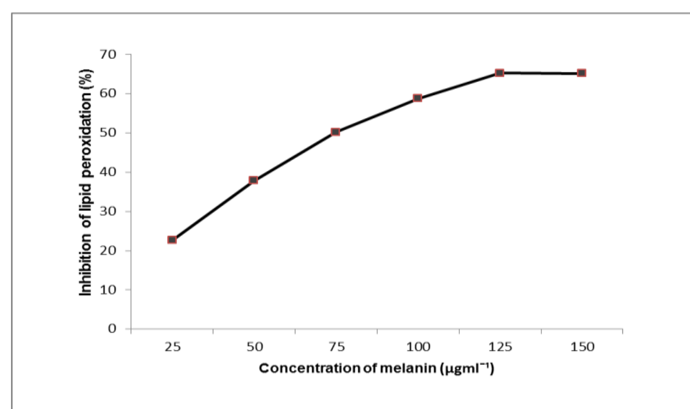


Fig. 5.11 Lipid peroxidation inhibition of crude M8 melanin

Table 5.6 Percentage inhibition of various concentrations of M8 melanin on lipid peroxidation

Concentration(μg/ml) Melanin	Inhibition% (I%)
25	22.67±4.72
50	37.90±2.47
75	50.24±2.75
100	58.75±7.32
125	65.29±2.15
150	65.1±1.05
IC_{50}	**73.70±12.6μg ml^{-1}**
BHT(5.5μg/ml)	70.84±0.11

Table 5.7 ABTS scavenging assay of M8 melanin and Trolox

Concentration M8 (µg/ml)	Inhibition% (I%)	Concentration Trolox (µg/ml)	Inhibition% (I%)
4	17.66±2.12	0.4	15.14±0.13
8	26.26±0.60	0.8	23.29±0.23
16	34.55±0.13	1.6	36.83±0.35
24	47.26±1.78	2.4	53.27±0.13
32	59.28±0.23	3.2	63.7±0.23
40	61.95±0.57	4	89.35±1.32
EC_{50}	**25.01±5.08 µg/ml**		**2.2±0.2 µg/ml**

Table 5.8 DPPH Scavenging assay of M8 melanin and Trolox

Concentration M8 (µg /ml)	Inhibition% (I%)	Concentration Trolox (µg/ml)	Inhibition% (I%)
20	36.92±1.36	0.5 µg	8.30±0.03
60	56.25±1.18	1.0µg	17.26±0.30
100	80.65±0.21	1.5	31.55±2.89
140	88.89±0.3	2.0	47.22±0.17
180	88.9±0.1	4.0	68.65±0.97
-	-	8.0	73.21±0
EC_{50}	**35.49±6.19 µg/ml**		**2.73±0.91 µg/ml**

5.3.6.2 ABTS radical scavenging activity

The scavenging ability of M8 melanin on ABTS radical is shown (Fig. 5.12). The scavenging effects of M8 melanin on ABTS radicals at a concentration of 4 µg/ml was 17.66 ± 2.12% which increased in a concentration dependent manner and attained an inhibition % of 61.95 ± 0.57% at a concentration of 40 µg/ml as shown (Table 5.7). The effective concentration (EC_{50}) of M8 melanin which effects scavenging of 50% ABTS radicals was calculated as 25.01±5.08 µg/ml. The EC_{50} of standard trolox on ABTS scavenging was calculated as 2.2 ± 0.2 µg/ml.

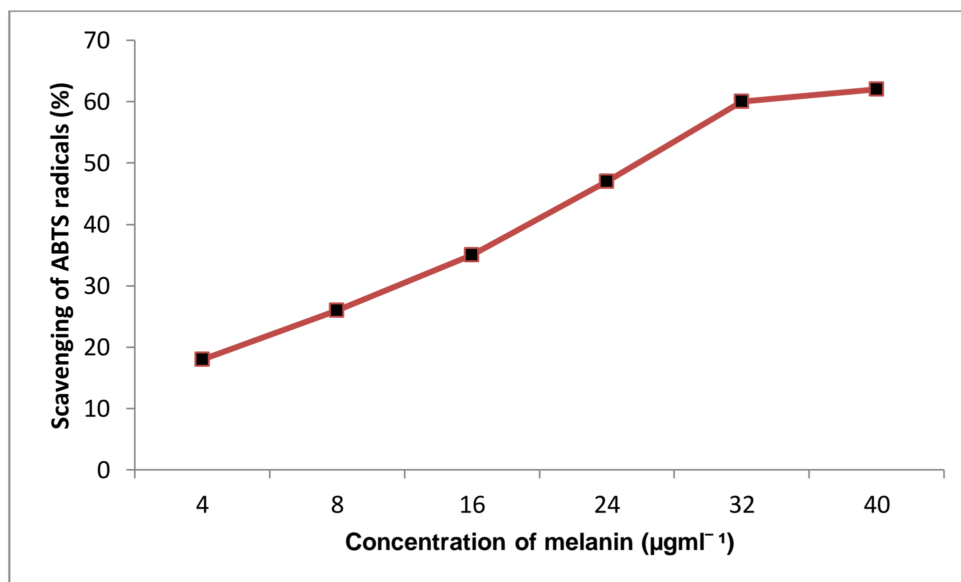


Fig. 5.12 Scavenging ability of M8 melanin on ABTS radicals

5.3.6.3 DPPH radical scavenging activity

Antioxidant compounds react with DPPH and convert it to 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine because of hydrogen donating ability. The scavenging potential of the antioxidant compounds is determined based on the extent of discoloration.

Scavenging ability of M8 on DPPH radicals was investigated (Fig. 5.13). At a concentration of 20 µg/ml, the inhibition % was 36.92 ± 1.36, which increased in a concentration dependent manner to 85.98 ± 1.29% at a concentration of 120 µg/ml. The effective concentration (EC_{50}) of M8 melanin which effects scavenging of 50% DPPH radicals was calculated as 35.49 ± 6.19 µg/ml. The EC_{50} of standard Trolox on DPPH scavenging was calculated as 2.73 ± 0.91 µg/ml (Table 5.8).

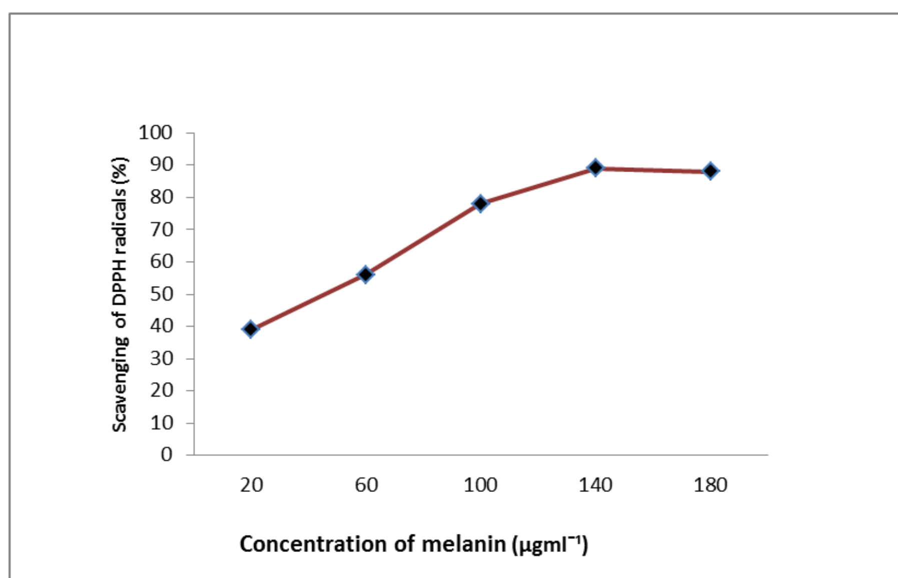


Fig. 5.13 Scavenging ability of M8 melanin on DPPH radicals

5.3.7 Biocompatibility and cytotoxicity of melanin in human HEp-2 cancer cells

The cell viability on treatment of HEp-2 cells with different concentrations of melanin was measured as a function of mitochondrial dehydrogenase activity, membrane integrity, and protein synthesis in healthy cells. Any decrease in the formation of formazan, neutral red uptake and protein synthesis were considered as an index of mitochondrial damage, membrane rupture and inhibition on protein synthesis (or cell death) respectively.

5.3.7.1 XTT assay

The viability of human HEp-2 cells in sequential cytotoxicity assays on exposure of cell lines to different concentrations of melanin is shown (Fig. 5.14). Melanin was non toxic to HEp-2 cells in culture up to a concentration of 156.25 μg ml^{-1}. At a concentration of 156.25 μg ml^{-1} only <5% inhibition of

mitochondrial dehydrogenase activity was observed and at 1250 µg ml^{-1} only <15% inhibition was observed. It may be noted that 1250 µg ml^{-1} is a concentration far higher than the dosage required for bioactivity.

5.3.7.2 Neutral red uptake (NRU) assay

In the case of neutral red uptake assay, the viability was maintained up to a concentration of 156.25 µg ml^{-1} and only a small percent loss of viability (8%) in terms of plasma membrane damage was observed. The viability in terms of neutral red uptake was found to be decreasing progressively with increasing concentration of melanin above 156.25 µg ml^{-1} and reached a maximum of ~25% reduction on cell viability at 1250 µg ml^{-1} concentration (Fig. 5.14).

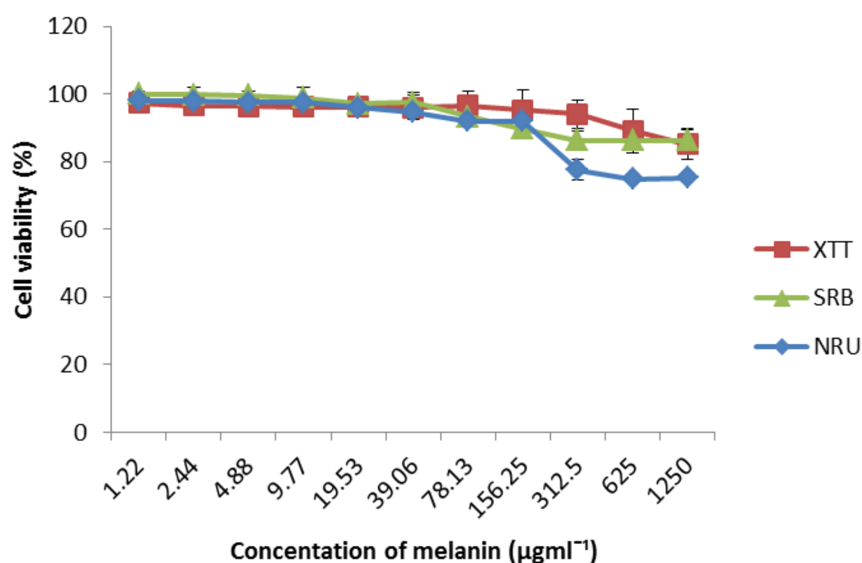


Fig. 5.14 Biocompatibility assay of melanin (3-bis [2- methyloxy 4 nitro-5-sulfophenyl]-2H-tetrazolium-5 carboxanilide (XTT), Neutral red uptake (NRU), and Sulforhodamine B (SRB) The Y axis shows the percentage of viable cells (n=3)

5.3.7.3 SRB assay

There was not much inhibition of protein synthesis up to a concentration of 156.25 µg ml^{-1}. Approximately, 10% inhibition of protein synthesis was observed at 156.25 µg ml^{-1} and reached a maximum of <15% inhibition of protein synthesis at 1250 µg ml^{-1} concentration (Fig. 5.14, Fig. 5.15)

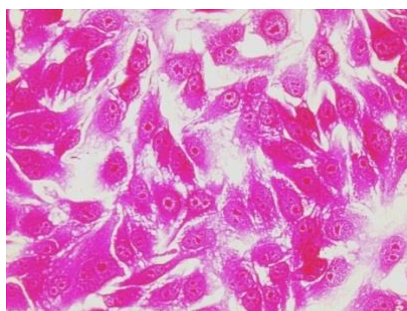


Fig. 5.15 Sulforhodamine B bound with cellular protein of HEp-2 cells

5.3.7.4 Superoxide anion (NBT reduction) assay of melanin treated HEp-2 cells

Exposure of HEp-2 cells to melanin resulted in the generation of free radicals in a dose dependent manner (Fig. 5.16). Basal production of free radicals by untreated cells was determined and this increased in the presence of M8 melanin to a maximum of ~600 % at the highest concentration of melanin (1250 µg ml^{-1}). The elevated production of free radicals was ~200% observed at a concentration of 156.25 µg ml^{-1}, which decreased to 32% at a concentration of 78.13 µg ml^{-1}

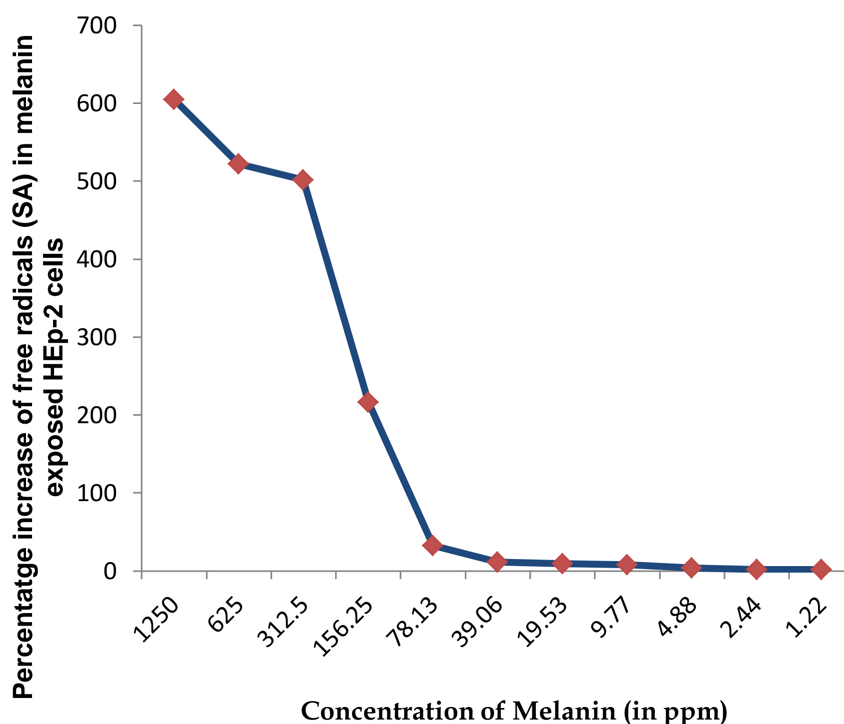


Fig. 5.16 Melanin induced free radical generation in human HEp-2 cancer cells. The Y axis shows percentage of super oxide anions generated (n=3)

5.3.7.5 Estimation of Superoxide dismutase activity

Marked elevation of super oxide dismutase (SOD) was observed in human HEp-2 cell line in response to the increase of melanin concentration (Fig. 5.17). Approximately 3% increase of SOD was observed at 156.25 µg ml^{-1} of melanin and 38% when the melanin concentration was 1250 µg ml^{-1}.

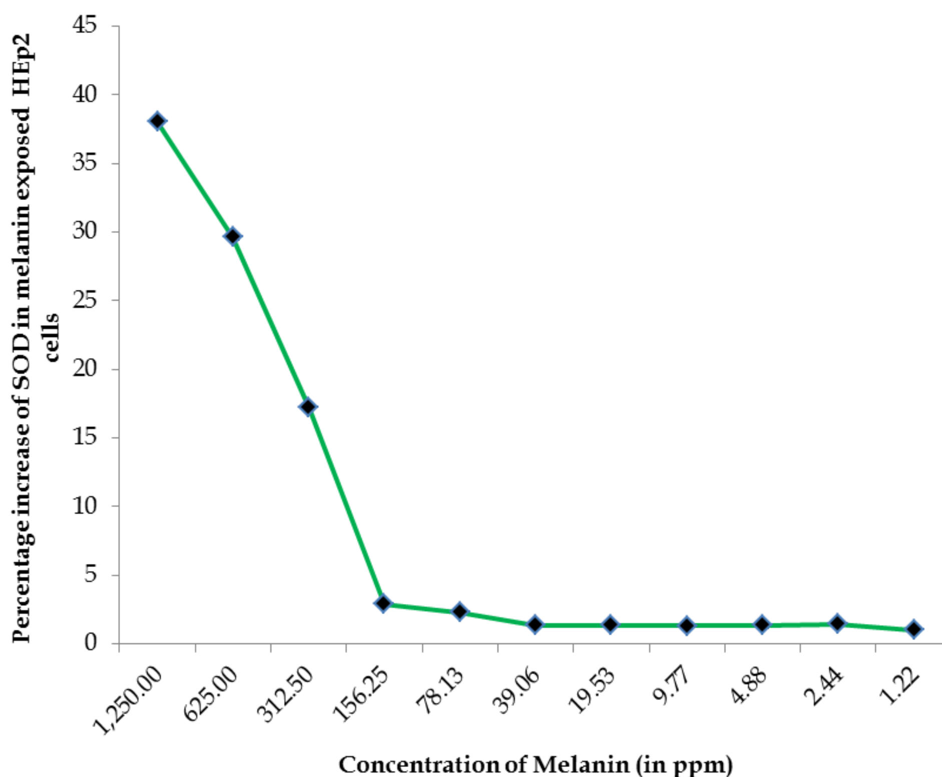


Fig. 5.17 Melanin induced Super oxide dismutase (SOD) production in human HEp-2 cancer cell line. The Y axis shows the percentage increase of SOD based on scavenging property on free radicals (n=3)

5.3.8 Phototoxicity assay of melanin (M8)

The exposure of HEp-2 cell lines to ultraviolet radiation, induced reactive oxygen species which was indicated by the increase in absorbance, approximately 1.5 times higher compared to control (Fig. 5.18). The reactive oxygen species (ROS) induced was 54% higher than the normal UV unexposed controls. On the other side the ROS production was significantly lower when cells were treated with melanin before giving UV exposure. Here, the melanin absorbed the incident UV, thereby protected the host cells from UV toxicity.

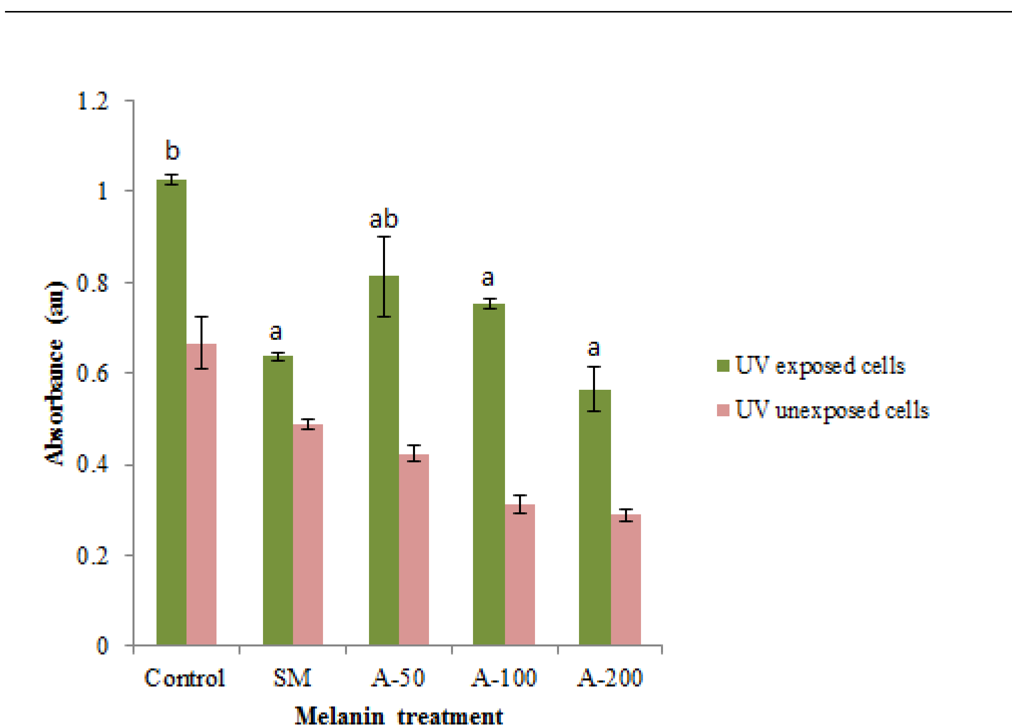


Fig. 5.18 Effect of melanin on scavenging ROS generated by UV. The absorbance (630 nm) indicated the mean concentration of ROS in HEp-2 cells in the presence of melanin. Control: HEp-2 Cells (without melanin) UV exposed and unexposed, SM: Cells in the presence of synthetic melanin (Positive control); A-50, A-100 and A-200: Cells in the presence of Actinomycete (M8) melanin- 50 µg ml^{-1}, 100 µg ml^{-1}; and 200 µg ml^{-1} respectively

There was not much significant variation in the ROS generated between synthetic melanin (50 µg ml^{-1}) treated HEp-2 cells and M8 melanin treated cells at concentrations 100 µg ml^{-1} and 200 µg ml^{-1} ($p<0.05$). The melanin (M8) treated cells scavenged the reactive oxygen species generated as a result of UV exposure. M8 melanin scavenged 20.74% ROS at 50 µg ml^{-1} and the synthetic melanin used as positive control, scavenged 37.93% of the ROS at the same concentration. At 100 µg ml^{-1} the scavenging of ROS by M8 melanin increased to 26.74%. The photoprotectant action of M8 melanin is evident from the reduction in absorbance, at a concentration of 200 µg ml^{-1} where M8

melanin scavenged 44.96% of the reactive oxygen species generated (Table 5.9).

Table 5.9 Phototoxicity assay of melanin (M8)

Melanin (µg ml^{-1})	Scavenging % of ROS (UV exposed)	Scavenging % of ROS (UV unexposed)
Synthetic - 50	37.93	26.6
M8 - 50	20.74	36.1
M8 - 100	26.74	48.4
M8 - 200	44.96	58.9

5.4 Discussion

The production of a diffusible dark brown pigment on complex organic media is so significant that it has long been regarded as a key characteristic for the identification and classification of *Streptomyce*s. The pigment has been referred to merely as a dark brown soluble pigment, as melanoid pigment, or as melanin until (Mencher and Heim, 1962), identified the pigment derived from *S. lavendulae* as dihydroxyphenyl alanine melanin.

The method of testing melanin formation by L-DOPA as substrate is used to confirm whether the diffusible pigments produced are melanoid (dark brown) or merely a brown substance, especially when complex organic media are employed (Dastager et al., 2006). The most effective carbon source in melanin production of strain M8 was starch followed by dextrin, glycerol, sucrose, and glucose. Similar results was observed by other workers (Dastager et al., 2006; Kshitija, 2012; Quadri and Agsar, 2012; Shaaban et al., 2013; Vasanthabharathi et al., 2011) who reported that starch was the most effective carbon source for the production of melanin, followed by glycerol and

fructose. Contrary to these results, it was found that *Kluyveromyces marxianus* and *Streptomyces chibaensis* produced the pigment with all carbon sources used and the main effective carbon source was D-xylose (Hewedy and Ashour, 2009). Starch and glycerol are the carbon sources most utilized by majority of *Streptomyces*, and these sources were used in the standard medium for the detection of tyrosinase activity (Arai and Mikami, 1972).

In the present study, comparative efficiency of various amino acids for the production of melanin revealed sodium glutamate as the most effective nitrogen source, followed by asparagine, proline, ornithine, arginine, citrulline and histidine. Sodium glutamate, L-asparagine, and basic amino acids, such as citrulline, L-glutamine, L-arginine, and L-ornithine, were found most effective nitrogen sources for the growth and the production of melanin pigment by several *Streptomyces* species including *S.phaeochromogenes* (Arai and Mikami, 1972). Later, citrulline, arginine, lysine and proline were reported as the most effective nitrogen source (Dastager et al., 2006). Simple nitrogen source tyrosine gave the maximum production of melanin followed by phenylalanine in the case of thermoalkaliphilic *Streptomyces* (Quadri and Agsar, 2012). Potassium nitrate was reported as the best nitrogen source for the experimental actinomycete isolate to produce melanin (Shaaban et al., 2013). Even though different *Streptomyces* species universally utilize starch as the sole carbon source in melanin production, the nitrogen source utilized varies among different species of *Streptomyce*s.

Actinomycete strain G-HD-4 isolated from soil sample collected from flower garden of Fujian Province, which produced the most melanin was identified as *Streptomyces capoamus* by taxonomic and phylogenetic analysis (Ai-Min et al., 2009). Recently, melanin producing *Streptomyces torulosus* (spiral spore chain and grey spore mass) was isolated from soil samples in

Egypt and the extracted melanin inhibited aflatoxin B production by *Aspergillus flavus* (Shaaban et al., 2013). Thermoalkaliphilic *Streptomyces* strains, able to produce melanin was isolated from limestone quarries of the Deccan traps (Quadri and Agsar, 2012). It was reported that *Streptomyces virginiae* produced a diffusible dark brown pigment on peptone yeast extract iron broth after 6 days of incubation on a rotary shaker (180 rpm) at 30°C (Amal et al., 2011). The melanogenic culture M8 in the present study was morphologically, biochemically and phylogenetically characterized as *Streptomyces bikiniensis*.

Melanins were extracted from black tea (BT-melanin), black soybean (BS-melanin) and black bone silky fowl (SF-melanin) and compared with synthetic melanin (SY-melanin) (Hsieh and Lien, 2012). In the present study, the extracted ratio of M8 melanin was 6.5% to that of dry biomass weight. The extracted ratio of BT-melanin was about 2% and that of BS melanin and SF melanin was 0.16% and 0.095% respectively. In the present study, the maximum yield of M8 melanin was 166 mg l^{-1} which is comparable with that of yeast melanin synthesized by *Yarrowia lipolytica* (160 mg l^{-1}) and tyrosine-mediated melanin production (130 mg l^{-1}) by *Klebsiella* sp. GSK (Apte et al., 2013; Sajjan et al., 2010). The BIOLOG SFP2 microplate test gave a characteristic reaction pattern called a “metabolic fingerprint” of the potential melanogenic culture. In the present study the extracted pigment was positive to all physical and chemical diagnostic tests for the standard melanin (Butler and Day, 1998; Sajjan et al., 2010). Wavelength scan of extracted melanin showed peaks in the UV region of the wave length ranging from 230-600 nm, but none in the visible region as already reported by Bell and Wheeler (1986) and Sajjan et al. (2010). The absorption was highest in the UV region of 230nm, but decreased towards the visible region, due to the presence of the

very complex conjugated structure, which is the characteristic property of melanin. Recently, purified melanin from saprophytic fungi *Lachnum* YM 404, also showed the absorption peak at 210nm, which is almost in agreement with the present finding (Ming et al., 2014).

Resonances between 8.0 and 6.0 ppm in NMR spectra are assigned to aromatic functionality, between 3.2 and about 4.3 ppm are assigned to protons attached to N and/or O, and at 0.7 ppm to methyl groups, based upon similar assignments in Sepia and human hair melanin (Aime et al., 1991; Katritzky et al., 2002). Resonances between 1.0 and 3.2 ppm are assigned to residual protein. NMR spectra for M8 melanin resemble spectra for human hair melanin except for the increased level of N and/or O attachment, less residual protein, and a lower level of methyl functionality relative to residual protein content. The four broad aromatic resonances in M8 at 7.60, 7.35, 7.00, and 6.60 ppm are assigned to indole and/or pyrrole repeat units of the melanin polymer (Katritzky et al., 2002).

One of the main tests for identifying melanin is the IR spectrum. The signals in the 3600-2800 cm^{-1} area are attributed to the stretching vibrations (O-H and N-H) of the carboxylic acid, phenolic and aromatic amino functions present in the indolic and pyrrolic systems (Magarelli et al., 2010). The N-H bending vibration peak at 1537.8, indicates that the pigment had typical indole structure of melanin. It was proposed that peaks at 1243 to 1305 cm^{-1} relates to the anhydride group (C-O) in synthetic melanin and all extracted microbial pigments (Hewedy and Ashour, 2009). The out of plane bending of the aromatic carbon hydrogen bond can be found in the 700-600 cm^{-1} area of Sepia melanin; also spectra of Sepia presented a bending vibration in the 1400-1300 cm^{-1} area, relevant for purified sample, which is not seen in M8 sample. The most noticeable differences between M8 melanin, Sepia and

synthetic melanin are the peaks at 1446.9, 1019.71 (COC asymmetrical stretching) and 1233.9 cm^{-1} which also implies the presence of a substantial amount of aliphatic groups in the M8 melanin structure. Free carboxylic groups (1715.4 cm^{-1}) found in synthetic melanin is not detected in M8 and Sepia melanin. The spectroscopic properties of the pigment extracted from *Streptomyces bikiniensis* M8 correlated with those of melanin produced by various microorganisms as reported previously as well as with the properties of Sepia melanin (Eisenman et al., 2007; Sajjan et al., 2010).

Scanning electron micrographs were used to examine the structure of natural and synthetic eumelanins and researchers have reported that the synthetic samples appear to be amorphous solids while the natural samples appear to be small spheres (Costa et al., 2012; Simon et al., 2000; Tarangini and Mishra, 2013). In the present study, melanin (M8) produced by *S.bikiniensis* appeared to be amorphous, which is in concordance with earlier reports. The natural Sepia melanin sample has a significant structural order with subunits that have a lateral dimension of ∼15 nm (Simon et al., 2000). Structural order is also lacking in the case of melanin produced by *S. bikiniensi*s.

The compositional variation of melanin pigments in energy dispersive spectrometer (EDS) analysis might be due to the change in the composition of production media used for various melanins (Tarangini and Mishra, 2013).

The ability of both eumelanin and pheomelanin to bind various metal ions is one of the most typical characteristics of the pigment (Meredith and Sarna, 2006). It is believed that this pigment can serve as a reservoir of metal ions, such as Ca (II) and Zn (II), and as a trap of heavy metal ions, such as Cu (II) and Fe (III) (Hong and Simon, 2006). The higher C: N ratio indicated that

the M8 melanin structure contains aliphatic groups almost similar to Sepia melanin. The C/H ratio of 1.21 indicates an aromatic nature (Koroleva et al., 2007). Higher carbon content and a higher C/H value indicate a higher level of aromaticity (Sava et al., 2001). The C: H ratio of M8 melanin was closer to Sepia melanin than synthetic melanin, hence the structural similarity to eumelanin.

Melanin is an amorphous dark colored pigment, insoluble in most solvents, bleached by oxidizing agents, and soluble in alkali and phenols (Krysciak, 1985). Phenolic compounds are large and an interesting group of natural antioxidants which can undergo reversible oxidation and reduction reactions and display the properties of donors or acceptors of electrons.

Lipid peroxidation inhibition ability of melanin is reported by various scientists. Earlier it was reported that melanin extracted from the eye could effectively inhibit lipid peroxidation to prevent uveitis (Bilgihan et al., 1995). MDA production and lipid peroxidation of liver cells when exposed to free radicals was inhibited by melanin extracted from black tea (Sava et al., 2001). Different groups of melanin extracted from black tea, black soya bean and black silky fowl showed apparent inhibition of lipid peroxidation (TBARS) of fibroblast cultures treated with melanin (Hsieh and Lien, 2012). It has been postulated that most microbial pigments evolved initially as a mechanism to combat environmental ROS for their persistence, but over time these compounds were adapted to serve divergent functions (Liu and Nizet, 2009).

Due to the complexity of melanin, multiple assays are employed to evaluate the antioxidant activity. The antioxidant activity of phenolic compounds is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donor and quenchers of singlet oxygen (Rice-

Evans et al., 1997). The lipid peroxidation inhibition results of the present study are in agreement with the results reported for melanin extracted from Taihe Black-bone silky fowl (TBSF) and synthetic DOPA melanin, where lipid peroxidation was dose dependent (Tu et al., 2009). In the present study, IC_{50} of crude M8 melanin extracted from *Streptomyces* was calculated as 73.7 ± 12.6 µg ml^{-1}, higher than that of Taihe Black-bone silky fowl (TBSF) melanin. Earlier it was reported that different doses of melanin extracted from silky fowl and DOPA melanin could both decrease lipid peroxidation of the egg (Tu et al., 2009). The potency of melanin to scavenge ROS to inhibit malondialdehyde formation and lipid peroxidation has been reported (Jee et al., 2009; Sava et al., 2001).The present study proves the dose dependent inhibition and shows that M8 melanin exhibits antioxidant potential in terms of inhibition of lipid peroxidation.

Effects of M8 melanin in scavenging ABTS radicals also proved the dose dependent scavenging ability. In the present study EC_{50} of extracted melanin (M8) on ABTS radicals (25.09 ± 5.08 µg ml^{-1}) was only 12 times lower than the standard trolox (2.2 ± 0.2 µg ml^{-1}). The ethyl acetate extract of *Streptomyces* sp. isolated from rhizosphere of curcumae longae exhibited an IC_{50} of 172.43 ± 22.19 µg ml^{-1} for ABTS, which was 220 times lower than the positive control (trolox 0.76 µg ml^{-1}). Hence it is clear from the above results that acid precipitation of melanin is highly effective than solvent extracts and further purification and evaluation of the compound from marine *Streptomyces* sp. can lead to a novel antioxidant with potential applications.

The method involving the use of DPPH as a stable free radical is widely used to measure the radical scavenging activity of polyphenolics and natural dyes (Blois, 1958). Melanin readily interacts with reactive oxygen species and free radicals due to the presence of unpaired electrons. The scavenging ability

of crude M8 melanin on DPPH and ABTS radicals illustrates that scavenging effects of M8 on stable free radicals was also dependent on the concentration as reported (Jasmine and Agastian, 2013). EC_{50} of M8 melanin for DPPH scavenging was determined as 35.49 ± 6.19 µg ml^{-1}. Similarly, ethyl acetate extracts of endophytic actinomycetes exhibited an inhibition percentage of 54.29 % at a conc of 1000 mg/ml on DPPH radicals (Jasmine and Agastian, 2013). At a concentration of 100 µg ml^{-1} the inhibition percentage exhibited by M8 melanin was 80.65 ± 0.21%. Recently, purified melanin extracted from marine actinobacteria, *Actinoalloteichus* sp. MA-32, exhibited DPPH radical-scavenging activity with an EC_{50} value of 85 µg ml^{-1} (Manivasagan et al., 2013). The EC_{50} exhibited by purified melanin (ABM) pigment from *A. bridgeri* exhibited a significant DPPH radical scavenging activity of 54.12 µg ml^{-1} (Kumar et al., 2011). Antioxidant activity for different concentrations of crude ethyl acetate extract of marine *Streptomyces* sp. isolated from west coast of India by DPPH assay exhibited an IC_{50} of 21.50µg/ml (Rajan et al., 2012). The results suggest that melanin synthesized by *S.bikiniensis* M8 exhibited potential radical scavenging ability more exaggerated to fungal and bacterial melanins.

Trolox equivalent antioxidant capacity (TEAC) values were calculated from the ratio of test compound reaction (measured as inhibition) to that of trolox reaction. In concept, *TEAC value (unit less) = Inhibition by test compounds / Inhibition by trolox* (Apak et al., 2013).

The EC_{50} of ethyl acetate extract of *Streptomyces* sp. isolated from rhizosphere of curcuma longae for DPPH radical scavenging was 842.18 ± 161.24 µg/ml which was 200 times lower than the trolox standard (Zhong et al., 2011). In the present study, the EC_{50} of extracted M8 melanin on DPPH radicals was only 13 times lower than the trolox standard which is much

higher than the EC_{50} of ethyl acetate extract of *Streptomyces* sp. isolated from rhizosphere of curcuma longae.

Biocompatibility and non toxicity of melanin are the prerequisites which determine the relevance of melanin in *in vitro* or *in vivo* applications. The biocompatibility of melanin analysed using XTT, NBT and NRU assays proved that melanin was nontoxic to HEp-2 cells in culture up to a concentration of 156.25 μg ml^{-1}. Slight loss in viability (< 15%) due to inhibition of mitochondrial activity and protein synthesis and membrane damage (< 25%) was observed at a concentration above 156.25 μg ml^{-1}upto a concentration of 1250 μg ml^{-1} which is far higher than the normal use. To ascertain the loss of viability, melanin induced free radical generation was checked in HEp-2 cells and it was observed that basal production of free radicals increase in the presence of melanin up to 600 % at the highest concentration of 1250 μg ml^{-1}. However a marked shift in elevated production of super oxide anions (~200) was observed at 156.25 μg ml^{-1}, while the value was only 32% when the concentration was 78.13 μg ml^{-1}.

Consequently, 3% increase of SOD was observed at 156.25 μg ml^{-1}, which further increased to 38% when the melanin concentration reached the maximum of 1250 μg ml^{-1}. Strikingly, it was found to be a triggering mechanism in melanin induced free radicals in HEp-2 cells that subsequently resulted in elevated production of SOD to scavenge the generated free radicals.

The EC_{50} of M8 melanin for various antioxidant assays viz., lipid peroxidation inhibition (73.7 ± 12.6 μg ml^{-1}), ABTS (25.01 ± 5.08 μg $ml^{-1)}$ and DPPH (35.49 ± 6.19 μg ml^{-1}) are well below the concentration of melanin

156.25 µg ml^{-1} where the toxicity is only < 10% (SRB assay). Hence it is proved that the melanin produced by *S. bikiniensis* (M8) is biocompatible as demonstrated by the various antioxidant and cytotoxicity assays.

The role of melanin as a photoprotectant is confirmed by the scavenging of reactive oxygen species generated as a result of UV exposure on HEp-2 cells. The reactive oxygen species present in melanin treated HEp-2 cells were significantly low compared to melanin untreated control cells ($p > 0.05$). There was not much significant variation in reactive oxygen species generated by synthetic melanin and M8 melanin at 100 and 200 µg ml^{-1}. Melanins confer resistance to UV light by absorbing a broad range of the electromagnetic spectrum and preventing photo induced damage (Hill, 1992). Consequently, melanins are used commercially in photoprotective creams and eye glasses. Melanin protects several fungal and bacterial species from UV, solar, or gamma radiation (Nosanchuk and Casadevall, 2003). *Streptomyces bikiniensis* (M8) melanin as photoprotectant has the ability to reduce photo oxidative damage that results from UV induced ROS production. Hence M8 melanin can act as UV filters (“sun screens”) designed to protect the skin from the harmful effects of solar radiation.

It is evident that M8 melanin in its crude form exhibited antioxidant potential comparable to trolox in the case of ABTS and DPPH radical scavenging assays. M8 melanin exhibited a significant antioxidant potential. Melanin production ability of *Streptomyces* which was till date considered a taxonomic criterion can be exploited in view of its antioxidant and photoprotectant role. In conclusion, melanin pigment from *Streptomyces bikiniensis* (M8) is very similar to eumelanin, which constitutes a diverse

group of aromatic polymers and a promising compound with potential applications in cosmetic and pharmaceutical industries.

6

Chitinolytic Streptomycetes as Biocontrol Agents

6.1 Introduction

Chitin is a type of marine polysaccharide, an abundant biomass resource in nature. It is noteworthy that enormous production of chitin occurs in the marine biosphere. Most of this production is as chitin in shells embedded in marine zooplankton and marine crustaceans such as shrimps, crabs and lobsters. Surprisingly, the chitin content in marine sediment is quite low due to bioconversion processes carried out by marine chitinolytic bacteria (Han et al., 2009) which transform this polysaccharide into simpler organic compounds that are subsequently used by other microorganisms as a source of carbon and nitrogen. Since most fungal cell walls and exoskeleton of insect species consists largely of chitin, it was postulated that chitinase could be involved in the control of insects and pathogenic fungi. Therefore, chitinolytic enzymes were considered as the criteria for the selection of potential biocontrol agents.

Production of inexpensive chitinase by the use of chitinous wastes (shrimp shells, chitin from seafood industry etc.) will be useful in bioremediation (Mukherjee and Sen, 2004). Different chitinous substrates that have been reported in the literature for chitinase production include fungal cell

walls, crab and shrimp shells, prawn wastes and flake chitin (Singh et al., 2005).

Chitinolytic enzymes have wide ranging applications such as preparation of pharmaceutically important chitooligosaccharides and N-acetyl-D-glucosamine, single cell protein, isolation of protoplasts from fungi and yeasts, control of pathogenic fungi, treatment of chitinous waste, and control of malaria transmission (Dahiya et al., 2006). Compared with chitinases derived from terrestrial organisms, marine chitinases with higher pH and salinity tolerance may contribute to special biotechnological applications.

6.1.1 Antifungal agents

Bacterial chitinases have been widely demonstrated as inhibiting fungal growth and can therefore be effective in controlling plant-pathogenic fungal diseases (Ordentlich et al., 1988). Chitinase producing organisms could be used directly in biological control of microorganisms or indirectly using their purified protein or through gene manipulation (Gupta et al., 1995; Singh et al., 1999). Actinobacteria have been considered as potential biocontrol agents of fungal pathogens. Several investigators have described the *in vitro* and *in vivo* antifungal activities of the actinobacteria. Their modes of action includes *via* enzymes such as cellulases, hemicellulases, chitinases, amylases and glucanases (Yuan and Crawford, 1995), competition with pathogens (Nishimura et al., 2002), antibiotic production (Igarashi, 2004), parasitism of hyphae (El-Tarabily and Sivasithamparam, 2006), and siderophore production (Khamna et al., 2009).

Many chitinases have been isolated and several genes that encode these enzymes have also been cloned from a variety of bacteria (Barboza-Corona et al., 2003; Duo-Chuan, 2006). A high correlation between chitinolysis and

antifungal properties has been reported (Hoster et al., 2005; Jung et al., 2003; Pleban et al., 1997). Chitinases (EC 3.2.1.14) play a vital role in the biological control of many plant diseases by degrading the chitin polymer in the cell walls of fungal pathogens (Ghasemi et al., 2010).

The cell wall degradation of *Penicillium chrysogenum* by chitinase system of *Streptomyces* sp. ATCC 1123 has been reported (Beyer and Diekmann, 1985). An extracellular chitinase from culture filtrates of *Streptomyces lydicus* WYEC108, a broad spectrum antifungal biocontrol agent, was characterized and purified (Mahadevan and Crawford, 1997). Two chitinolytic bacteria (*Paenibacillus* sp. and *Streptomyces* sp.) were reported to suppress *Fusarium* wilt of cucumber (Singh et al., 1999).

Tsujibo et al. (2000) reported that *Streptomyces thermoviolaceus* OPC-520 was the major source of thermophilic chitinases. Chitinase was isolated from the culture filtrate of *Streptomyces* sp. M-20 and the purified chitinase showed antifungal activity against Botrytis cinerea (Kim et al., 2003). Characterization of chitinase genes from an alkaliphilic actinomycete, *Nocardiopsis prasina* and chitinase exhibiting anifungal activity against *Trichoderma* sp. has been reported (Tsujibo et al., 2003). Chitinase that showed antagonism against phytopathogenic fungi was reported from endophytic *Streptomyces aureofaciens* CMUAc130 and *Streptomyces hygroscopicus* isolated from Thailand soils (Prapagdee et al., 2008; Taechowisan et al., 2003). The obvious antifungal activity of chitinase from the novel *Streptomyces* strain was proved against *Aspergillus nidulans* and phytopathogens such as *Botrytis cinerea*, *Fusarium culmorum*, and *Sclerotia sclerotiorum* (Hoster et al., 2005). Chitinase of *Streptomyces* sp. DA11 isolated from South China sponge *Craniella australiensis*, was investigated for antifungal activity and proved to be a novel enzyme (Han et al., 2009).

Chitinase showing selective inhibition of the insect N-acetyl-D-glucosamine (GlcNAcase) was studied from *Streptomyces griseoloalbus* JCM4480, *Streptomyces clavifer* JCM5059, *Streptomyces anulatus* NBRC13369 and *S. griseus* (Usuki et al., 2006). During the last decade, chitinases have received increased attention because of their wide range of applications (Chye et al., 2005; Tiffin, 2004).

Streptomyces viridificans was found to be a good chitinase producer, and its crude and purified enzyme has potential cell wall lysis of many fungal pathogens (Gupta et al., 1995). Similar results were observed with crude and purified enzymes from *Streptomyces aureofaciens*, resulting in cell wall lysis in many phytopathogenic fungi (Taechowisan et al., 2003). Several *Streptomyces* and non *Streptomyces* bacteria are well known as producers of chitinase and exercising antagonistic potential against *Sclerotinia minor*, the pathogen of the basal drop of lettuce. Two of these isolates; *S. viridodiasticus* and *Micromonospora carbonacea* are known to produce a high level of chitinase and thus significantly reduced the growth of *S.* minor *in vitro* and reduced the incidence of the disease under controlled green house conditions (El-Tarabily et al., 2000). Purified chitinases synthesized by *Stenotrophomonas maltophilia* inhibited the growth of fungal phytopathogens belonging to the genera *Fusarium*, *Rhizoctonia* and *Alternaria* (Jankiewicz et al., 2012).

Streptomyces sp. TH-11 isolated from the sediment of the Tou-Chien River, Taiwan and identified as *Streptomyces viridochromogenes* produced several chitinolytic enzymes (Hoang et al., 2011). Soil *Streptomyces* isolated from local environment in Saudi Arabia with potential chitinolytic activity and antagonistic to plant pathogenic fungi were identified based on 16S rDNA sequence analysis as *Streptomyces tendae*, *S. griseus*, *S.variabilis*, *S. endus*

and *S. violaceusniger* (Gherbawy et al., 2012). A novel antifungus phenazine derivative was isolated from saline culture broth of marine actinomycete (BM-17) isolated from a sediment sample collected in the Arctic Ocean. The strain was identified as *Nocardia dassonvillei* based on morphological, cultural, physiological, biochemical characteristics, along with the cell wall analysis and 16S rDNA gene sequence analysis (Gao et al., 2012).

6.1.2 Chitinolytic actinomycetes as biocontrol agents

Biological control or 'biocontrol' is the use of natural enemies to manage pest populations. There are several types of biological control including the direct introduction of parasites, pathogen and predators to target insect pests or using the spores of the soil bacteria and actinomycetes which will interfere in the digestive systems of mosquitoe larvae (El-Khawagh et al., 2011).

Many reports indicated that actinomycetes play an important role in the biological control against insects including the cotton leaf worm, *Spodopetra littoralis* (Bream et al., 2001), house fly, *Musca domestica* (Hussain et al., 2002), *Culex quinquefasciatus* (Sundarapandian et al., 2002), *Drosophila melanogaster* (Gadelhak et al., 2005), *Helicoverpa armigera* (Osman et al., 2007), *Anopheles mosquito* larvae (Dhanasekaran et al., 2010) and *Culex pippiens* (El-Khawagh et al., 2011). Investigations on the biological activity of secondary metabolites of some actinomycete isolates on last instar larvae of the cotton leaf worm *Spodoptera littoralis* indicated that *Streptomyces* and *Streptoverticillum* were the most potent actinomycetes, which cause larval and pupal mortality (Bream et al., 2001). It was reported that the pellets of some *Strepyomyces* isolates were more active against cotton leaf worm than culture filtrate (Osman et al., 2007).

Marine microorganisms can be considered an important source of insecticidal antibiotics and they have potential for the application as insecticides. A new member of the tartrolone series of macrodiolides, tartrolone C, was isolated from a *Streptomyces* species on the basis of its insecticidal activity (Lewer et al., 2003). An actinomycete, *Streptomyces* sp. 173, isolated from seawater and sediments, was found to have strong insecticidal activity against both brine shrimp and *H. armigera*, similar to that of avermectin B1 (Xiong et al., 2004).

In a study involving 41 soil actinomycete isolates, *Streptomyces* and *Streptoverticillium* were recorded as the most effective genera against third larval instar of the house fly, *Musca domestica* (Ghazal et al., 2001). The potential of using chitinase producing non streptomycete actinomycetes belonging to the genus *Actinoplanes* for the biological control of insects having chitin as a major component of their cuticle in Arabian Gulf area has been investigated (Gadelhak et al., 2005). The application of either *Actinoplanes philippinensis* or *Actinoplanes missouriensis* gave a good effect, shown as lowest pupal formation percentages, being 39.43 and 31.75%, respectively (Gadelhak et al., 2005). Insecticidal action of Quinomycin A from *Streptomyces* sp. KN-0647, isolated from a forest soil displayed inhibition on pathogenetic insects, such as *Spodoptera exigua*, *Dendrolimus punctatus*, *Plutella xylostella*, *Aphis glycines* and *Culex pipiens* (Liu et al., 2008).

Drosophila melanogaster (“Fruit fly”) was chosen as insect model as it is one of the most studied model organisms in biological research, particularly in genetics, physiology, cytology and developmental biology. The systematic position of *Drosophila* is included in phylum Arthropoda, class insecta and order diptera to which the common insect vector mosquito belongs. They are

considered the most popular insect model, because of their small size, easily culturable, has a short generation time, and its complete genome has been sequenced. There is a lack of published information with regard to the use of actinomycetes particularly, marine Streptomyces, as biocontrol agents. Hence a preliminary investigation into the chitinolytic ability of marine *Streptomyces* was carried out to ascertain the possibility of exploiting marine *Streptomyces* sp. as biocontrol agents of fungal pathogens and insect pests.

6.2 Materials and methods

6.2.1 Chitinolytic marine actinomycetes as antifungal and insecticidal agents

Selection and culture of chitinolytic actinomycetes

Spore suspension of selected chitinolytic actinomycetes (Refer section 2.2.7.8 and 2.3.6) J18, R25, M32, L128 and L92 (zone of inhibition >15mm) were inoculated into chitinase production medium i.e., mineral basal medium supplemented with 1% crude chitin and incubated for 7 days (Fig. 6.1). Cell free supernatant was used for antifungal assay.

6.2.1.1 Inhibition of pathogenic fungi

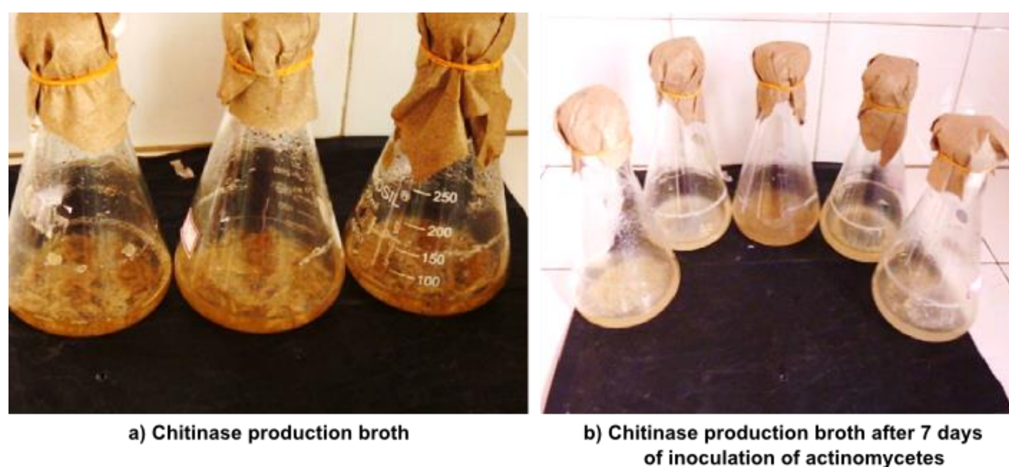
Fungal pathogens

Fungal pathogens tested included both yeast and filamentous fungi. Yeasts included *Candida* tropicalis (230), *Candida wickerhamii* (3013),

Saccharomyces cerevasiae (173), *Kluveromyces marxianus* (1388) and *Candida capsulata* (203). Filamentous fungi included, *Aspergillus versicolor* (SF2), *Penicillium* sp.(J49), *Cladosporium* sp.(R32) *Penicillium verrucosum* (SF45), *Penicillium citrinum* (2553), *Penicillium commune* (SF46), *Geotrichum candidum* (SF135), *Paeciliomyces liliacinum* (SF166), *Mucor* sp. (SF112), and *Scopulariopsis brevicaulis* (1469). The yeast cultures were obtained from IMTECH Chandigarh and the filamenous fungal cultures from the culture stock maintained at the Department of Microbiology, School of Marine Sciences, and National Centre for Aquatic Animal Health (NCAAH), CUSAT.

Antifungal assay

Inhibition against yeasts was done by disc diffusion assay as per Kirby and Bauer (1966) using malt extract agar. Antifungal activity was performed according to hyphal extension inhibition assay (Tsujibo et al., 2000). The mycelium of filamentous fungi were directly inoculated on to the centre of malt extract agar plates and the plates were incubated at 28°C for 3 days. Discs containing the crude enzyme (20 µl) of R25, J18, L128, and M32, L92 were placed around the edge of the fungal growth and incubated at 28°C for 3-5 days. Inhibition of hyphal extension around the discs was examined and recorded.



a) Chitinase production broth

b) Chitinase production broth after 7 days of inoculation of actinomycetes

Fig. 6.1 Screening of marine actinomycetes for chitinolytic ability in crude chitin medium

6.2.1.2 Chitinolytic marine actinomycetes as biocontrol agents of insect pests

Actinomycete isolates with chitinolytic activity were tested for their potential to inhibit the development of insect, *Drosophila melanogaster* under controlled laboratory conditions.

6.2.1.2.1 Collection and maintenance of Drosophila melanogaster

Wild type (Red eyed-strain CSBZ) *Drosophila melanogaster* was procured from fly facility, Centre for cellular and molecular platforms, National Centre for Biological Sciences (NCBS), Bangalore. The stock cultures were maintained in sterile bottles with fly media of the following composition. (Corn flour 8 g; D glucose 2 g; sugar 4 g; agar 8 g; yeast 1.5 g; propionic acid 0.4 ml; distilled water 100 ml). The fly cultures were maintained at an ambient temperature of 25 ± 2°C (Strickberger, 1962).

6.2.1.2.2 Rearing and counting of Drosophila eggs

Drosophila eggs were collected using cut bottles fitted with 60mm sterile petridish containing egg laying media (Agar 3g; acetic acid 1.5 ml; alcohol 2.5 ml; distilled water 100 ml) and yeast paste as feed in the centre of the petridish. Adult male and female flies were allowed into the bottle for oviposition. Flies were left for 3-4 hours at 28ºC and then transferred to a fresh culture bottle. A female *Drosophila* may lay about 50-75 eggs/day (Strickberger, 1962). The petridish with the medium and laid eggs were carefully removed. Eggs were counted using a Binocular Microscope and were gently removed with a soft brush.

6.2.1.2.3 Selection of chitinolytic actinomycetes

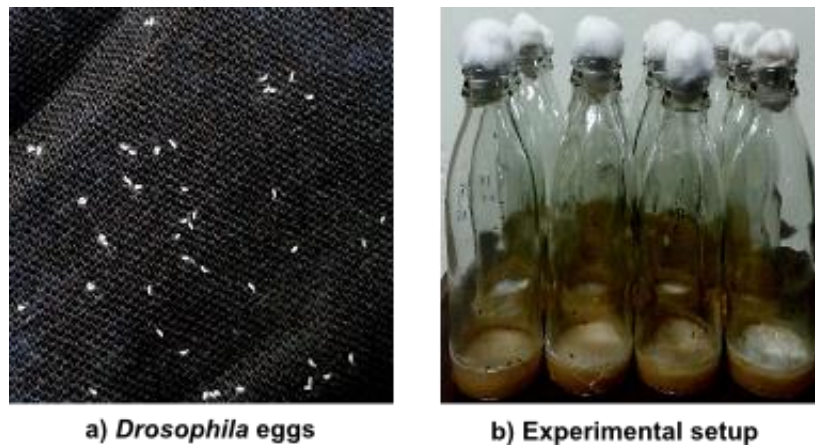
The three potential chitinolytic actinomycete isolates (J18, M32, L128) (clear zone >15 mm) in chitin agar plates, were tested for their ability to inhibit the growth and development of *Drosophila* under controlled laboratory conditions.

6.2.1.2.4 Actinomycete preparation

Actinomycetes were cultured in colloidal chitin agar and incubated at 28ºC until sporulation. Spores of the isolates (J18, M32 and L128) were harvested individually and suspended in 7 ml of sterile distilled water to obtain an OD of 1.0 ($3x10^7$spores/ml). The 1 OD spore suspension of each strain was diluted tenfold in distilled water to obtain spore suspensions of optical density 0.1 ($3x10^6$ spores/ml) and 0.01($3x10^5$ spores/ml). All the three different concentration of spores suspensions were used for the experiment. Five ml of the spore suspensions of each isolate were autoclaved to kill the spores to be used as control.

6.2.1.3 Application of chitinolytic actinomycete on D.melanogaster

The actinomycete (J18, M32 and L128) spore suspensions (1 ml) both active and heat killed (autoclaved) were added individually to 10 ml of cooled, molten *Drosophila* media, mixed thoroughly, poured into petridish and allowed to set. *Drosophila* eggs were carefully rinsed in 70% alcohol and then in distilled water to remove any contaminant microflora. *Drosophila* eggs were counted, and placed gently on the prepared spore inoculated and control media under sterile conditions. A total of 18 treatment groups for all the three isolates and a distilled water control were set up. All the treatments were conducted in triplicates and were incubated at 25ºC for approximately 9-12 days, until pupation. Successful pupations were counted at the end of the experiment (Fig. 6.2).



a) *Drosophila* eggs b) Experimental setup

Fig. 6.2 Screening of marine actinomycetes for insecticidal activity

6.2.1.3.1 Toxicological activity

The larvae were observed daily until pupation and adult emergence, to estimate the following parameters:

Larval mortality percent was estimated using the following equation:

$$Larval\ mortality\ \% = \left(A - \frac{B}{A}\right) \times 100$$

Where,
A *is the number of tested larvae and* ***B****is the number of tested pupa (Briggs, 1960).*

Pupation rate: The pupation percent was estimated by using the following equation:

$$Pupation\ \% = \frac{A}{B} \times 100$$

Where,
A *is the number of pupae and* ***B*** *is the number of tested larvae.*

6.2.2 Characterization of actinomycete isolates exhibiting fungicidal (J18) and insecticidal (M32) activity

Morphological (Refer section 2.2.3), /Biochemical/ Physiological (Refer section 2.2.4) sensitivity to antibiotics (refer section 4.2.2.2) and Molecular characterization (Refer section 3.2.2, 3.2.3) of the isolate J18 and M32 were done as per the methods given in the sections referred.

6.2.2.1 Phylogenetic analysis and GenBank accession

The sequences retrieved on BLAST analysis of the *Streptomyces* spp. J18 and M32 were multiple aligned using the programme Clustal W (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the actinomycete genera from GenBank. Phylogenetic tree was inferred by the Neighbor joining (NJ) method (Saitou and Nei, 1987), with MEGA 5.0 package (Tamura et al., 2011). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

6.2.2.2 Carbon Utilization profile

The carbon utilization/nutritional profile of chitinolytic strain J18 and M32 were done using BIOLOG SFP2™ microplate* as per the manufacturer's protocol (Refer section 4.2.4).

6.2.3 Statistical analysis

All the tests were performed in triplicates, and values were represented as means ± SD. Data were subjected to analysis of variance (ANOVA) and significant differences between means were determined. Significance level of the analyses was set to $p < 0.05$. The LC_{50} values were calculated by probit analysis. Statistical analyses was done using software SPSS (version 17.0 for Windows, SPSS Inc.).

6.3 Results

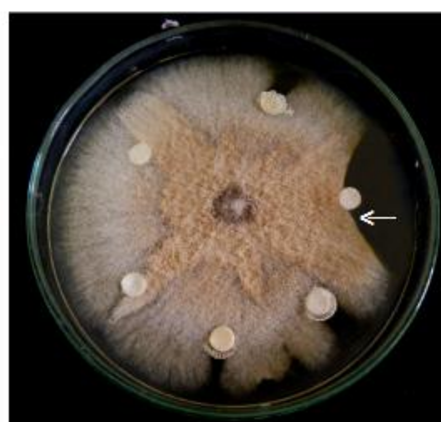
6.3.1 *Invitro* antagonism against pathogenic fungi

Isolate J18 exhibited antifungal activity against almost all the yeast and filamentous fungi as given in (Table 6.1). The antifungal activity was observed as cresentic area of hyphal extension inhibition around the disc (Fig. 6.3). All the isolates exhibited activity against *Penicillium verrucosum* except strain M32. Isolate R25 inhibited five different fungal pathogens viz., *Penicillium commune*, *Penicillium citrinum*, *Geotrichum candidum*, *Paeciliomyces liliacinum* and *Aspergillus versicolor*. Isolate L128 exhibited activity against *Penicillium verrucosum*, *Penicillium commune*, *Geotrichum candidum*, and *Scopulariopsis brevicoli*s. Chitinase extract from L92 and M32 exhibited activity against only a single fungal pathogen, *Penicillium verrucosum* and *Scopulariopsis brevicoli*s respectively.

Table 6.1 Antifungal activity of marine actinomycetes

Strain	J18	L128	R25	M32	L92
Candida tropicalis (230)	+	-	-	-	-
Candida intermedius (1904)	+	-	-	-	-
Kuraishia *capsulata* (2703)	+	-	-	-	-
Saccharomyces cerevesiae (170)	+	-	-	-	-
Kluveryomyces *marxianus* (1388)	+	-	-	-	-
Penicillium verrucosum (SF45)	+	+	+	-	+
Aspergillus versicolor (SF2)	+	-	-	-	-
Penicillium citrinum (2553)	+	-	+	_	_
Penicillium commune (SF46)	+	+	-	-	-
Geotrichum candidum (SF135)	+	+	+	-	-
Paeciliomyces liliacinum (SF166)	+	-	+	_	_
Mucor sp. (SF112)	+	-	-	-	-
Scopulariopsis brevicoli (1469)	+	+	-	+	

Pencillium Verrucosum (SF45)



Scopulariopsis brevicaulis (1469)

Fig. 6.3 Antifungal activities of chitinolytic marine actinomycete (J18) by hyphal extension inhibition assay (indicated by an arrow)

6.3.2 Chitinolytic marine actinomycetes (*Streptomyces* sp.) as biocontrol agents of insect pests

Spore suspensions of marine actinomycetes J18 incorporated into *Drosophila* rearing media exhibited significant reduction in pupation compared to controls in the larval development of *Drosophila melanogaster*.

The comparison of various spore concentrations of actinomycete J18, on larval development of *Drosophila* are given in Table 6.2. However a significant reduction in pupation was observed with different concentration of viable spores compared with the killed spores ($p < 0.05$). The pupation percentage in media treated with different spore concentrations (0.01, 0.1 and 1 OD) of J18 was 88.33%, 42.71% and 7.87% respectively (Fig. 6.4). The pupation percentage with various concentrations of autoclaved/killed spores of J18 in *Drosophila* media remained more or less same as that of the water control.

Table 6.2 Comparison of various spore concentrations of strain J18 on larval development of *D. melanogaster*

Treatment groups (J18)	Pupation %	Mortality %	Larval duration
Water control	91.58 ± 1.54^{c}	8.42 ± 1.54^{a}	6.66 ± 0.47^{a}
Killed spore (0.01OD)	90.89 ± 2.54^{c}	9.11 ± 2.54^{a}	6.3 ± 0.47^{a}
Viable spore(0.01 OD)	88.33 ± 6.03^{c}	11.67 ± 6.03^{a}	7.6 ± 0.47^{ab}
Killed spore (0.1 OD)	90.20 ± 3.39^{c}	9.80 ± 3.39^{a}	6.3 ± 0.47^{a}
Viable spore (0.1 OD)	42.17 ± 5.89^{b}	57.83 ± 5.89^{b}	8.6 ± 0.94^{b}
Killed spore (1 OD)	88.94 ± 1.21^{c}	11.06 ± 1.21^{a}	7 ± 0.81^{a}
Viable spore (1 OD)	7.87 ± 3.41^{a}	92.13 ± 3.41^{c}	8.6 ± 0.47^{b}
IC_{50}=0.07±0.02 (1.9 x 10^{6}) spores /ml			

Values with the same superscript do not vary significantly ($p > 0.05$)

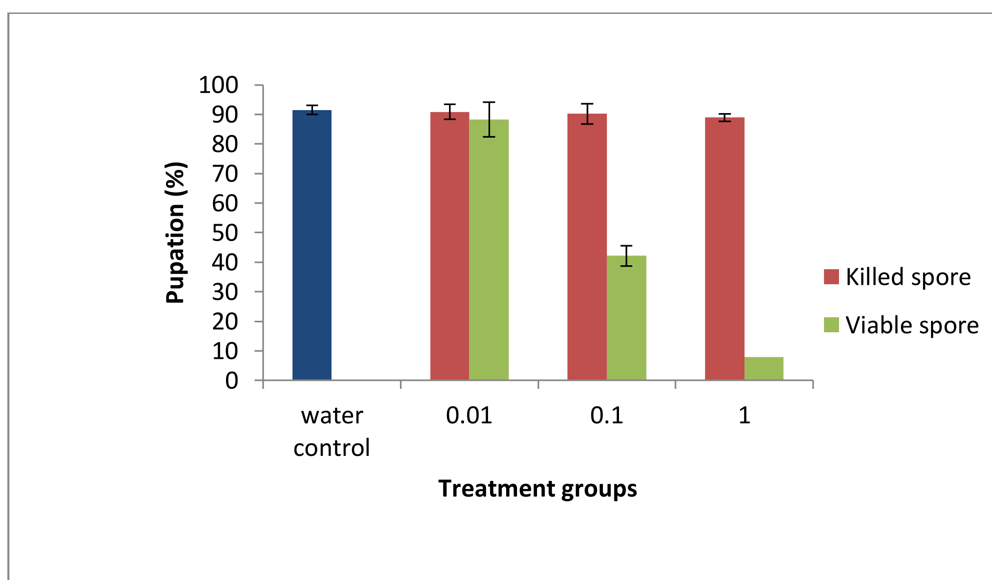


Fig. 6.4 Effect of different spore concentrations of J18 on pupation of *Drosophila melanogaster*

The toxicological activity of different spore concentrations of actinomycete strain M32, on larval development of *Drosophila* are given (Table 6.3). The pupation percentage in *Drosophila* media with various concentrations of autoclaved/killed spores of strain M32 did not vary significantly from the water control ($p > 0.05$). At various concentrations of killed spores (0.01, 0.1, 1 OD), the pupation percentage remained the same (Fig. 6.5). However a significant reduction in pupation was observed with different concentration of viable spores compared with the respective killed spore control ($p < 0.05$). The pupation percentage in media treated with different spore concentrations (0.01, 0.1 and 1 OD) of strain M32 was 77.67%, 35.29%, and 6.67% respectively. The mean difference for all treatment groups was significant at the 0.05 level.

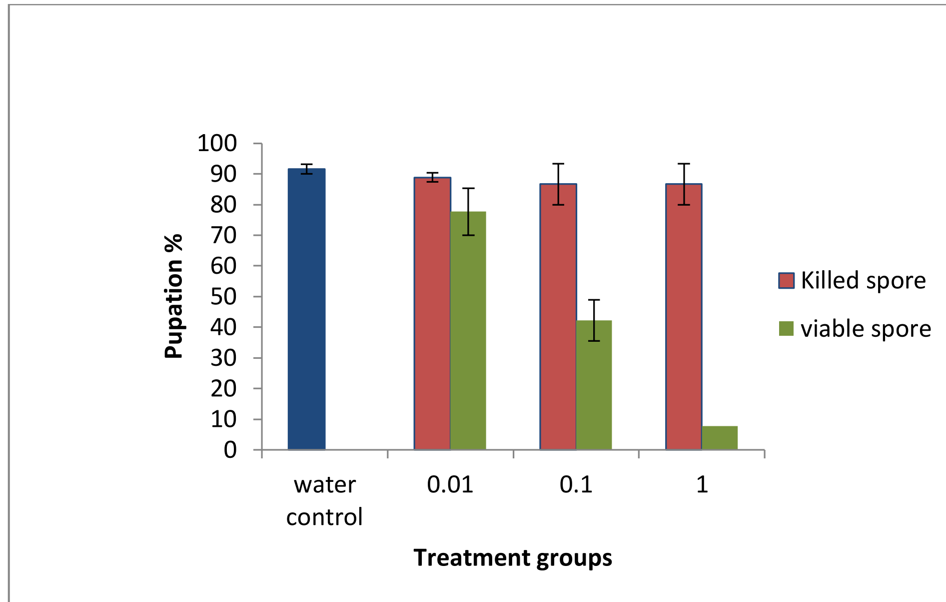


Fig. 6.5 Effect of different spore concentrations of M32 on pupation of *Drosophila melanogaster*

Table 6.3 Comparison of various spore concentrations of strain M32 on larval development of *Drosophila melanogaster*

Treatmentgroups (M32)	Pupation %	Mortality %	Larval duration (Days)
Water control	91.58 ± 1.54^{d}	8.42 ± 1.54^{a}	6.66 ± 0.47^{a}
Killed spore (0.01OD)	88.87 ± 1.43^{d}	11.13 ± 1.43^{a}	6.33 ± 0.47^{a}
Viable spore(0.01OD)	77.67 ± 4.04^{c}	22.33 ± 4.04^{b}	7.33 ± 0.47^{a}
Killed spore (0.1 OD)	86.63 ± 6.65^{cd}	13.37 ± 6.65^{ab}	6.66 ± 0.47^{a}
Viable spore (0.1 OD)	35.29 ± 5.89^{b}	64.71 ± 5.89^{c}	8.33 ± 0.47^{b}
Killed spore (1 OD)	86.63 ± 6.65^{cd}	13.37 ± 6.65^{ab}	7 ± 0^{a}
Viable spore (1 OD)	6.67 ± 6.67^{a}	93.33 ± 6.67^{d}	8.6 ± 0.47^{b}
IC_{50}= 0.045±0.02 OD (1.5 x 10^{6} spores/ml)			

Values with the same superscript do not vary significantly ($p > 0.05$)

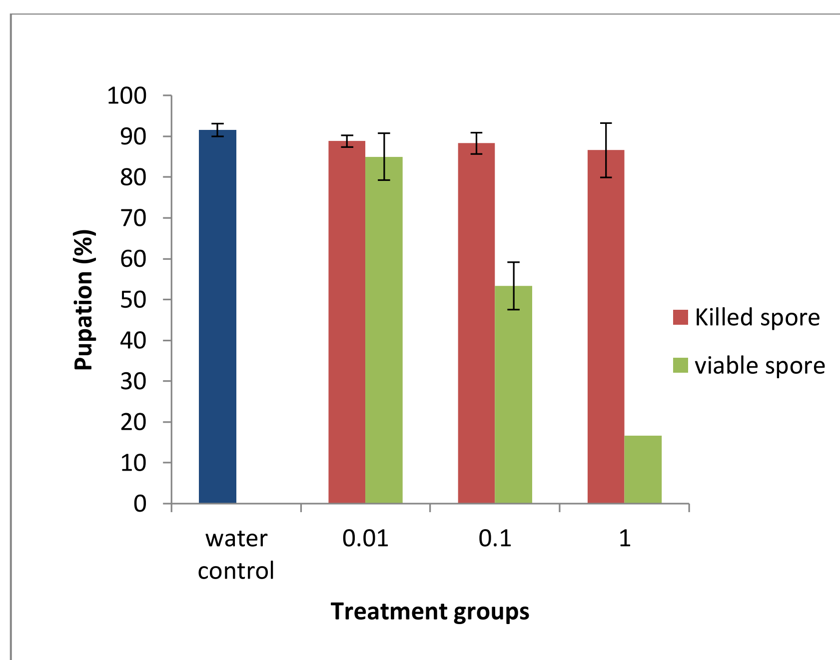


Fig. 6.6 Effect of different spore concentrations of strain L128 on pupation of *Drosophila melanogaster*

Table 6.4 Comparison of various spore concentrations of strain L128 on larval development of *D. melanogaster*

Treatment groups (L128)	Pupation %	Mortality %	Larval duration
Water control	91.58 ± 1.54^{c}	8.42 ± 1.54^{a}	6.66 ± 0.47^{ab}
Killed spore (0.01OD)	88.87 ± 1.43^{c}	11.13 ± 1.43^{a}	6.33 ± 0.47^{a}
Viable spore(0.01 OD)	85.00 ± 5.00^{c}	15.00 ± 5.00^{a}	6.66 ± 0.47^{ab}
Killed spore (0.1 OD)	88.30 ± 2.61^{c}	11.70 ± 2.61^{a}	6.33 ± 0.47^{a}
Viable spore (0.1 OD)	53.33 ± 5.77^{b}	46.67 ± 5.77^{b}	7.33 ± 0.47^{ab}
Killed spore (1 OD)	86.63 ± 6.65^{c}	13.37 ± 6.65^{a}	7 ± 0.00^{ab}
Viable spore (1 OD)	16.67 ± 5.77^{a}	83.33 ± 5.77^{c}	7.6 ± 0.47^{b}
IC_{50}=0.088±0.03 (2.6 x 10^{6}spores/ml)			

Values with the same superscript do not vary significantly ($p > 0.05$)

The toxicological activity of different spore concentrations of actinomycete strains L128, on larval development of *Drosophila* are given (Table 6.4). The pupation percentage in *Drosophila* media with various concentrations of autoclaved/killed spores of strain L128 did not vary significantly from the water control (Fig. 6.6). However a significant reduction in pupation was observed with different concentration of viable spores compared with the respective killed spore control ($p<0.05$). But, pupation percentage in viable spores at 0.01OD (85%) did not significantly vary from the control (91.58%) ($p > 0.05$). The pupation percentage in media treated with different spore concentrations (0.01, 0.1 and 1 OD) of strain L128 was 85%, 53.33%, and 16.67% respectively. The mean difference for all treatment groups is significant at the 0.05 level.

The larval duration was prolonged for the treatment group with spores of strain M32 and strain J18, compared with the control. Treatment group with strain L128 did not show much variation in larval duration compared with the control. Pupal duration for all the treatment groups with different actinomycete strains did not show significant difference compared with the control. The efficacy of actinomycete spore suspension which causes 50% inhibition of pupation is expressed as IC_{50} value. The IC_{50} expressed in terms of OD was calculated as 0.045 ± 0.02 approximately 1.5×10^6 spores/ml, for strain M32, 0.07 ± 0.02 approximately 1.9×10^6 spores /ml for strain J18 and 0.088 ± 0.03 approximately 2.6×10^6spores/ml for L128 respectively. Eventhough all the three isolates inhibited larval development of *Drosophila melanogaster*, based on the above results strain M32 was considered the most potent in biocontrol of insects (Fig. 6.7).

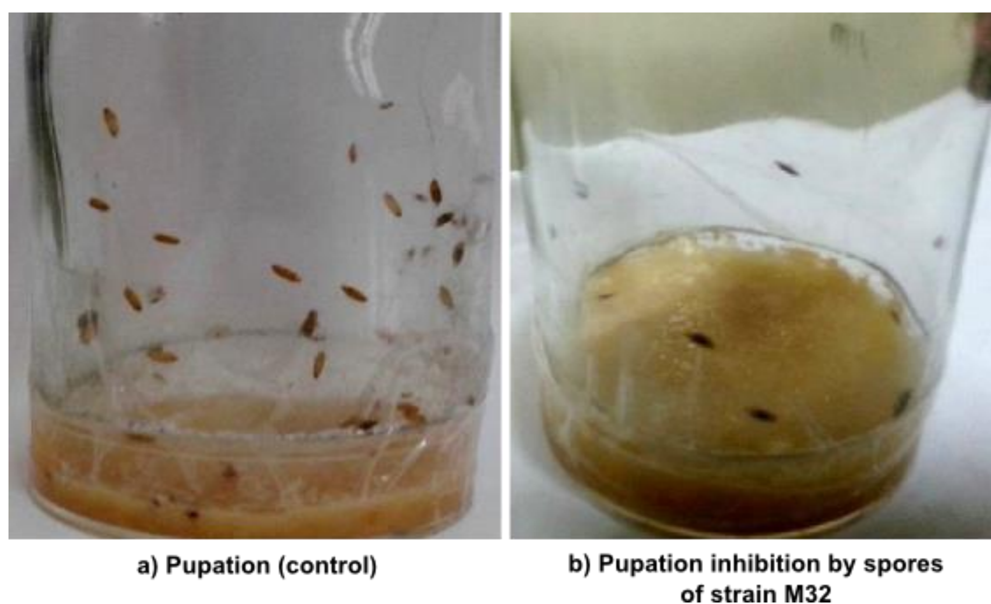


Fig. 6.7 Reduction in pupation by spores of strain M32 compared with control

6.3.3 Characterization of actinomycete isolates with potent fungicidal (J18) and insecticidal activity(M32)

6.3.3.1 Morphological characterization

The isolate J18 exhibited good growth on starch casein agar, glycerol asparagine agar, yeast extract malt extract agar and nutrient agar. The aerial mycelium was dull white with dark maroon substrate mycelium and no diffusible pigments were produced in any of the media. Microscopic observation of the coverslip culture of the strain revealed the sporophores as rectiflexibiles (Fig. 6.8).

The strain M32 with potent insecticidal activity was characterized based on polyphasic approach. The strain was slow growing, exhibited moderate growth on all four media with white aerial mycelium and dull white substrate

mycelium (Fig. 6.9). Micromorphology of the strain revealed sporophores as rectiflexibiles.

6.3.3.2 Biochemical and Physiological characterization

The strain J18 did not produce melanin on tyrosine or peptone yeast extract iron agar, had the ability to degrade esculin, urea, casein, tyrosine, xanthine, hypoxanthine. It was resistant to lysozyme, reduced nitrate, utilized citrate and produced H_2S. The strain produced acids from carbohydrates viz., mannitol, glucose, arabinose, galactose, rhamnose, trehalose and xylose. Inositol and sorbitol was not utilized and the strain was resistant to antibiotics penicillin and cephaloridine and sensitive to rifampicin, streptomycin, neomycin, gentamicin, tobramycin and vancomycin. Apart from chitinase activity the culture exhibited protease, amylase, lipase, phosphatase and DNase activity.

The strain M32 did not produce melanin on tyrosine or peptone yeast extract iron agar, had the ability to degrade esculin, urea, tyrosine, xanthine and hypoxanthine. It was resistant to lysozyme, reduced nitrate; citrate was not utilized and did not produce H_2S. The strain utilized lactose, mannitol, arabinose, rhamnose, galactose, and produced acids from carbohydrates glucose, and rhamnose, the strain did not produce acids from lactose, mannitol and galactose. Inositol, sorbitol and xylose were not utilized and the strain was resistant to antibiotics penicillin and cephaloridine sensitive to rifampicin, streptomycin, neomycin, gentamicin, tobramycin and vancomycin. Apart from chitinase, the culture exhibited protease, amylase, phosphatase and pectinase activity (Table 6.5).

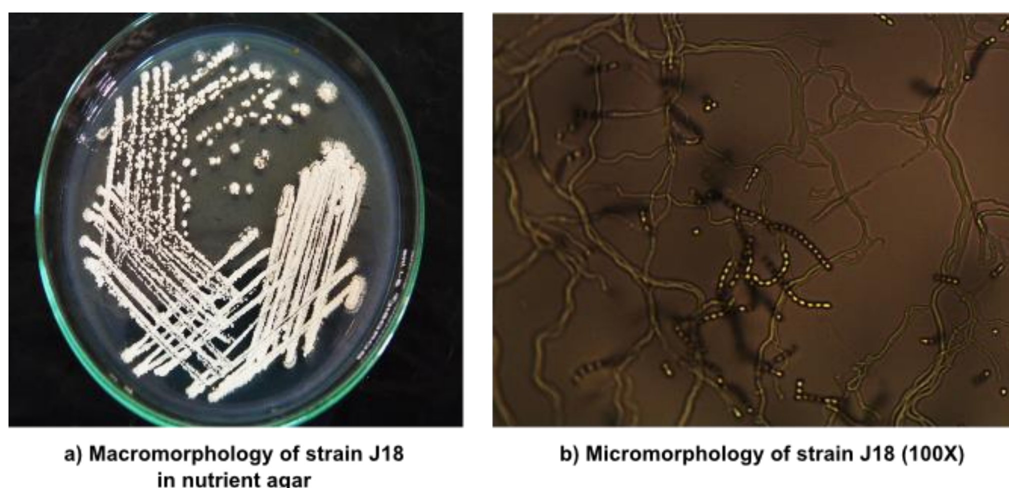


Fig. 6.8 Morphology of antifungal strain J18

6.3.3.3 Molecular characterization and phylogenetic analysis

Molecular characterization and phylogenetic analysis of the culture J18 with broad spectrum antifungal activity revealed the species identity as 99% similar to *Streptomyces sampsonii* of the *S. albidoflavus* cluster. The culture M32 with potent insecticidal activity was identified as 99% similar to *Streptomyces radiopugnans* of the thermophilic clade (Fig. 6.10).

6.3.3.4 Sequence Accession no.

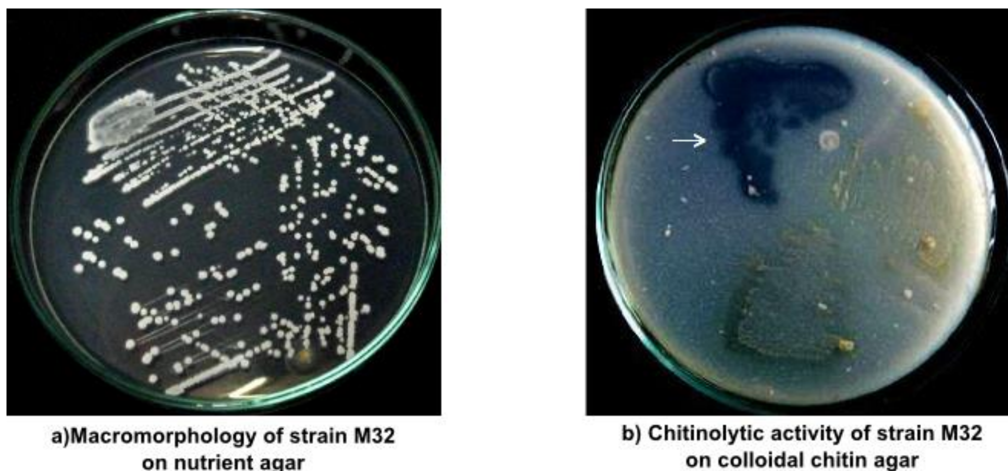
The 1049 bp sequence of culture J18 and the 1023 bp sequence of strain M32 determined were submitted to GenBank database under the accession number **KJ158472** and **KC570321** respectively.

6.3.3.5 Carbon utilization profile

Nutritional/Carbon utilization profile obtained for cultures J18, and M32 based on BIOLOG SFP2 microtitre plates is given (Table 6.6). The carbon utilization profile obtained using BIOLOG SFP2 revealed that the isolate J18

could utilize 29 of the 95 carbon compounds tested, which include dextrin, trehalose, xylose, β-methyl-D-glucoside, D-piscose, rhamnose, ribose, stachyose, arabinose, cellobiose, fructose, D-galactose, α-methyl-D-galactoside, β- methyl-Dgalactoside, maltotriose, D mannose, D-gentiobiose, carboxylic acids such as galacturonic acid, D-gluconic acid, amino acids viz., L-glutamic acid, asparagine, sugar alcohols glycerol, D arabitol, D mannitol, salicin, ester viz.,succinic acid monomethyl ester and aromatic compound arbutin and tween 80.

The strain M32 was slow growing and utilized minimal carbon sources which include the sugars α-cyclodextrin, dextrin, arabinose, rhamnose, galactose, D-trehalose, turanose, palatinose, raffinose, D-mannose and glucose., amino acids such as alanine, L- alanyl glycine, utilized carboxylic acid viz., malic acid, sugar alcohol; glycerol, polymer such as tween 80, tween 40 and ester pyruvic acid methyl ester.



a)Macromorphology of strain M32 on nutrient agar

b) Chitinolytic activity of strain M32 on colloidal chitin agar

Fig. 6.9 Morphology and chitinolytic activity of strain M32

Table 6.5 Morphological and biochemical characteristics of actinomycete J18 and M32

Characteristic		Result	
		J18	M32
Morphological	Spore mass colour	Dull white	White
	Aerial mycelium	Dull white	White
	Substrate mycelium	Dull white	Off
	Spore chain morphology	Rectiflexibiles	Spirals
	Diffusible pigment production	No diffusible	No
Biochemical reactions	Melanin production (Peptone yeast extract iron agar)	-	-
	Melanine Production (Tyrosine agar)	-	-
	Esculin decomposition	-	+
	Casein decomposition	+	-
	Tyrosine decomposition	+	+
	Hypoxanthine decomposition	+	-
	Xanthine decomposition	+	-
	Lysozyme resistance (0.05%)	+	+
	Nitrate reduction	+	+
	H_2S production	+	-
	Urea Hydrolysis	+	+
	Citrate utilization	+	-
Acid Production from carbohydrates	Lactose	-	-
	Mannitol	+	-
	Glucose	+	+
	Arabinose	+	-
	Rhamnose	+	+
	Galactose	+	+
	Inositol	-	-
	Trehalose	+	-
	Sorbitol	-	-
	Xylose	+	-
Resistance to Antibiotics	Penicillin G (10 i.u)	+	+
	Tobramycin(10μg)	-	-
	Rifampicin (30 μg)	-	-
	Cephaloridine(30μg)	+	+
	Streptomycin (25 μg)	-	-
	Neomycin (30 μg)	-	-
	Gentamicin (10 μg)	-	-
	Vancomycin (30 μg)	-	-
Hydrolytic Enzyme Activity	Protease	+	+
	Amylase	+	+
	Lipase	+	-
	DNase	+	_
	Phosphatase	+	+
	Pectinase	-	+
	Ligninase	-	-
	Cellulase	-	-
	Aryl sulphatase	-	-
	Chitinase	+	+

Table 6.6 Carbon utilization profile of *Streptomyces sampsonii* (J18) (Acc.no: KJ158472) and *Streptomyces radiopugnans* (M32) (Acc.no: KC570321)

Carbon Source	Result		Carbon Source	Result		Carbon Source	Result	
	J18	M32		J18	M32		J18	M32
α-Cyclodextrin	-	+	L-Arabinose	+	+	α-D-Lactose	-	-
β-Cyclodextrin	-	-	D-Arabitol	+	-	Lactulose	-	-
Dextrin	+	+	Arbutin	+	-	Maltose	-	-
Glycogen	-	-	D- cellobiose	+	-	Maltotriose	+	-
Inulin	-	-	D- fructose	+	-	D-mannitol	+	-
Mannan	-	-	L-fucose	-	-	D-Mannose	+	+
Tween 40	-	+	D- galactose	+	-	D-melezitose	-	-
Tween80	+	+	D-Galacturonic acid	+	-	D-Melibiose-	-	-
N-Acetyl- D glucosamine	-	-	Gentiobiose	+	-	α-Methyl-D galactoside	+	-
N-Acetyl β-	-	-	D-Gluconic acid	+	-	B-Methyl-D-galactoside	+	-
Amygdalin	-	-	α-D- Glucose	+	+	3-Methyl-D glucose	-	-
D-Tagatose	-	-	m-Inositol	-	-	α-Methyl-D glucoside	-	-
D-Trehalose	+	+	L-Alaninamide	-	-	Adenosine	-	-
Turanose	-	+	D-Alanine	-	-	2-Deoxy adenosine	-	-
Xylitol	-	-	L-Alanine	-	+	Inosine	-	-
D-Xylose	+	-	L-Alanyl glycine	-	+	Thymidine	-	-
Acetic acid	-	-	L- Asparagine	+	-	Uridine	-	-
α-Hydroxybutyric acid	-	-	L-Glutamic acid	+	-	Adenosine-5	-	-
β-Hydroxybutyric acid	-	-	Glycyl –L- Glutamic acid	-	-	Thymidine5-monophosphate	-	-
ϒ-Hydroxybutyric acid	-	-	L-Pyroglutamic acid	-	-	Uridine-5-monophosphate	-	-
P-Hydroxyphenyl acetic	-	-	L -Serine	-	-	D-Fructose-6-Phosphate	-	-
α-Ketoglutaric acid	-	-	Putrescine	-	-	α-D-Glucose-1 phosphate	-	-
α-Ketovaleric acid	-	-	2,3-Butanediol	-	-	D-glucose-6- phosphate	-	-
β-methyl-D glucoside	+	-	Glycerol	+	+	D-L-α-glycerol Phosphate	-	
α-Methyl- D mannoside	-	-	Lactamide	-	-	Succinicacid-mono	+	-
Palatinose	-	+	D-Lacticacid methyl	-	-	Propionic acid	-	-
D-Psicose	+	-	L-Lacticacid	-	-	Pyruvic acid	-	-
D-Raffinose	-	+	D-Malic acid	-	-	Succinamic acid	-	-
L-Rhamnose	+	+	L-Malic acid	-	+	Succinic acid	-	-
D-Ribose	+	-	Pyruvic acid methyl	-	+	Sedoheptulosan	-	-
Salicin	+	-	N-acetyl-L glutamic	-	-	Stachyose	+	-
D- Sorbitol	-	-	Sucrose	-	-			

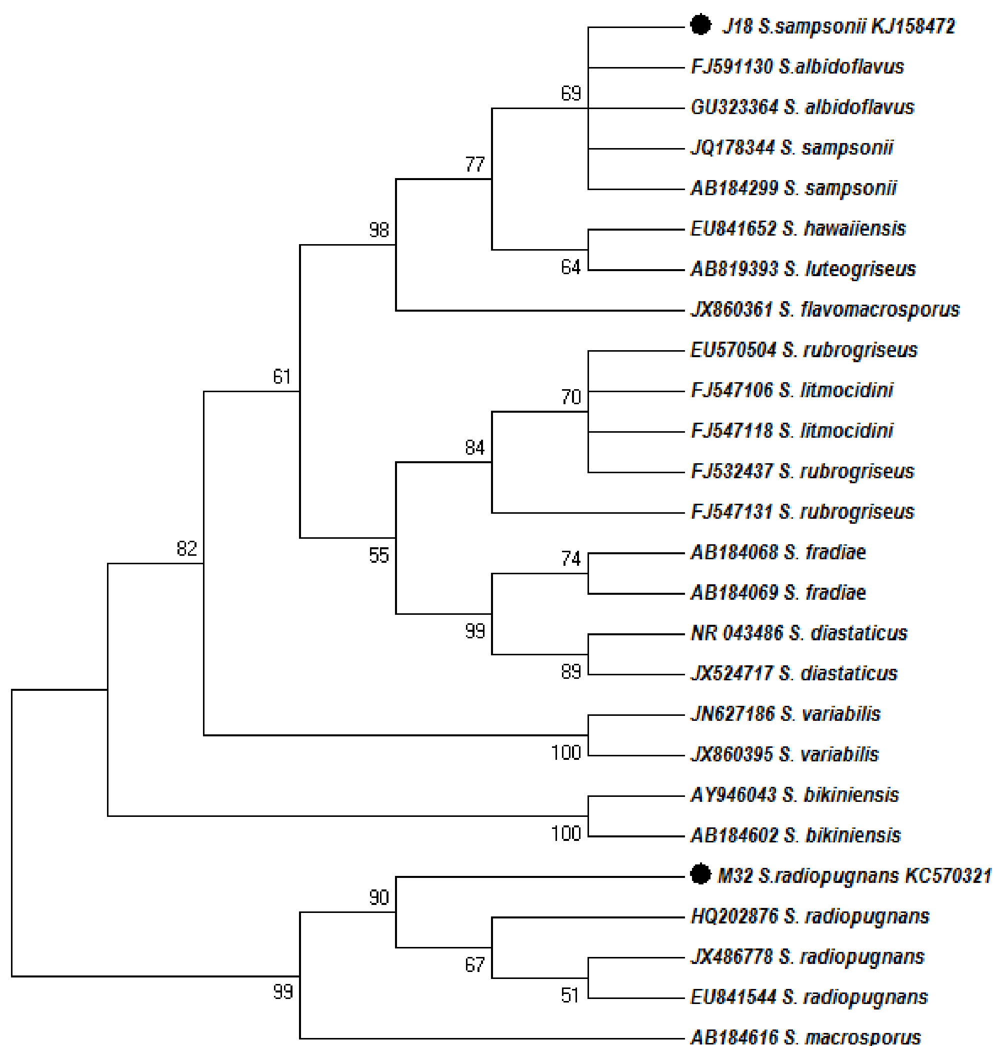


Fig. 6.10 Neighbor-joining phylogenetic tree of chitinolytic marine actinomycete strains constructed using MEGA 5.0. Numbers at nodes indicate levels of bootstrap support (%) based on a Neighbor-joining analysis of 1000 resampled datasets; only values >50% are given

6.4 Discussion

Chitinases are a class of antifungal proteins, and are of particular interest owing to their resistance against both insects as well as fungal pathogens

(Vogelsang and Barz, 1993; Ye et al., 2005). Chitin is an important component of the cell wall of fungi and occurs in combination with other polymers, such as proteins. In the present study, out of the 63% chitinolytic marine actinomycetes, potent five were selected for antifungal assay. Eventhough all the five strains selected for the antifungal assay elaborated significant chitinolytic activity, varied response of fungi to different actinomycete chitinases were observed. Earlier, different response of fungi to *S. griseus* chitinase was reported (Anitha and Rabeeth, 2010). It was also suggested that the resistance to chitinolytic degradation is attributed to the chitin composition in the cell walls of different pathogenic fungi. One possible explanation for fungal inhibition is the action of chitinases on fungal cell walls (Inbar and Chet, 1991). However, chitinases are not fully effective in all circumstances owing to different environmental conditions (Gohel et al., 2006). In our study, antifungal activity of *Streptomyces sampsonii* (J18) was observed as an inward growth of hyphae around the perimeter of the disc (Fig.6.3) containing the crude enzyme. According to Roberts and Selitrennikoff (1986), bacterial chitinases from *Streptomyces griseus*, acted as an exoenzyme and had no effect on hyphal extension of test fungi like *Trichoderma reesei* and *Phycomyces blackesleeanus*. In contrary, they also reported that plant chitinases isolated from the grain of wheat, barley, and maize functioned as endochitinases and inhibited hyphal elongation of test fungi.

The present findings agree that extracellular secondary metabolites or hydrolytic enzymes including chitinase play a crucial role in fungal growth inhibition as reported by Julaluk and Hataichanoke (2012).

In the present study marine actinomycete strain J18 identified as *Streptomyces sampsonii* of the *S.albidoflavus* group was identified as the most

potent antifungal agent. Previously, terrestrial actinomycete with broad spectrum antifungal activity against unicellular and filamentous fungi were reported as closely related to *S. albidoflavus* cluster (Atta et al., 2011; Narayana et al., 2007). Recently, chitinolytic actinomycete isolated from agricultural soil which inhibited the growth of the fungal phytopathogens *Fusarium solani* and *Alternaria alternata* was phylogenetically identified as *Streptomyces rimosus* (Brzezinska et al., 2013). Similar reports of chitinolytic soil actinomycetes that inhibited phytopathogens include *Streptomyces griseoflavus which* restricted the radial growth of *Fusarium oxysporum f.*sp. *lycopersici* (Julaluk and Hataichanoke, 2012) and *Streptomyces roseolus* against *Aspergillus* and *Penicillium* hyphal extensions (Xiayun et al., 2012).

The antifungal activity of *S. sampsonii J18* from marine sediments can be extended to clinical as well as agricultural pathogens as the antifungal activity of *Streptomyces* VITSVK5 sp. against drug resistant *Aspergillus* clinical isolates (Kumar and Kannabiran, 2010). Nowadays, agriculture relies on biofungicides containing various microorganisms (e.g., *Bacillus subtilis* GB03, *Pseudomona*s *aureofaciens* Tx-1, and *Streptomyces griseoviridis* K61), (Copping, 2004), the marine *Streptomyces sampsonii* (J18) can also be a welcome addition to this group.

In the present study, the spore suspensions of chitinolytic marine actinomycetes J18, M32 and L128 were screened for potential insecticidal activity using *Drosophila melanogaster* as the insect model. The spore suspensions of actinomycete isolates were all considerably effective compared

to their controls. The strain M32 caused significant reduction in *D. melanogaster* pupation under controlled laboratory conditions and significantly reduced the percentage of pupal formation under the same conditions. The inability of the autoclaved preparation of each chitinolytic actinomycete to inhibit pupation indicates that the biocontrol may be associated with the chitinolytic enzyme production as reported earlier (Gadelhak et al., 2005). One probable postulate for inhibition of pupation is that, the cuticle of insect species consists largely of chitin, and chitinase produced by the actinomycetes could be involved in insect control. Microbial chitinolytic enzymes have been considered important in the biological control of many insects because of their ability to interfere with chitin deposition (Tripathi et al., 2002). Besides, chitinase induced damage to the peritrophic membrane in the insect gut causes a significant reduction in nutrient utilization and consequently affect insect growth (Terra and Ferreira, 2005). During the lifecycle, *Drosophila* eggs hatch out within 12-15hrs and the molting 2nd and 3rd stage larva feeds on microorganisms and their spores. As the larval feeding stage lasts for 4-6 days, some of the spores ingested by the larva utilize the chitin present in the gut lining for their germination, leading to the loss of chitin in the gut lining, which could also be an attribute to inhibition of pupation.

Both the isolates J18 and M32 could significantly increase the larval mortality by >50% when applied to the medium individually at a concentration of 0.1 OD which corresponds to $3x10^6$ spores/ml. The pupation percentage

was lowest for strain M32 (35.29%), followed by 42.22% and 53.33% for J18 L128 respectively. The pupation percentage was concordant with the highest larval mortality calculated as 64.71% for M32 and the least for L128 (46.67%). The present findings are in validation with significant mortality observed on the application of chitinase producing non streptomycetes to the rearing medium of the fruit fly, *D. melanogaster* (Gadelhak et al., 2005). Contrary to the mortality caused by chitinase, there are few reports where bacterial chitinases are generally ineffective in certain assays. No mortality of the nymphal stages of the rice brown plant hopper (*Nilaparvata lugens*) occurred when 0.09% (w/v) *S. griseus* chitinase was added to an artificial diet (Powell et al., 1993). Similarly, *Serratia* and *Streptomyces* chitinases at 1–2% levels in the diet of the merchant grain beetle (*Oryzaephilus Mercator*) caused no mortality (Kramer et al., 1997). The inactivity of many of these chitinase preparations was explained by the presence of primarily exochitinases being substantially less effective than the endochitinases in degrading chitin.

However, the *Streptomyces* sp. isolated from marine sediments in the present study viz., *Streptomyces radiopugnans* (M32) and *Streptomyces sampsonii* (J18) are highly effective in the control of pupal formation of *Drosophila* as that of non *streptomycete Actinoplanes* sp.(39.43%) than soil *Streptomyces clavuligerus* (55.71 ± 5.56%) in a previous report by Gadelhak et al. (2005). With regard to the development, the larval duration was significantly prolonged with isolates M32 and J18 in comparison with that of the control. No significant effect was exhibited for pupal durations. Similar

effects on prolonged larval duration were reported for soil actinomycetes on lepidopteran *Spodoptera littoralis*, and also various soil *Streptomyces* sp. on 3rd instar larva of mosquitoe *Culex pipiens* (Bream et al., 2001; El-Khawagh et al., 2011).

The marine actinomycete isolates M32, J18, L128 with insecticidal property were identified based on polyphasic approach as belonging to the dominant *Streptomyces* sp. viz., *S.radiopugnans*, *Streptomyces sampsonii*, and *S.luteogriseu*s. Similarly, actinomycetes from desert soil of Egypt exhibiting insecticidal activity on *Culex pipiens* were identified as *Streptomyces* sp. viz., *Streptomyces fungicidicus*, *Streptomyces griseus*, *Streptomyces albus*, *Streptomyces rochei*, *Streptomyces violaceus*, *Streptomyces alboflavus* and *Streptomyces griseofuscus* (El-Khawagh et al., 2011). The present study provides an insight into the efficacy of marine *Streptomyces radiopugnans* and *S. sampsonii as* biocontrol agents unlike the terrestrial counterparts. The BIOLOG system originally developed to assist with taxonomic description of bacteria is based on the ability of the bacteria to oxidise up to 95 different carbon substrates. In the present investigation it was found that the potent insecticidal strain (M32) utilized only 25% of the carbon sources. It could be due to a fact that not all the organic substrates in the BIOLOG is ecologically relevant for the particular habitat where the organism thrives.

The chitinolytic marine actinomycetes can definitely open new perspectives as biocontrol agents of fungal pathogens and insect vectors. It is imperative that new and effective bioinsecticides are identified suitable and

adaptive to ecological conditions, unlike conventional insecticides based on a single active chemical compound. To conclude, it has been proved that actinomycetes, isolated from marine sediments have promising potential as biocontrol agents.

7

Summary and Conclusion

Microbial natural products have become a prime resource for novel drugs; yet microbes inhabiting the largest habitat, covering 70% on earth's planet, the oceans remain underexploited. Among the potential sources of microbes, actinomycetes have been proven to be a prolific contributor accounting for the vast majority of the products. Actinomycetes are unique among prokaryotes due to their filamentous nature, differentiated life cycle and metabolic versatility. As marine environmental conditions are extremely challenging, marine microbes have evolved the greatest genetic and metabolic diversity. Successful and effective exploitation of actinomycetes relies in characterization of actinomycetes from unexplored habitats which in turn unravels the ecological and biotechnological perspectives of the organism.The present work was focused on the biochemical, molecular and functional characterization of marine actinomycete isolates from sediments of Arabian Sea and Bay of Bengal for biotechnological applications.

Marine actinomycetes already isolated from continental shelf and slope sediments of Arabian Sea and Bay of Bengal and maintained in the

Microbiology Laboratory of School of Marine Sciences, CUSAT were used for the study. The isolates were identified up to generic level based on morphological, physiological and biochemical characteristics. They were further clustered based on biochemical characteristics and subjected to molecular identification. Amplified ribosomal DNA restriction analysis followed by sequencing of representative isolates helped to identify the isolates up to species level. The antimicrobial and biogranulation property of actinomycetes were studied and the efficacy of actinomycete biogranules for *in vitro* exclusion of pathogenic vibrios were analysed. The melanin production ability of marine streptomycetes was tested and the role of melanin as antioxidant and photoprotectant was studied. Biocompatibility of melanin was tested in HEp-2 cell lines by XTT and sequential cytotoxicity assays. The marine actinomycetes were screened for hydrolytic enzyme production and preliminary investigations of potent chitinolytic strains in biocontrol of fungal pathogens and insect pests were carried out. The salient findings of the study are as follows:

- ❖ Morphological, biochemical and physiological characterization of marine actinomycetes (230 Nos.) revealed *Streptomyces* (76%) as the dominant genera followed by *Nocardiopsis* sp. (24%).
- ❖ White spore mass (58%) was predominant among the marine actinomycetes followed by grey, yellow, green and pink spore series. Also spiral spore chain (34%) was predominant followed

by rectiflexibiles (28%) long chain of spores (24%) and retinaculiaperti (14%) spore chain morphology.

- Pentose sugar rhamnose induced heavy growth and sporulation in marine actinomycetes.
- *Streptomyces* and *Nocardiopsis* were almost equivocal in their biochemical and decomposition profile.
- Self-immobilization property (biogranulation) was exhibited by marine actinomycetes (82%) in shake flask cultures in nutrient broth.
- Actinomycetes isolated from the sediments elaborated a wide array of enzymes, ranging from gelatinase (99.13%) to ligninase (15.22%). DNase activity was exhibited by 96.09%, followed by lipase (86.96%), phosphatase (84.78%), amylase (76.96%) chitinase (63.48%), and pectinase (22.17%). Compared to *Nocardiopsis*, the *Streptomyces* isolates displayed better ability to degrade compounds such as chitin, pectin and lignin.
- Melanin production ability was exhibited by only genus *Streptomyces* (4%).
- 16S rDNA amplification using universal eubacterial primers: 27 F and 1492R yielded a single amplicon of approximately 1500 bp for all the marine actinomycete isolates.

- A total of eight polymorphic patterns were obtained for *Sau3*A1, ten patterns for *Taq*1 and only six patterns for *Hin*f1 digestions of 16S amplicons of the actinomycete isolates.
- Sequencing of representative isolates based on polyphasic approach resulted in 23 species representing 2 genera *i.e*., Nineteen species could be identified under the genus *Streptomyces* and 4 species under *Nocardiopsi*s.
- The most abundant species in marine sediment was identified as *Streptomyces albidoflavus* (20.42%) followed by *Nocardiopsis alba* (17.82%), *S. radiopugnans* (8.26%), *S. variabilis* (5.22%) and other minor species.
- *Streptomyces radiopugnans* (28.78%) was the most abundant among the actinomycete isolates (66) from Bay of Bengal and *Streptomyces albidoflavus* (26.82%) from Arabian Sea. *Streptomyces thermolineatus* (10.66%), *S. acrimycini* (12.12%), *S. rubrolavendulae* (7.66%), *S. bikiniensis* (7.57%) were found only in the sediments of Bay of Bengal.
- Actinomycete isolate *Streptomyces rubrolavendulae* M56 from Bay of Bengal exhibited broad spectrum antibacterial activity.
- *Streptomyces rubrolavendulae* (M56) could significantly inhibit *Vibrio* spp. by *in vitro* co-culture exclusion experiments.
- Marine actinomycete *S. bikiniensis* M8 isolated from sediments of Bay of Bengal produced melanin like pigment. The yield of

melanin extracted from peptone yeast extract iron broth culture of strain M8 was 0.166g dry wt/L.

- NMR spectra for M8 melanin resembled spectra for human hair melanin except for the increased level of N and/or O attachment, less residual protein, and a lower level of methyl functionality relative to residual protein content.
- FTIR spectra of melanin showed the presence of peaks at 3277.63 (OH or NH stretching), 1633.76 (C = C stretching or C=O stretching), 1537.8 (NH bending) and 1446 cm^{-1} (CH_2-CH_3 bending) characteristic of melanin pigment.
- M8 melanin synthesized by *S. bikiniensis* appeared to be amorphous by SEM imaging, similar to synthetic melanin, while natural Sepia melanin had a defined spherical structure. The surface elemental composition of M8 melanin consisted of 6 elements, carbon as the most abundant followed by moderate amounts of oxygen and iron, small amounts of phosphorous followed by sulphur and a trace of aluminum is also detected.
- The most abundant element detected in M8 melanin is iron followed by calcium, sodium, potassium and magnesium.
- Starch was found to be the most effective carbon source and sodium glutamate the most effective nitrogen source, for melanin production of melanogenic strain (M8).

- Melanin exhibited significant antioxidant activity as exemplified by lipid peroxidation inhibition (IC_{50}=73.70 ± 12.6 µg ml^{-1}), ABTS (IC_{50} = 25.09 ± 5.08 µg ml^{-1}) and DPPH (IC_{50} =35.49 ± 6.19 µg ml^{-1}) radical scavenging assays. IC_{50} of the melanin (M8) on ABTS radicals was only 12 times lower than the standard trolox (2.2 ± 0.2 µg ml^{-1}); the activity can be improved on purification.
- The biocompatibility study of M8 melanin tested by standard assays proved that melanin was non toxic to HEp-2 cells up to a concentration of 156.25 µg ml^{-1}.
- Antioxidant activity of melanin (M8) in human cancer cells (HEp-2) was confirmed by super oxide dismutase and super oxide anion assays.
- Crude Melanin (M8) at 200 µg ml^{-1}could scavenge 44 % reactive oxygen species of HEp-2 cells generated on UV exposure, which was comparable to positive control synthetic melanin at 50 µg $ml^{-1.}$
- Crude melanin extracted from *S. bikiniensis* (M8) has proved to be a promising antioxidant and photoprotectant.
- Chitinolytic actinomycete J18 from sediments of Arabian Sea identified as *Streptomyces sampsonii* exhibited remarkable antifungal property.

- Actinomycete M32 isolated from Bay of Bengal identified as *Streptomyces radiopugnans* exhibited potent insecticidal activity with an IC_{50} of 1.5 $x10^{6}$ spores/ml.
- From the present study, the following marine *Streptomyces* isolates viz., *S.rubrolavendulae* M56 (Anti vibrio), *S.bikiniensis* M8 (Melanin producing), *S. sampsonii* J18 (Antifungal) and *S. radiopugnans* M32 (Insecticidal) were found to be excellent candidates with potent industrial applications.

Conclusion

The present study provides an insight into the various actinomycetes occurring in the sediments of Arabian Sea and Bay of Bengal. *Streptomyces* was found to be the dominant group followed by *Nocardiopsi*s. Eventhough generic level identification is possible by traditional phenotypic methods, species level identification necessitate a polyphasic approach including both phenotypic and genotypic characterization. Antibiotic production coupled with biogranulation property helped in the effective utilization of the actinomycetes for the control of vibrios. Melanin from *Streptomyces bikiniensis* was proved to be a promising antioxidant and photoprotectant. Marine actinomycetes were found to be a good source of hydrolytic enzymes and the chitinolytic isolates could be explored as biocontrol agents in terms of antifungal and insecticidal property. The present study explored the potential of marine actinomycetes

especially Streptomycetes as a promising source of bioactive molecules for application in aquaculture and pharmaceutical industry.

Scope for future research

- Testing the efficacy of *Streptomyces rubrolavendulae* M56 as an antivibrio agent in *P.monodon* culture system for the exclusion of vibrios.
- Process optimization for the production and down stream processing of melanin from *Streptomyces bikiniensis* M8.
- Testing the potential of *Streptomyces sampsonii* J18 for the control of fungal pathogens.
- Testing the potential of *S.radiopugnans* M32 in mosquito control.

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Appendix 1: *List of the Cruises of Fisheries and Oceanographic Research Vessel (FORV) Sagar Sampada and the area of sample (sediment) collection giving details of the source of isolation of actinomycetes used in the present study.*

Cruise No:	Area of sample collection	Latitude, longitude
Cruise No. 228	West coast of India: Cape Comorin to Porbander (200,500 and 1000m depth)	7°10'00''N to 21°29'00''N and 77°20'00''E to 67°46'00''E
Cruise No. 233	South West Coast of India: Cape Comorin to Coondapore (200, 500 and 1000m depth)	7°10'00N to 13°29'00''N and 77°20'00''E to 73°17'00''E
Cruise No. 236	East coast of India: Karaikal to Paradip (200,500 and 1000m depth)	10°34'00''N to 20°01'00''N and 80°26'00''E to 87°30'00''E
Cruise No. 245	East coast of India: Karaikal to Paradip (200,500 and 1000m depth)	10°35'7N to19°59'46''N and 80°27'27''E to 87°19'75''E
Cruise No. 254	West coast of India :Cape Comorin to Porbander (200,500 and 1000m depth)	7°01'00''N to 21°30'00''N and 77°15'00''E to 67°28'00''E
Cruise No. 255	South West coast of India: Trivandrum to Kannur (50,100 and 200m depth)	8°29'28'N to 11°59'08''N and 76°43'00''E to 74°25'76''E

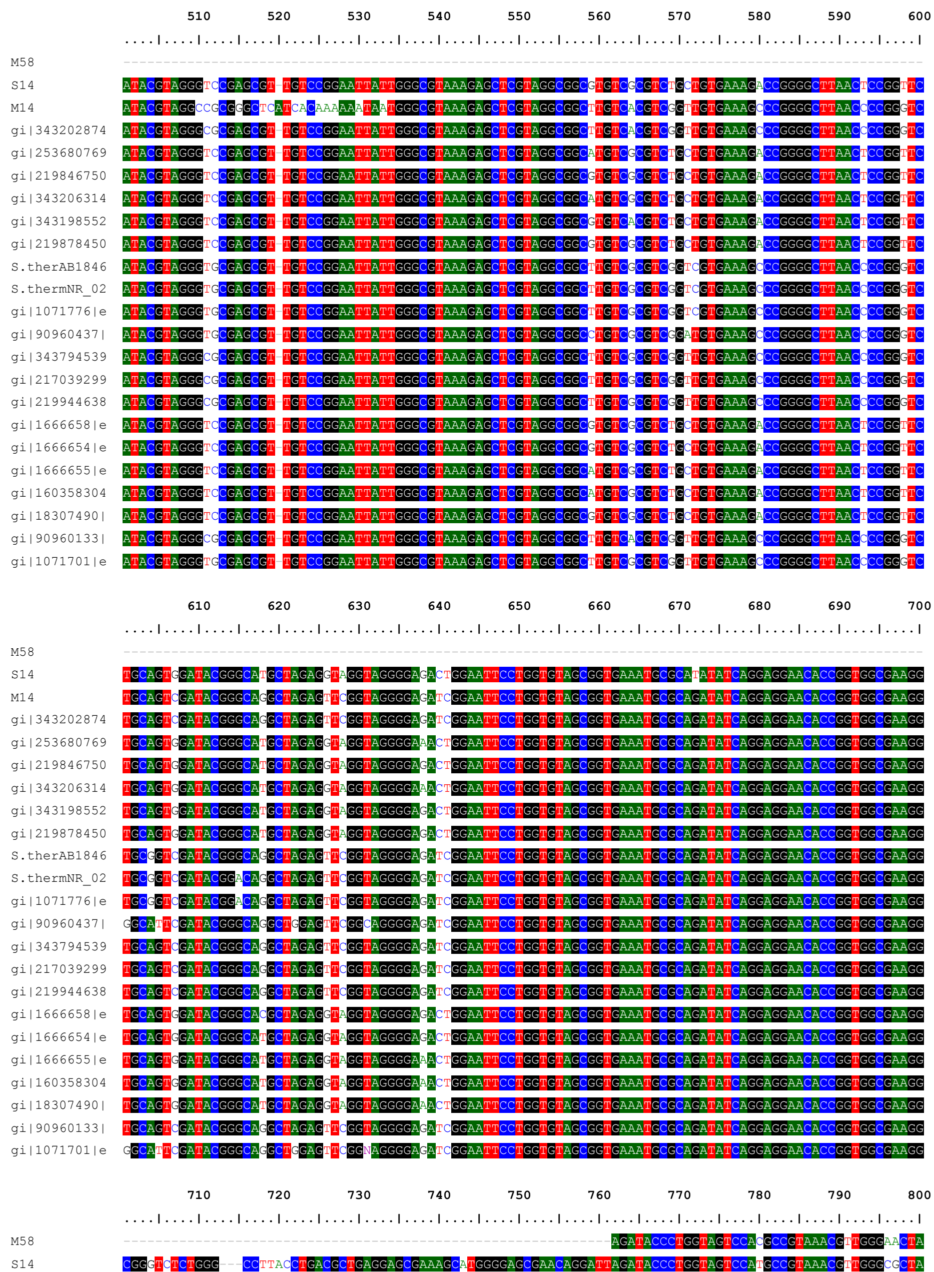
Appendix 2: *Average Vibrio count (Logarithmic value of cfu/ml) during different time intervals in the co-culture experiment*

Co- culture	0hour	24hr	48hr	72hr
V.harveyi (control)	6.63 ± 0.02^{a}	7.66 ± 0.027^{e}	8.202 ± 0.002^{e}	8.732 ± 0.013^{d}
V.harveyi+M56	6.63 ± 0.03^{a}	7.03 ± 0.04^{d}	2.544 ± 0.06^{a}	0.000^{a}
V.parahaemolyticus (control)	6.93 ± 0.008^{a}	8.37 ± 0.50^{f}	7.890 ± 0.006^{d}	7.527 ± 0.02^{b}
V.parahemolyticus+M56	6.83 ± 0.02^{a}	3.91 ± 0.10^{a}	2.439 ± 0.18^{a}	0.000^{a}
V.alginolyticus (control)	7.38 ± 0.46^{b}	8.29 ± 0.001^{f}	8.382 ± 0.002^{e}	7.735 ± 0.01^{c}
V.alginolyticus+M56	6.87 ± 0.03^{a}	5.37 ± 0.06^{c}	3.754 ± 0.19^{c}	0.000^{a}
V.fluvialis (control)	6.85 ± 0.01^{a}	8.46 ± 0.004^{f}	7.931 ± 0.01^{d}	7.727 ± 0.01^{c}
V.fluvialis+M56	6.67 ± 0.04^{a}	4.96 ± 0.04^{b}	3.069 ± 0.08^{b}	0.000^{a}

Values in the same column with the same superscript are not significantly different by one way ANOVA ($p < 0.05$).

Appendix 3 – Multiple sequence alignments obtained using BioEdit v7.2.5

A. *Multiple sequence alignment of nucleotide sequences of actinomycetes with green spore mass with closely related Streptomyces and Nocardiopsis sp. Conserved regions are indicated in colour.*



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M14            CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
gi|343202874   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
gi|253680769   CGGGTNTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAANAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|219846750   CGGGTCTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|343206314   CGGGTTTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|343198552   CGGGTCTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|219878450   CGGGTCTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
S.therAB1846   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTA
S.thermNR_02   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTA
gi|1071776|e   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTA
gi|90960437|   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTA
gi|343794539   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
gi|217039299   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
gi|219944638   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
gi|1666658|e   CGGGTCTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|1666654|e   CGGGTCTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|1666655|e   CGGGTTTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|160358304   CGGGTTTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA-CGTTGGGCGCTA
gi|18307490|   CGGGTTTCTGGG---CCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTA
gi|90960133|   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
gi|1071701|e   CGGATCTCTGGG---CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTA

                        810       820       830       840       850       860       870       880       890       900
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M58            GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
S14            GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
M14            GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|343202874   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|253680769   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|219846750   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|343206314   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|343198552   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|219878450   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
S.therAB1846   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
S.thermNR_02   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|1071776|e   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|90960437|   GGTGTGGGCGGCATTCCACGTCGT-CCGTGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|343794539   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|217039299   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|219944638   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|1666658|e   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|1666654|e   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|1666655|e   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|160358304   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|18307490|   GGTGTGGG-GACTTTCCACGGTTT-CCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|90960133|   GGTGTGGGCGACATTCCACGTCGT-CCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC
gi|1071701|e   GGTGTGGGCGGCATTCCACGTCGT-CCGTGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT-TGAC

                        910       920       930       940       950       960       970       980       990      1000
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M58            GGGGGCCCGCACAAGAGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACTGGCAGAGATGTCAGC
S14            GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
M14            GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGC
gi|343202874   GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGC
gi|253680769   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|219846750   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGCAGAGATGTGGGG
gi|343206314   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|343198552   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|219878450   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGCAGAGATGTGGGG
S.therAB1846   GGGGGCCCGCACAAGAGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACTGGCAGAGATGTCAGC
S.thermNR_02   GGGGGCCCGCACAAGAGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACTGGCAGAGATGTCAGC
gi|1071776|e   GGGGGCCCGCACAAGAGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACTGGCAGAGATGTCAGC
gi|90960437|   GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGCCCAGAGATGGGTGC
gi|343794539   GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGC
gi|217039299   GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGC
gi|219944638   GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGC
gi|1666658|e   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|1666654|e   GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
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gi|1666655|e    GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|160358304    GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|18307490|    GGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGG
gi|90960133|    GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGC
gi|1071701|e    GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGCCCAGAGATGGGTGC

                        1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M58             CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
S14             TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCC
M14             CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|343202874    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|253680769    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
gi|219846750    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCC
gi|343206314    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCC
gi|343198552    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
gi|219878450    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTATTCC
S.therAB1846    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
S.thermNR_02    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
gi|1071776|e    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
gi|90960437|    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|343794539    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|217039299    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|219944638    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|1666658|e    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCC
gi|1666654|e    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCC
gi|1666655|e    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCC
gi|160358304    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
gi|18307490|    TCATTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
gi|90960133|    CCCCTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC
gi|1071701|e    CCCCTTG--TGGTCGGTGTATAGGTGGTGCATGGCTGTCGTCAGCTCGTGT-CGTGAGATGTTG--GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCC

                        1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M58             GTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
S14             ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGAC-------------------------------------------------------
M14             GTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|343202874    GTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|253680769    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|219846750    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|343206314    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|343198552    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|219878450    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
S.therAB1846    GTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
S.thermNR_02    GTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|1071776|e    GTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|90960437|    GTGTTGCCAGCGTGCCCTTCGGGGTGACGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|343794539    GTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|217039299    GTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|219944638    GTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCCAGTCATCATG---
gi|1666658|e    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|1666654|e    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|1666655|e    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|160358304    ATGTTGCCAGCA-------CGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|18307490|    ATGTTGCCAGCA-------CGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|90960133|    GTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---
gi|1071701|e    GTGTTGCCAGCGTGCCCTTCGGGGTGACGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG----ACGACGTCAAGTCATCATG---

                        1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M58             CCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGC----------------------------------------------
S14             ----------------------------------------------------------------------------------------------------
M14             CCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGG
gi|343202874    CCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGG
gi|253680769    CCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGG
gi|219846750    CCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTGAGGTGGAGCGAATCCCTAAAAGCCGGTCTCAGTTCGG
gi|343206314    CCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGG
gi|343198552    CCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGG G
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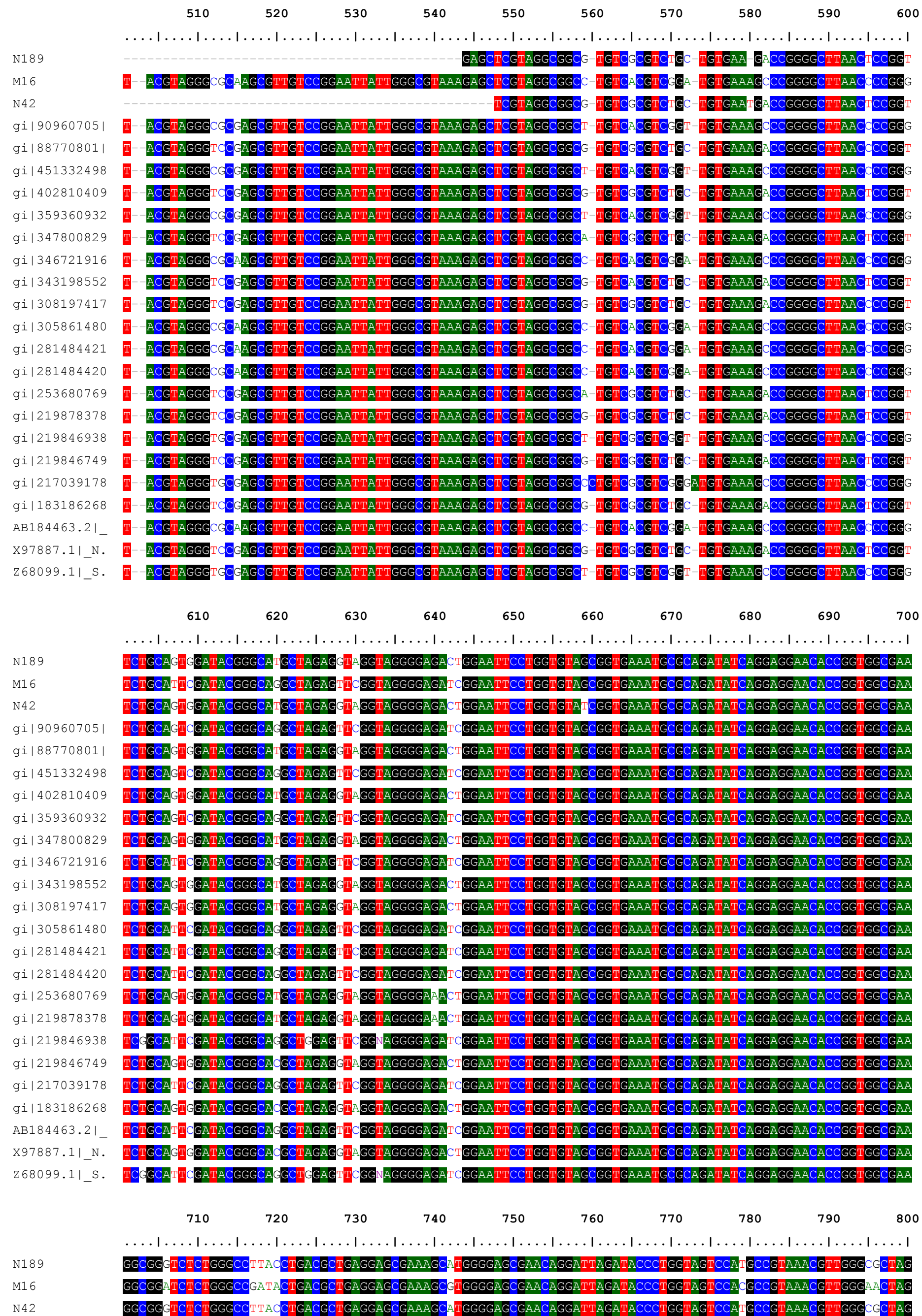
***B.** Multiple sequence alignment of nucleotide sequences of pink spore mass actinomycetes with other actinomycetes. Conserved sequences are indicated in colour.*



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gi|88770801|   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|451332498   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAG
gi|402810409   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|359360932   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAG
gi|347800829   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|346721916   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAG
gi|343198552   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|308197417   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|305861480   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAG
gi|281484421   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAG
gi|281484420   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAG
gi|253680769   GGCGGGTNTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAANAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|219878378   GGCGGGTTTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|219846938   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAG
gi|219846749   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
gi|217039178   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAG
gi|183186268   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
AB184463.2|_   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAG
X97887.1|_N.   GGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAG
Z68099.1|_S.   GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAG

                       810       820       830       840       850       860       870       880       890       900
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
N189           GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
M16            GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
N42            GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTT-CTAACGCATTAAGCGCCCCGCCTGGGGATTACGGCCGCAAGGCTAAAACTCACAGGAATTGACGGG
gi|90960705|   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|88770801|   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|451332498   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|402810409   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|359360932   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|347800829   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|346721916   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|343198552   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|308197417   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|305861480   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|281484421   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|281484420   GTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|253680769   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|219878378   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
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gi|219846749   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|217039178   GTGTGGGCGGCATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
gi|183186268   GTGTGGG-GACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
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                       910       920       930       940       950       960       970       980       990      1000
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M16            GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCC
N42            GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTARAGATACAGGGTCA
gi|90960705|   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCC
gi|88770801|   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
gi|451332498   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCC
gi|402810409   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
gi|359360932   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCC
gi|347800829   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
gi|346721916   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCC
gi|343198552   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
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gi|305861480   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCC
gi|281484421   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCC
gi|281484420   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCC
gi|253680769   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
gi|219878378   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
```

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gi|219846938   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGCCCAGAGATGGGTGCCCC
gi|219846749   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
gi|217039178   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCC


gi|183186268   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
AB184463.2|_   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCC
X97887.1|_N.   GGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCA
Z68099.1|_S.   GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGCCCAGAGATGGGTGCCCC

                      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
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N189           TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
M16            CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
N42            TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
gi|90960705|   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
gi|88770801|   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
gi|451332498   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
gi|402810409   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
gi|359360932   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
gi|347800829   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
gi|346721916   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
gi|343198552   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTATGTTG
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gi|305861480   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
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gi|219878378   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTATGTTG
gi|219846938   CTTG--TGGTCGGTGTATAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
gi|219846749   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
gi|217039178   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTG
gi|183186268   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
AB184463.2|_   CTTG--TGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
X97887.1|_N.   TTTAGTTGGTGGGTG-ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTG
Z68099.1|_S.   CTTG--TGGTCGGTGTATAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG

                      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
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M16            CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
N42            CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|90960705|   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|88770801|   CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|451332498   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
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gi|359360932   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|347800829   CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|346721916   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
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gi|308197417   CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|305861480   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|281484421   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
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gi|253680769   CCAGCAC-------GTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|219878378   CCAGCAC-------GTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|219846938   CCAGCGTGCCCTTCGGGGTGACGGGGACTCACGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|219846749   CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|217039178   CCAGCGTGCCTTTCGGGGTGACGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
gi|183186268   CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
AB184463.2|_   CCAGCAGGCCCTT-GTGGTGCTGGGGACTCACGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
X97887.1|_N.   CCAGCAC-------GTAATGGTGGGGACTCATGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
Z68099.1|_S.   CCAGCGTGCCCTTCGGGGTGACGGGGACTCACGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
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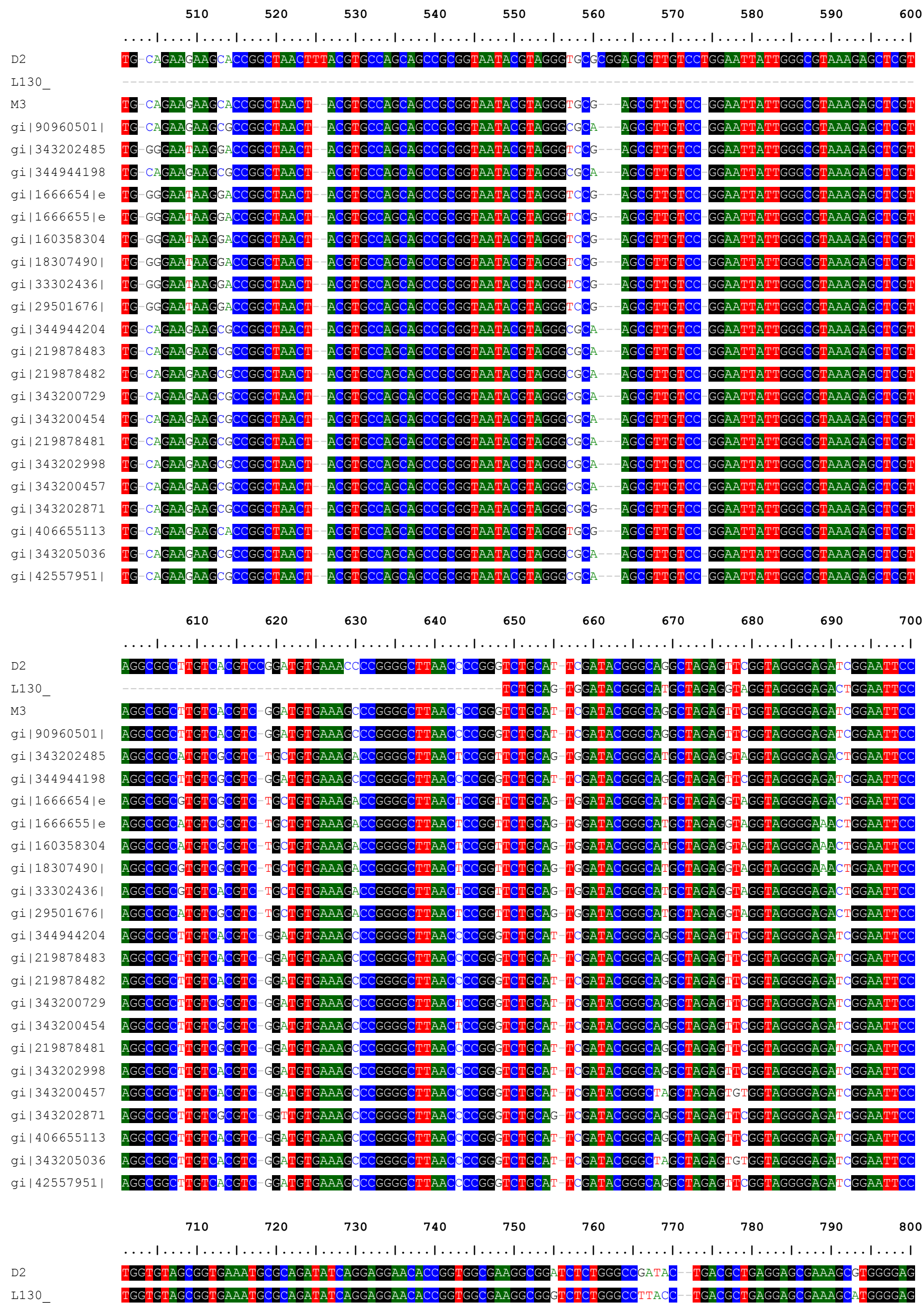
***C.** Multiple sequence alignment of nucleotide sequences of yellow spore series actinomycetes with other Streptomyces and Nocardiopsis sp. Conserved regions are indicated in colour.*



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gi|90960501| TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|343202485 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGTAG
gi|344944198 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|1666654|e TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGGAG
gi|1666655|e TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTTTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGGAG
gi|160358304 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTTTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGGAG
gi|18307490| TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTTTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGGAG
gi|33302436| TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGGAG
gi|29501676| TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACC--TGACGCTGAGGAGCGAAAGCATGGGTAG
gi|344944204 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|219878483 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|219878482 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|343200729 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|343200454 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|219878481 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|343202998 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
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gi|406655113 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
gi|343205036 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTAC--TGACGCTGAGGAGCGAAAGCGTGGGGAG
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                     810       820       830       840       850       860       870       880       890       900
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D2           CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCC-ACGTCGTCCGTGCCGCAGCTAACGCATTAAGT
L130_        CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTATCTAACGCATTAAGC
M3           CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGT
gi|90960501| CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCC-ACGTCGTCCGTGCCGCAGCTAACGCATTAAGT
gi|343202485 CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTAGCTAACGCATTAAGC
gi|344944198 CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCC-ACGTCGTCCGTGCCGCAGCTAACGCATTAAGT
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gi|1666655|e CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTAGCTAACGCATTAAGC
gi|160358304 CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA-CGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTAGCTAACGCATTAAGC
gi|18307490| CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTAGCTAACGCATTAAGC
gi|33302436| CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTAGCTAACGCATTAAGC
gi|29501676| CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGG-GACTTTCC-ACGGTTTCCGCGCCGTAGCTAACGCATTAAGC
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gi|343200457 CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCC-ACGTCGTCGGTGCCGCAGCTAACGCATTAAGT
gi|343202871 CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCC-ACGTCGTCCGTGCCGCAGCTAACGCATTAAGT
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gi|343205036 CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCC-ACGTCGTCGGTGCCGCAGCTAACGCATTAAGT
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                     910       920       930       940       950       960       970       980       990      1000
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M3           TCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCGGCGGAGCATGTGGCTTAATTCGACG
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gi|343200454  TCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCGGCGGAGCATGTGGCTTAATTCGACG
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gi|343202998  TCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCGGCGGAGCATGTGGCTTAATTCGACG
gi|343200457  TCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCAGCGGAGCATGTGGCTTAATTCGACG
gi|343202871  GCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCGGCGGAGCATGTGGCTTAATTCGACG
gi|406655113  TCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCGGCGGAGCATGTGGCTTAATTCGACG
gi|343205036  TCCCCGCCTGGGGAGTACGGCC------GCAAGGCTAAAACTCAAAGGAATTGA-CGGGGGCCCGCA--CAAGCAGCGGAGCATGTGGCTTAATTCGACG
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                      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
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D2            CAACGCGAAGAACCTT-----ACCAAAGGCTTTGACATACATCGGAAAGCCGTAGAGATACGGCCCCCCCTTGTGGTCGGTGTACAGGTGGTGCAT----
L130_         CAACGCGAAGAACCTT-----ACCAAGG--TTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCATTTGGTTGGCGGTGACAGGTGGTGCAT----
M3            CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATACATCGGAAAGCCGTAGAGATACGGCCCCCC-TTGTGGTCGGTGTACAGGTGGTGCAT----
gi|90960501|  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCC-TTGTGGTCGGTGTACAGGTGGTGCAT----
gi|343202485  CAACGCGAAGAACCTT-----ACCAAGG--TTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCATTTGGTTGGCGGTGACAGGTGGTGCAT----
gi|344944198  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATACACCGGAAACCTCTGGAGACAGGGGCCCCC-TTGTGGTCGGTGTACAGGTGGTGCAT----
gi|1666654|e  CAACGCGAAGAACCTT-----ACCAAGG--TTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCATTTAGTTGGTGGTGACAGGTGGTGCAT----
gi|1666655|e  CAACGCGAAGAACCTT-----ACCAAGG--TTTGACATCACCCGTGGACCTGTAGAGATACAGGGTCATTTAGTTGGTGGTGACAGGTGGTGCAT----
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gi|343202998  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCC-TTGTGGTCGGTGTACAGGTGGTGCAT----
gi|343200457  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCC-TTGTGGTCGGTATACAGGTGGTGCAT----
gi|343202871  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCC-TTGTGGTCGGTGTACAGGTGGTGCAT----
gi|406655113  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATACATCGGAAAGCCGTAGAGATACGGCCCCCC-TTGTGGTCGGTGTACAGGTGGTGCAT----
gi|343205036  CAACGCGAAGAACCTT-----ACCAAGG--CTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCC-TTGTGGTCGGTATACAGGTGGTGCAT----
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                      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
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D2            GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
L130_         GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGTTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCA-------CGTATTGGTGG
M3            GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|90960501|  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGG
gi|343202485  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCA-------CGTAGTGGTGG
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gi|219878482  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGG
gi|343200729  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|343200454  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|219878481  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGG
gi|343202998  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|343200457  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|343202871  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGTGTTGG
gi|406655113  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|343205036  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGG
gi|42557951|  GGCTGTCGTCA-----GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGG
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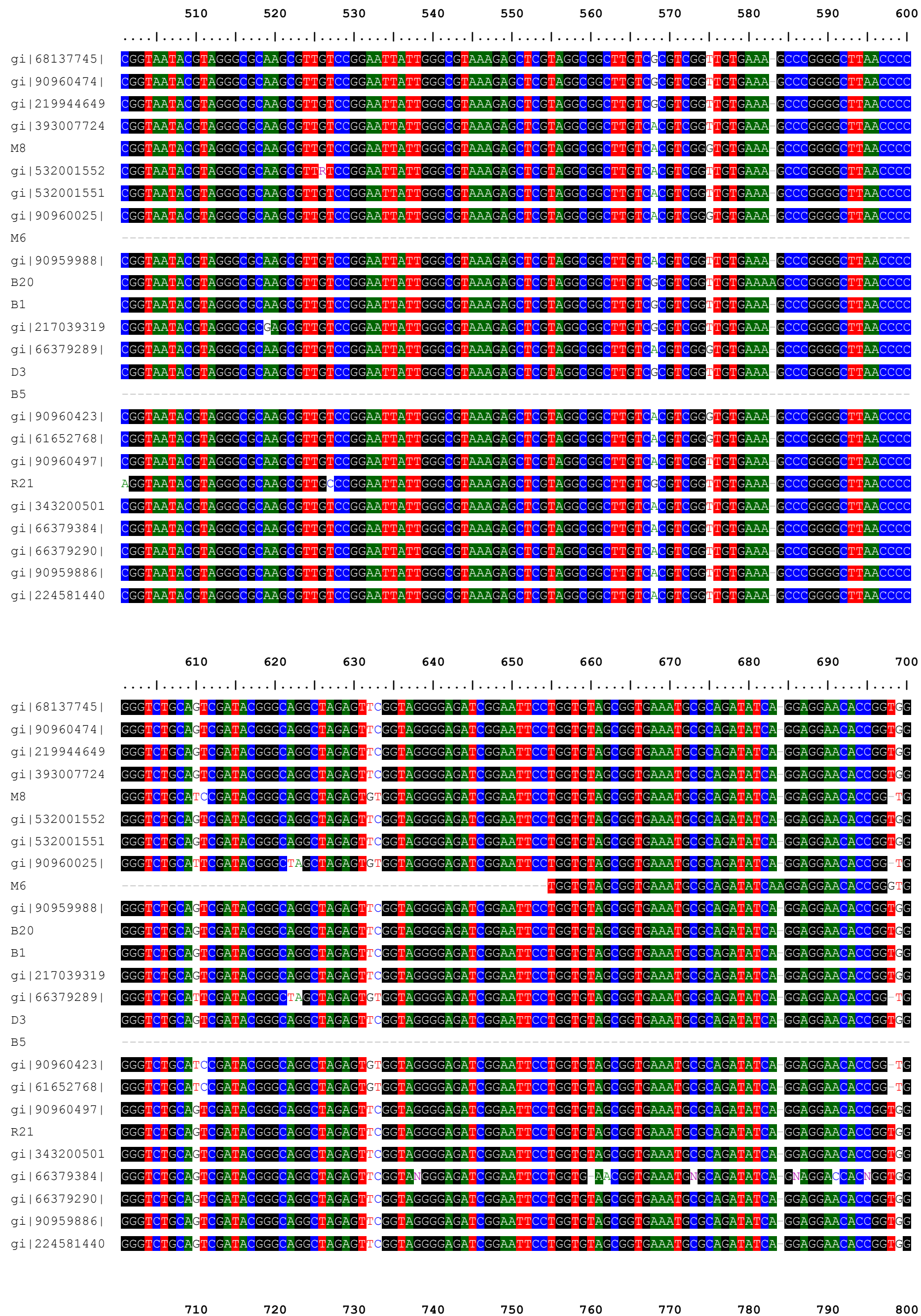
***D.** Multiple sequence alignment of nucleotide sequences of grey spore and red substrate mycelium, pigment producing actinomycetes with other Streptomyces sp. Conserved sequences are indicated in colour.*



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gi|90960474|  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|219944649  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|393007724  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
M8            GCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACG-CCGTAAA-CGTTG
gi|532001552  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|532001551  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|90960025|  GCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACG-CCGTAAA-CGGTG
M6            GCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAACAGGAATAGATACCCTGGTAGTTCACGTCCGTAAAACGTTG
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B20           -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCCCGTAAA-CGGTG
B1            -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|217039319  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|66379289|  GCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACG-CCGTAAA-CGGTG
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B5            -------------------------------------------------------------------------CTGGTAGTCCACGCC-GTAA--CGGTG
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gi|61652768|  GCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACG-CCGTAAA-CGTTG
gi|90960497|  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
R21           -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|343200501  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|66379384|  GCGAAGGCGGATCTTTGGGCNGATACTGACG-TGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCN-GTAAA-CGGTG
gi|66379290|  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|90959886|  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG
gi|224581440  -CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA-CAGGATTAGATACCCTGGTAGTCCACGCC-GTAAA-CGGTG

                       810       820       830       840       850       860       870       880       890       900
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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gi|90960474|  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|219944649  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|393007724  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
M8            GGAACTAGG-TGTTGGCGACATTGCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|532001552  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|532001551  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|90960025|  GGAACTAGG-TGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
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B20           GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
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gi|217039319  GGCACTAGG-TGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|66379289|  GGAACTAGG-TGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
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B5            GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
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gi|61652768|  GGAACTAGG-TGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|90960497|  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
R21           GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
gi|343200501  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
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gi|90959886|  GGCACTAGG-TGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
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gi|219944649  ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGAT
gi|393007724  ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGAT
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gi|532001551  ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGAT
gi|90960025|  ATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGAT
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gi|217039319   ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGAT
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gi|66379290|   ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGAT
gi|90959886|   ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGAT
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gi|219944649   GGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCC
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M6             GGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCC
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D3             GGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCC
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gi|61652768|   GGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCC
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B1             CGTGTTGCCAGCAA-CTCTTCGGAG-GTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTT
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D3             CGTGTTGCCAGCAA-CTCTTCGGAG-GTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTT
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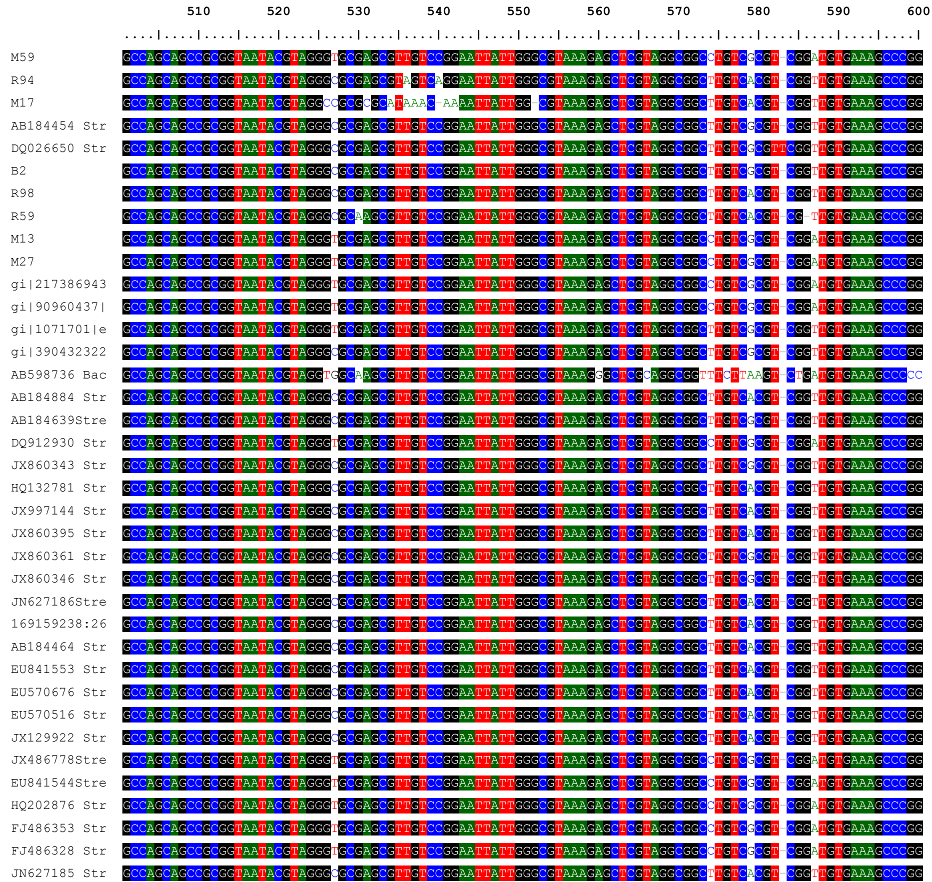
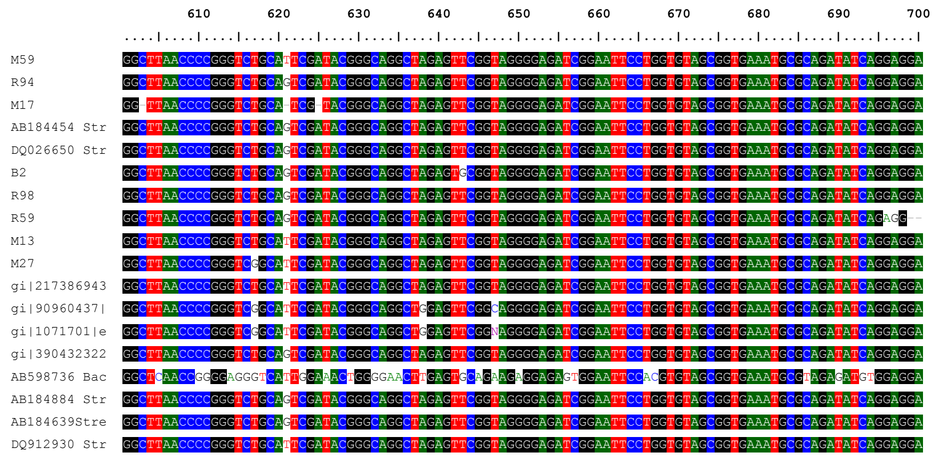
***E.** Multiple sequence alignment of nucleotide sequences of grey spore actinomycetes with other Streptomyces sp. Conserved sequences are indicated in colour.*





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HQ132781 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
JX997144 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
JX860395 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
JX860361 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
JX860346 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
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AB184464 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
EU841553 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
EU570676 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
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JX129922 Str  GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
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HQ202876 Str   ACGTTGGGCACTAGGTGTGGGCGGCATT-CCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
FJ486353 Str   ACGTTGGGCACTAGGTGTGGGCGGCATT-CCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
FJ486328 Str   ACGTTGGGCACTAGGTGTGGGCGGCATT-CCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
JN627185 Str   ACGGTGGGCACTAGGTGTGGGCGACATT-CCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
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M17            AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
AB184454 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAAC-CC
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gi|1071701|e   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGCCC
gi|390432322   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAAC-CC
AB598736 Bac   AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATC-CT
AB184884 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
AB184639Stre   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACG-TC
DQ912930 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCC
JX860343 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACG-TC
HQ132781 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCC
JX997144 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
JX860395 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
JX860361 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACG-TC
JX860346 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACG-TC
JN627186Stre   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
169159238:26   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
AB184464 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
EU841553 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATT
EU570676 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATT
EU570516 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATT
JX129922 Str   AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
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JX486778Stre  AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCC
EU841544Stre  AAAGGAATTGACGGGGGCCCGCCCAAGCGGCGGAGCATGTGGCTTAATTGGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGCCC
HQ202876 Str  AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCC
FJ486353 Str  AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCC
FJ486328 Str  AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCC
JN627185 Str  AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC

                      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M59           G--GAGACACTTGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
R94           A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
M17           A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
AB184454 Str  T-GGAGACAGGGTCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
DQ026650 Str  T-GGAGACAGGCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
B2            TAGGAGACAGCGGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCATCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
R98           A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATG--------------------------
R59           A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
M13           G--GAGACTCGTGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
M27           A--GAGATGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
gi|217386943  A--GAGATGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
gi|90960437|  A--GAGATGGGTGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
gi|1071701|e  A--GAGATGGGTGCCCCCTTGTGGTCGGTGT-ATAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
gi|390432322  T-GGAGACAGGGTCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
AB598736 Bac  AGAGATAGGACGTCCCCTTCGGGGCAGAGTGACAGGTGGTGCATGG-TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
AB184884 Str  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
AB184639Stre  T-GGAGACAGGCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
DQ912930 Str  A--GAGATGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX860343 Str  T-GGAGACAGGCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
HQ132781 Str  A--GAGATGGGCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX997144 Str  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX860395 Str  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX860361 Str  T-GGAGACAGGCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX860346 Str  T-GGAGACAGGCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JN627186Stre  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
169159238:26  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
AB184464 Str  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
EU841553 Str  A--GAGATAGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
EU570676 Str  A--GAGATAGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
EU570516 Str  A--GAGATAGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX129922 Str  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JX486778Stre  A--GAGACGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
EU841544Stre  G--GAGACGTTTGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
HQ202876 Str  A--GAGATGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
FJ486353 Str  A--GAGATGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
FJ486328 Str  A--GAGATGGTCGCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
JN627185 Str  A--GAGATGGTGCCCCCCTTGTGGTCGGTGT-ACAGGTGGTGCATGG-CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA

                      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
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M59           ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
R94           ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
M17           ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
AB184454 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
DQ026650 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
B2            ACCCTTGTCCCGTGTTGCCAGCACGCCCTT---GTGGTGTTGGGGCACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
R98           ----------------------------------------------------------------------------------------------------
R59           ACCCTTGTCCCGTGTTGCCAGCAAGCCCCTTCGGGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
M13           ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
M27           ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
gi|217386943  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
gi|90960437|  ACCCTTGTCCCGTGTTGCCAGCGTGCCCTTC--GGGGTGACGGGG-ACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
gi|1071701|e  ACCCTTGTCCCGTGTTGCCAGCGTGCCCTTC--GGGGTGACGGGG-ACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
gi|390432322  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
AB598736 Bac  ACCCTTGATCTTAGTTGCCAGCATTCAGTT------------GGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAAT
AB184884 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
AB184639Stre  ACCCTTGTCCCGTGTTGCCAGCAAGCCCTTC--GGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
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DQ912930 Str  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX860343 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
HQ132781 Str  ACCCTTGTCCCGTGTTGCCAGCAAGCCCTTC--GGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX997144 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX860395 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX860361 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX860346 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JN627186Stre  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
169159238:26  ACCCTTGTCCCGTGTTGCCAGCAAGCCCCTTCGGGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
AB184464 Str  ACCCTTGTCCCGTGTTGCCAGCAAGCCCCTTCGGGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
EU841553 Str  ACCCTTGTCCCGTGTTGCCAGCAAGCTCCTTCGGGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
EU570676 Str  ACCCTTGTCCCGTGTTGCCAGCAAGCTCCTTCGGGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
EU570516 Str  ACCCTTGTCCCGTGTTGCCAGCAAGCTCCTTCGGGGGTGTTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX129922 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JX486778Stre  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
EU841544Stre  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
HQ202876 Str  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
FJ486353 Str  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
FJ486328 Str  ACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGG-ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
JN627185 Str  ACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGG-ACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT

                       1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M59           CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
R94           CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
M17           CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
AB184454 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
DQ026650 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
B2            CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
R98           ----------------------------------------------------------------------------------------------------
R59           CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
M13           CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
M27           CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
gi|217386943  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
gi|90960437|  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGAGGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCA
gi|1071701|e  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGANGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCA
gi|390432322  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
AB598736 Bac  CATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCA
AB184884 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
AB184639Stre  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
DQ912930 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JX860343 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
HQ132781 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JX997144 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTA-AAT-----------------------------------------------
JX860395 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JX860361 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JX860346 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JN627186Stre  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
169159238:26  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
AB184464 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
EU841553 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
EU570676 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
EU570516 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JX129922 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JX486778Stre  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
EU841544Stre  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
HQ202876 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
FJ486353 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
FJ486328 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
JN627185 Str  CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
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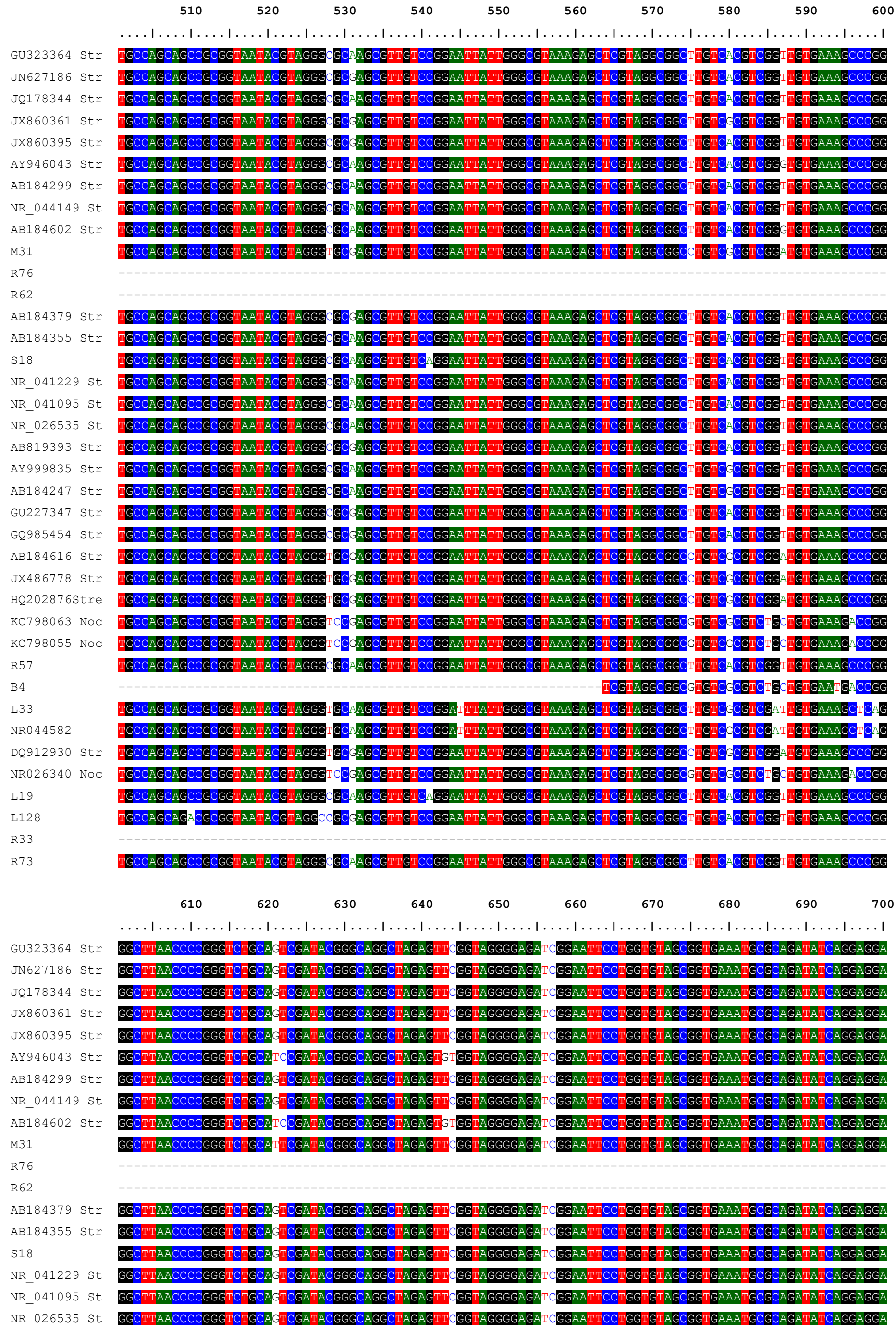
***F.* Multiple sequence alignment of nucleotide sequences of white spore actinomycetes with other related actinomycetes. Conserved sequences are indicated in colour.**



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AY999835 Str   GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAAGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
AB184247 Str   GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
GU227347 Str   GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
GQ985454 Str   GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
AB184616 Str   GGCTTAACCCCGGGTCGGCATTCGATACGGGCAGGCTGGAGTTCGGCAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
JX486778 Str   GGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
HQ202876Stre   GGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
KC798063 Noc   GGCTTAACTCCGGTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
KC798055 Noc   GGCTTAACTCCGGTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
R57            GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
B4             GGCTTAACTCCGGTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTATCGGTGAAATGCGCAGATATCAGGAGGA
L33            GGCTTAACCCTGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
NR044582       GGCTTAACCCTGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
DQ912930 Str   GGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
NR026340 Noc   GGCTTAACTCCGGTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
L19            GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
L128           GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
R33            ----------------------------------------------------------------------------------------------------
R73            GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
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                   710       720       730       740       750       760       770       780       790       800
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GU323364 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
JN627186 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
JQ178344 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
JX860361 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
JX860395 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AY946043 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AB184299 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
NR_044149 St   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AB184602 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
M31            ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
R76            ------------------------------------------------------------------------------ATACCCTGGTAGTCCACGCCGT
R62            ---------------------------------------------------------TGGGGAGCGAACAGGATTACCATACCCTGGTAGTCCACGCCGT
AB184379 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AB184355 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
S18            ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
NR_041229 St   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
NR_041095 St   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
NR_026535 St   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AB819393 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AY999835 Str   ACACCGGTGGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AB184247 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
GU227347 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
GQ985454 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
AB184616 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
JX486778 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
HQ202876Stre   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
KC798063 Noc   ACACCGGTGG-CGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCATGCCGT
KC798055 Noc   ACACCGGTGG-CGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCATGCCGT
R57            ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
B4             ACACCGGTGG-CGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCATGCCGT
L33            ACACCGGTGG-CGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCTGT
NR044582       ACACCGGTGG-CGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCTGT
DQ912930 Str   ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
NR026340 Noc   ACACCGGTGG-CGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCATGCCGT
L19            ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
L128           ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
R33            -------------------------------------------------------------------------------TACCCTGGTAGTCCACGCACG
R73            ACACCGGTGG-CGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG-ATACCCTGGTAGTCCACGCCGT
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                   810       820       830       840       850       860       870       880       890       900
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GU323364 Str   AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
JN627186 Str   AAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
JQ178344 Str   AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
JX860361 Str   AAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
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JX860395 Str  AAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AY946043 Str  AAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTC-GTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB184299 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
NR_044149 St  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB184602 Str  AAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTC-GTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
M31           AAACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
R76           AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
R62           AAACGGTGGGCACTAAGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB184379 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB184355 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
S18           AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
NR_041229 St  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
NR_041095 St  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
NR_026535 St  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB819393 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AY999835 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB184247 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
GU227347 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
GQ985454 Str  AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
AB184616 Str  AAACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTC-GTCCGTGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
JX486778 Str  AAACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
HQ202876Stre  AAACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
KC798063 Noc  AAACGTTGGGCGCTAGGTGTGGG-GACTTTCCACGGT-TTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
KC798055 Noc  AAACGTTGGGCGCTAGGTGTGGG-GACTTTCCACGGT-TTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
R57           AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCC-GCCTGGGGAGTACGGGCCGCAAGGCTAAAA
B4            AAACGTTGGGCGCTAGGTGTGGG-GACTTTCCACGGT-TTCCGCGCCGTT-CTAACGCATTAAGCGCCCCGCCTGGGGATTACGG-CCGCAAGGCTAAAA
L33           AAACGGTGGGAACTAGGTGTGGGCAGCATTCCACGTT-GTCTGTGCCGTAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
NR044582      AAACGGTGGGAACTAGGTGTGGGCAGCATTCCACGTT-GTCTGTGCCGTAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
DQ912930 Str  AAACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTC-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
NR026340 Noc  AAACGTTGGGCGCTAGGTGTGGG-GACTTTCCACGGT-TTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
L19           AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
L128          AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCA-GGCTAAAA
R33           TAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA
R73           AAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTT-GTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG-CCGCAAGGCTAAAA

                      910       920       930       940       950       960       970       980       990      1000
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GU323364 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
JN627186 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
JQ178344 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
JX860361 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
JX860395 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AY946043 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATATACCGGAA
AB184299 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
NR_044149 St  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AB184602 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATATACCGGAA
M31           CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
R76           CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
R62           CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCAACGCAACGCGAACAACCTTACCAA-GGCTTGACATACACCGGAA
AB184379 Str  CTCAAAGGAATT-GACGGGGGCCC-GCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AB184355 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
S18           CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
NR_041229 St  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
NR_041095 St  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
NR_026535 St  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AB819393 Str  CTCAAAGGAATT-GACGGGGGCCC-GCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AY999835 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AB184247 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
GU227347 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
GQ985454 Str  CTCAAAGGAATT-GACGGGGGCCC-GCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
AB184616 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
JX486778 Str  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
HQ202876Stre  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
KC798063 Noc  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGTTTGACATCACCCGTGG
KC798055 Noc  CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGTTTGACATCACCCGTGG
R57           CTCAAAGGAATT-GACGGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAAGGCTTGACATACACCGGAA
B4            CTCACAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGTTTGACATCACCCGTGG
L33           CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACGCCGGAA
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NR044582       CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACGCCGGAA
DQ912930 Str   CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
NR026340 Noc   CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGTTTGACATCACCCGTGG
L19            CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
L128           CTCAAAGGAATTTGACGGGGGCCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
R33            CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA
R73            CTCAAAGGAATT-GACGGGGG-CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA-GGCTTGACATACACCGGAA

                        1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GU323364 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
JN627186 Str   AGCATCAGAGATGGTGCCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
JQ178344 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
JX860361 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
JX860395 Str   AGCATCAGAGATGGTGCCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AY946043 Str   AGCATCAGAGATGGTGCCCCCCTTG--TGGTCGGTATACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AB184299 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
NR_044149 St   AACCCTGGAGACAGGGTCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AB184602 Str   AGCATCAGAGATGGTGCCCCCCTTG--TGGTCGGTATACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
M31            ACACGCGGAGACACGTGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
R76            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
R62            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCATGA-GATGTTGGGTT-AAGTCCCGCAA
AB184379 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AB184355 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
S18            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
NR_041229 St   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
NR_041095 St   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
NR_026535 St   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AB819393 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AY999835 Str   AACCCTGGAGACAGGGTCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AB184247 Str   AACCCTGGAGACAGGGTCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
GU227347 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
GQ985454 Str   ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
AB184616 Str   ACGCCCAGAGATGGGTGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
JX486778 Str   ACGGCCAGAGACGGTCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
HQ202876Stre   ACGGCCAGAGATGGTCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
KC798063 Noc   ACCTGTAGAGATACAGGGTCATTTAGTTGGTGGGTG-ACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
KC798055 Noc   ACCTGTAGAGATACAGGGTCATTTAGTTGGTGGGTG-ACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
R57            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
B4             ACCTGTARAGATACAGGGTCATTTAGTTGGTGGGTG-ACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
L33            AACCATGGAGACATGGTCCCTCTTT--GAGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
NR044582       AACCATGGAGACATGGTCCCTCTTT--GAGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
DQ912930 Str   ACGGCCAGAGATGGTCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
NR026340 Noc   ACCTGTAGAGATACAGGGTCATTTAGTTGGTGGGTG-ACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
L19            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
L128           ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGGGTGGTGCATGGCTGTCGTCAGCCTCGTGTCGTGAAGATGTTGGGTTTAAGTCCCGCAA
R33            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA
R73            ACGTCTGGAGACAGGCGCCCCCTTG--TGGTCGGTGTACAGG-TGGTGCATGGCTGTCGTCAGC-TCGTGTCGTGA-GATGTTGGGTT-AAGTCCCGCAA

                        1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GU323364 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
JN627186 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
JQ178344 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
JX860361 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
JX860395 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AY946043 Str   CGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTC--GGGGTGATGGGGACTCACAGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AB184299 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
NR_044149 St   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AB184602 Str   CGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTC--GGGGTGATGGGGACTCACAGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
M31            CGAGCGCAACCCTTGTTCTGTGTTGCCAGCGTGCCCTTC--GGGGTGACGGGGACTCACAGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
R76            CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
R62            CGAGCGCAACCCTTGTCCCGTGTTGCCAACAGGCCCTT---GTGGTGCTGGGGACTCACGGGAAAACACCCAAGGGCAACTCGGAGGCAGGTGGGGACA
AB184379 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AB184355 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
S18            CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
NR_041229 St   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
NR_041095 St   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
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NR_026535 St   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AB819393 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AY999835 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AB184247 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
GU227347 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
GQ985454 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
AB184616 Str   CGAGCGCAACCCTTGTCCCGTGTTGCCAGCGTGCCCTTC--GGGGTGACGGGGACTCACGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
JX486778 Str   CGAGCGCAACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGGACTCACAGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
HQ202876Stre   CGAGCGCAACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGGACTCACAGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
KC798063 Noc   CGAGCGCAACCCTTGTTCCATGTTGCCAGCAC---------GTAATGGTGGGGACTCATGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
KC798055 Noc   CGAGCGCAACCCTTGTTCCATGTTGCCAGCAC---------GTAATGGTGGGGACTCATGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
R57            CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
B4             CGAGCGCAACCCTTGTTCCATGTTGCCAGCAC---------GTAATGGTGGGGACTCATGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
L33            CGAGCGCAACCCTTATCCTGTGTTGCCAGCAACTCTCTTCGGAGGGGTTGGGGACTCACGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
NR044582       CGAGCGCAACCCTTATCCTGTGTTGCCAGCAACTCTCTTCGGAGGGGTTGGGGACTCACGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
DQ912930 Str   CGAGCGCAACCCTTGTTCTGTGTTGCCAGCGTGCCTTTC--GGGGTGACGGGGACTCACAGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
NR026340 Noc   CGAGCGCAACCCTTGTTCCATGTTGCCAGCAC---------GTAATGGTGGGGACTCATGGG--AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
L19            CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
L128           CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGG--------------------------
R33            CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
R73            CGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTT---GTGGTGCTGGGGACTCACGGG--AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG

                       1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GU323364 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
JN627186 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
JQ178344 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
JX860361 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
JX860395 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
AY946043 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGC-TGCGATGCCGCGAGGCGGAGCGAATCTCAAAAA
AB184299 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
NR_044149 St   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
AB184602 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGC-TGCGATGCCGCGAGGCGGAGCGAATCTCAAAAA
M31            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATGCCGCGAGGTGGAGCGAATCTCAAAAA
R76            ACGTCAAGTCATCATGCCCCTTATGT--------------------------------------------------------------------------
R62            ACCTC-----------------------------------------------------------------------------------------------
AB184379 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
AB184355 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGNGAGGTGGAGCGAATCTCAAAAA
S18            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
NR_041229 St   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
NR_041095 St   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGNGAGGTGGAGCGAATCTCAAAAA
NR_026535 St   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGNGAGGTGGAGCGAATCTCAAAAA
AB819393 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGTGAGGTGGAGCGAATCTCAAAAA
AY999835 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
AB184247 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
GU227347 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
GQ985454 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
AB184616 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGAGGCCGCGAGGCGGAGCGAATCTCAAAAA
JX486778 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATGCCGTGAGGTGGAGCGAATCTCAAAAA
HQ202876Stre   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATGCCGTGAGGTGGAGCGAATCTCAAAAA
KC798063 Noc   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAA
KC798055 Noc   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAA
R57            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
B4             ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAA
L33            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCTGGTACAATGAGC-TGCGATGCCGTGAGGTGGAGCGAATCTCAAAAA
NR044582       ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCTGGTACAATGAGC-TGCGATGCCGTGAGGTGGAGCGAATCTCAAAAA
DQ912930 Str   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATGCCGTGAGGTGGAGCGAATCTCAAAAA
NR026340 Noc   ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAA
L19            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
L128           ----------------------------------------------------------------------------------------------------
R33            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATAC-------------------------
R73            ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGC-TGCGATACCGCGAGGTGGAGCGAATCTCAAAAA
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Appendix 4 – GenBank Submissions

1. *Streptomyces bikiniensis* strain M8 16S ribosomal RNA gene, partial sequence. GenBank: **JX657681.1**
2. *Streptomyces thermolineatus* strain M58 16S ribosomal RNA gene, partial sequence. GenBank: **KC551929.1**
3. *Streptomyces bikiniensis* strain M6 16S ribosomal RNA gene, partial sequence. GenBank: **KC570315.1**
4. *Streptomyces rubrolavendulae* strain M16 16S ribosomal RNA gene, partial sequence. GenBank: **KC570317.1**
5. *Streptomyces radiopugnans* strain M59 16S ribosomal RNA gene, partial sequence. GenBank: **KC570318.1**
6. *Streptomyces radiopugnans* strain M13 16S ribosomal RNA gene, partial sequence. GenBank: **KC570319.1**
7. *Streptomyces acrimycini* strain M14 16S ribosomal RNA gene, partial sequence. GenBank*:* **KC570320.1**
8. *Streptomyces radiopugnans* strain M32 16S ribosomal RNA gene, partial sequence. GenBank*:* **KC570321.1**
9. *Streptomyces chungwhensis* strain M41 16S ribosomal RNA gene, partial sequence. GenBank: **KC570322.1**
10. *Streptomyces radiopugnans* strain M27 16S ribosomal RNA gene, partial sequence. GenBank: **KC570323.1**
11. *Streptomyces variabilis* strain M17 16S ribosomal RNA gene, partial sequence. GenBank: **KC570324.1**
12. *Streptomyces chungwhensis* strain M3 16S ribosomal RNA gene, partial sequence. GenBank: **KC570325.1**
13. *Nocardiopsis prasina* strain S14 16S ribosomal RNA gene, partial sequence. GenBank: **KF713523.1**
14. *Streptomyces chungwhensis* strain D2 16S ribosomal RNA gene, partial sequence. GenBank: **KF713524.1**
15. *Streptomyces tendae* strain B5 16S ribosomal RNA gene, partial sequence. GenBank: **KF713525.1**
16. *Nocardiopsis alkaliphila* strain L130 16S ribosomal RNA gene, partial sequence. GenBank: **KF713526.1**
17. *Streptomyces variabilis* strain R94 16S ribosomal RNA gene, partial sequence. GenBank: **KF713527.1**
18. *Streptomyces variabilis* strain R98 16S ribosomal RNA gene, partial sequence. GenBank: **KF713528.1**

19. *Streptomyces flavomacrosporus* strain B2 16S ribosomal RNA gene, partial sequence. GenBank: **KF713529.1**
20. *Streptomyces violorubens* strain R59 16S ribosomal RNA gene, partial sequence. GenBank: **KF713530.1**
21. *Nocardiopsis alba* strain B4 16S ribosomal RNA gene, partial sequence. GenBank: **KF713531.1**
22. *Streptomyces rubrolavendulae* strain M56 16S ribosomal RNA gene, partial sequence. GenBank: **KJ403746.1**
23. *Streptomyces griseoaurantiacus* strain: D3, 16S ribosomal RNA gene,partial sequence GenBank: **KJ158466.1**
24. *Streptomyces rubrogriseus* strain: B1, 16S ribosomal RNA gene partial sequence. GenBank: **KJ158468.1**
25. *Streptomyces sampsonii* strain: R76, 16S ribosomal RNA gene partial sequence. GenBank: **KJ158470.1**
26. *Streptomyces exfoliatus* strain: R73,16S ribosomal RNA gene, partial sequence. GenBank: **KJ158473.1**
27. *Streptomyces albidoflavus* strain: S18, 16S ribosomal RNA gene partial sequence. GenBank: **KJ158474.1**
28. *Nocardiopsis listeri* strain: N189,16S ribosomal RNA gene, partial sequence. GenBank: **KJ158482.1**
29. *Streptomyces luteogriseus* strain: L128, 16S ribosomal RNA gene, partial sequence. GenBank: **KJ158476.1**
30. *Streptomyces champavatii* strain: R33,16S ribosomal RNA gene, partial sequence. GenBank: **KJ158471.1**
31. *Streptomyces minutiscleroticus* strain: R62, 16S ribosomal RNA gene, partial sequence. GenBank: **KJ158480.1**

Appendix 5– List of Publications

1. **Deepthi Augustine**, Jimly C Jacob, Ramya K D, Rosamma Philip. **2013**. Actinobacteria from sediment samples of Arabian Sea and Bay of Bengal: Biochemical and physiological characterization. **International Journal of Research in Marine Sciences** 2(2): 56-63
2. Anil Kumar.V, **Deepthi Augustine,** Asmita Mehta, Kavitha R Dinesh, Darsana Viswam, Rosamma Philip. **2012**. *Leuconostoc garlicum*: An unusual pathogen in the era of vancomycin therapy. **The Indian journal of chest diseases and allied sciences** 54, 127-130
3. Anil Kumar.V, **Deepthi Augustine,** Sudhindran S, Anu M Kurian, Kavitha R Dinesh, Shamsul Karim, Rosamma Philip. **2011**. *Weissella confusa :a rare cause of vancomycin resistant Gram-positive bacteraemia*. **Journal of Medical Microbiology** 60**,** 1539-1541
4. Anil Kumar.V, **Deepthi Augustine**, Dilip Paniker, Aswathy Nandakumar, Kavitha R Dinesh, Shamsul karim, Rosamma Philip. **2011**. *Brevibacterium casei* as a cause of brain abscess in an immunocompetent patient. **Journal of Clinical Microbiology** 49, 4374-4376

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