

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

*Thesis submitted to*

**Cochin University of Science and Technology**

*in partial fulfillment of the requirements for the degree of*

**DOCTOR OF PHILOSOPHY**

**in**

**MICROBIOLOGY**

**UNDER THE FACULTY OF MARINE SCIENCES**

**By**

**DEEPTHI AUGUSTINE**



DEPARTMENT OF MARINE BIOLOGY, MICROBIOLOGY AND BIOCHEMISTRY  
SCHOOL OF MARINE SCIENCES  
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY  
KOCHI- 682016, INDIA.

**MAY 2014**



**To the Lord Almighty...**



**Dr. Rosamma Philip**  
Associate Professor

Department of Marine Biology, Microbiology and Biochemistry  
Cochin University of Science and Technology  
Finearts Avenue, Kochi-16

## Certificate

*This is to certify that the thesis entitled ~~Actinob~~mycete 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~~ ~~of~~ Philosophy in Microbiology of Cochin University of Science and Technology, and no part ~~there~~ has been presented for the award of any other degree, diploma or associateship in any university.*

*Dr. Rosamma Philip*

*Kochi- 1 6*

*May 2014*



## Declaration

*I hereby declare that the thesis entitled Actinomyceete 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 provision and guidance of 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.*

*Deepthi Augustine*

*Kochi-16*

*May 2014*





**Dr. Rosamma Philip**  
Associate Professor

Department of Marine Biology, Microbiology and Biochemistry  
Cochin University of Science and Technology  
Finearts Avenue, Kochi-16

## Certificate

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

*Dr. Rosamma Philip*

*Kochi 16*

*May 2014*



## Acknowledgements

---

*It is a privilege to acknowledge one and all who have been instrumental in the successful completion of this thesis. I was amazingly fortunate to have the most vibrant and enthusiastic guide Dr. Rosamma Philip. At this juncture, I wish to express my deep sense of appreciation, gratitude and love to you mam, for your valuable guidance and undaunting support throughout my research tenure. Your true interest and vigilance in research coupled with pleasant nature, openness and accessibility was truly motivational and helped me to pursue my research even in the midst of adversaries.*

*I thankfully acknowledge Dr. A. A. Mohamed Hatha, Head, Department of Marine Biology, Microbiology and Biochemistry for providing all help and support for the smooth conduct of my research. My sincere thanks to Dr. C. A. Babu, Director, School of Marine Sciences for facilitating my research under the Department.*

*I gratefully appreciate all the teachers in the Department of Marine Biology, especially Dr. Babu Philip, Dr. A.V. Saramma, Dr. Aneykutty Joseph and Dr. S. Bijoy Nandan for their sociable nature, precious advice, and support.*

*I gratefully acknowledge the librarian and other staff members of School of Marine Sciences library for their valuable cooperation. I would like to thank all the office and technical staff of the department for their timely help and assistance rendered.*

*I wish to place on record my sincere thanks and whole hearted gratitude to Dr. I.S. Bright Singh, Co-ordinator, National Centre for Aquatic Animal*

*Health, for his encouragement, help and facilities provided throughout my work period.*

*I acknowledge with thanks the timely help and statistical assistance received from Dr. Valsamma Joseph, NCAAAH faculty, and Dr.Venu, for his valuable suggestions.*

*I specially acknowledge Dr. Annies Joseph, who paved the way for my research career. Mere words of thanks are inadequate for Dr. Swapna P. Antony, for sparing her valuable time, sharing the basics of molecular biology, her truly scientific intuition has inspired and enriched me as a researcher.*

*I am extremely thankful to Dr. Manjusha K., Dr. Sreedevi N. Kutty, for their companionship as well as insightful comments and constructive criticisms. I deeply appreciate the extreme sincerity and valuable suggestions by Dr. Simi Joseph P, while proof reading my thesis.*

*I express my sincere gratitude to Dr. Selvan, Dr. Priyaja and Dr. Vrinda for the timely assistance received during the final stages of my work.*

*I deeply appreciate Dr. Jayesh Puthumana for his whole hearted assistance in cell line work and his valuable suggestions. I thankfully acknowledge the technical expertise, timely help and suggestions received from Mr. Anil Kumar P.R. and Mr. Naveen Satyan. My whole hearted thanks to Ms.Chaithanya E.R, Ms. Jini Jacob and Mrs. Aishwarya Nair for their timely help and support.*

*My sincere thanks to Mrs. Aneesa and Mrs. Susan for the support and assistance received during the experiments in biochemistry.*

*I am much obliged to Ms. Divya T. Babu for her whole hearted assistance on all occasions. Mere words of thanks are inadequate for Ms. Ramya K.D. who has always rendered a helping hand whenever I needed. I gratefully acknowledge Ms. Jimly C. Jacob and Ms. Ramya for proof reading my thesis meticulously.*

*I am much indebted to Dr. Reema Kuriakose for her sisterly affection, encouragement and moreover her spiritual advice, helped me overcome the hurdles during this exhausting journey of research.*

*I cherish the friendship of fellow colleagues Mrs. Nifty John, Mrs. Divya P.S, for their timely help and moral support. I deeply appreciate the invaluable assistance received from Mr. Anit M. Thomas and Mr. Harisanker H.S. during the final stages of my work. My immense thanks to Mrs. Emilda Rosmine and Ms. Deborah Alexander for the assistance received.*

*I would like to specially mention a cheerful group of juniors, Ms. Manomi S, Ms. Bhavya Kachiprath, Mr. Jayanath G, Mr.Solly Solomon, Mrs. Neema Job and Mrs. Wilsy Wilson for their constant help, support and encouragement during various stages.*

*I would like to thank my colleagues from my early days of research tenure, Dr.Lakshmi Nair, Dr.Smitha S.L., Dr. Neil Scholastic Correya, Dr. Sini P.J., Mrs. Jisha V.K., Mrs. Jisha Jose, for their help and advice.*

*My special thanks to all the research fellows of the Dept. of Marine Biology, especially, Dr. Mujeeb Rahman, Mr. Afsal V.V, Mr. Jabir T, Mr. Nashad M, Ms. Sruthy K.S, Mrs. Neethu, Mrs. Ally, Mrs. Jesmi Youssaf, Mrs. Philomina Joseph and Ms Reshma S. for all the help rendered.*

*I acknowledge with thanks the support and encouragement received from my friends Mrs. Julie Varghese, Mrs. Deepa Elizabeth, and Dr.Rekha Kiron.*

*I take this opportunity to acknowledge Cochin University of Science and Technology and the Department of Science and Technology for the fellowship granted during the tenure of my research.*

*This thesis would not have been completed without the collaboration of people from various institutions. I sincerely acknowledge the assistance received from Dr. Douglas Lawman for NMR analysis, Mr. Adarsh (STIC) and Mr. Vinay Vikas, (NCBS), for the completion of research. The assistance received from Mr. Mathew Kuriakose to buy BIOLOG plates is gratefully acknowledged.*

*I feel the affection and concern of my parents, which has been a motivating factor in pursuing my research career. Their abundant prayers and blessings showered on me has led to the successful completion of my thesis. The love and moral support of my family members and in-laws have helped me in coping with the hardships during the tenure of this work. I take this opportunity to express my gratitude and a million thanks to them.*

*Finally, I must accolade my husband, Mr. Jerry George, whose patience, adjustment, encouragement and support helped me to fulfill this much yearned endeavour. I dedicate this work to the boundless affection and sacrifice of my precious daughters Michelle and Maria who have done remarkably well in my absence.*

*I surrender myself to the Lord Almighty for granting me wisdom, health and strength to undertake this research task and enabling me to its completion.*

*Deepthi Augustine*

# Table of Contents

---

|          |   |           |
|----------|---|-----------|
| <b>1</b> | <b>General Introduction .....</b>   | <b>1</b>  |
| 1.1      | Introduction .....  | 1         |
| 1.2      | Distribution of marine actinobacteria .....   | 3         |
| 1.3      | Diversity of actinomycetes in the marine environment .....  | 4         |
| 1.4      | Marine actinobacteria as a source of secondary metabolites .....  | 7         |
| 1.5      | Systematics in biodiscovery .....   | 10        |
| 1.6      | Relevance of the Present Study.....   | 11        |
| 1.7      | Objectives.....   | 12        |
| 1.8      | Outline of the Thesis .....   | 12        |
| <b>2</b> | <b>Morphological, Biochemical and Physiological Characterization of Marine Actinomycete Isolates.....</b> | <b>13</b> |
| 2.1      | Introduction .....  | 13        |
| 2.1.1    | The Polyphasic taxonomy.....  | 14        |
| 2.1.2    | Taxonomy of Actinomycetes.....  | 15        |
| 2.1.3    | Actinomycete genera in marine sediments.....  | 18        |
| 2.1.4    | Dominant genera - <i>Streptomyces</i> .....   | 20        |
| 2.1.5    | Nocardiopsis.....   | 20        |
| 2.1.6    | Biogranulation property .....   | 21        |
| 2.1.7    | Antibacterial activity of marine actinomycetes .....  | 22        |
| 2.1.8    | Hydrolytic Enzymes.....   | 23        |
| 2.2      | Materials and methods.....  | 28        |
| 2.2.1    | Microorganisms used for the study .....   | 28        |
| 2.2.2    | Purification of Isolates .....  | 28        |
| 2.2.3    | Morphological and Cultural Characterization.....  | 28        |
| 2.2.4    | Biochemical and Physiological characterization .....  | 30        |
| 2.2.5    | Screening of marine actinomycetes for biogranulation property .....                                       | 34        |
| 2.2.6    | Screening of marine actinomycetes for antibacterial activity .....  | 35        |
| 2.2.7    | Screening for hydrolytic enzyme production .....  | 36        |
| 2.3      | Results .....   | 39        |
| 2.3.1    | Morphological and Cultural characterization.....  | 39        |
| 2.3.2    | Generic composition .....   | 42        |
| 2.3.3    | Biochemical and physiological characterization.....   | 43        |
| 2.3.4    | Screening of marine actinomycetes for biogranulation property .....                                       | 46        |
| 2.3.5    | Screening of marine actinomycetes for antibacterial activity .....  | 47        |
| 2.3.6    | Screening for hydrolytic enzyme potential .....   | 48        |
| 2.4      | Discussion .....  | 49        |
| <b>3</b> | <b>Molecular Identification and Phylogenetic Analysis of Marine Actinomycete Isolates .....</b>           | <b>59</b> |

---

|            |   |            |
|------------|---|------------|
| <b>3.1</b> | <b>Introduction .....</b>   | <b>59</b>  |
| 3.1.1      | Molecular detection methods.....  | 61         |
| 3.1.2      | Nucleic acid sequence comparisons .....   | 65         |
| 3.1.3      | Phylogenetic studies.....   | 69         |
| <b>3.2</b> | <b>Materials and methods.....</b>   | <b>72</b>  |
| 3.2.1      | Selection of actinomycete isolates.....   | 72         |
| 3.2.2      | Genomic DNA extraction from actinomycete isolates .....   | 72         |
| 3.2.3      | Amplification of 16S rRNA gene.....   | 73         |
| 3.2.4      | Amplified ribosomal DNA restriction analysis (ARDRA) .....  | 74         |
| 3.2.5      | Gene sequence analysis and species identification .....   | 75         |
| 3.2.6      | Nucleotide Sequence Accession Numbers .....   | 75         |
| <b>3.3</b> | <b>Results .....</b>  | <b>75</b>  |
| 3.3.1      | Amplification of 16S rRNA gene.....   | 75         |
| 3.3.2      | ARDRA analysis .....  | 76         |
| 3.3.3      | Gene sequence analysis and species identification .....   | 78         |
| 3.3.4      | Species Composition of Actinomycetes in Arabian Sea and Bay of Bengal .....                               | 98         |
| <b>3.4</b> | <b>Discussion .....</b>   | <b>102</b> |
| <b>4</b>   | <b>Antimicrobial Property of Marine Actinomycetes with Special Reference to Antivibrio Activity .....</b> | <b>113</b> |
| <b>4.1</b> | <b>Introduction .....</b>   | <b>113</b> |
| 4.1.1      | Vibriosis in aquaculture .....  | 114        |
| 4.1.2      | Antivibrio activity of marine actinomycetes.....  | 117        |
| 4.1.3      | Application of Aerobic Granulation Technology .....   | 118        |
| <b>4.2</b> | <b>Materials and Methods .....</b>  | <b>120</b> |
| 4.2.1      | Selection of marine actinomycetes for exclusion experiments .....   | 120        |
| 4.2.2      | Characterization of potent antibacterial strain <i>Streptomyces</i> sp. M56.....                          | 120        |
| 4.2.3      | Phylogenetic analysis .....   | 120        |
| 4.2.4      | Carbon utilization profile.....   | 121        |
| 4.2.5      | Application potential of biogranule in aquaculture .....  | 121        |
| 4.2.6      | Statistical analysis .....  | 123        |
| <b>4.3</b> | <b>Results .....</b>  | <b>124</b> |
| 4.3.1      | Characterization of the potent antibacterial strain M56.....  | 124        |
| 4.3.2      | <i>In vitro</i> exclusion of <i>Vibrio</i> spp. by actinomycete biogranule .....                          | 131        |
| <b>4.4</b> | <b>Discussion .....</b>   | <b>133</b> |
| <b>5</b>   | <b>Characterization and Evaluation of Bioactivity of Melanin from Marine Actinomycetes .....</b>          | <b>137</b> |
| <b>5.1</b> | <b>Introduction .....</b>   | <b>137</b> |
| 5.1.1      | Melanins in Actinomycetes .....   | 139        |
| 5.1.2      | Enzymes of microbial melanogenesis .....  | 141        |
| 5.1.3      | Types of melanin.....   | 142        |
| 5.1.4      | Chemical characterization.....  | 142        |
| 5.1.5      | Functions of melanin.....   | 143        |
| 5.1.6      | Microbial melanin as antioxidants.....  | 145        |



---

|            |  |            |
|------------|--|------------|
| <b>5.2</b> | <b>Materials and Methods .....</b>   | <b>147</b> |
| 5.2.1      | Selection of melanogenic actinomycete .....  | 147        |
| 5.2.2      | Melanin Formation test .....   | 148        |
| 5.2.3      | Characterization of Melanogenic strain (M8) .....  | 149        |
| 5.2.4      | Carbon utilization profile of melanogenic strain M8.....   | 151        |
| 5.2.5      | Extraction of melanin.....   | 151        |
| 5.2.6      | Physicochemical characterization of extracted melanin .....  | 151        |
| 5.2.7      | Assay for antioxidant activity.....  | 154        |
| 5.2.8      | Biocompatibility and cytotoxicity assays of melanin in human HEp-2 cancer cells .....                      | 157        |
| 5.2.9      | Phototoxicity assay of M8 melanin .....  | 160        |
| 5.2.10     | Statistical analysis .....   | 161        |
| <b>5.3</b> | <b>Results .....</b>   | <b>161</b> |
| 5.3.1      | Effect of carbon source on melanin production .....  | 161        |
| 5.3.2      | Effect of nitrogen source on melanin production.....   | 162        |
| 5.3.3      | Characterization of melanogenic culture (M8) .....   | 162        |
| 5.3.4      | Extraction of melanin.....   | 168        |
| 5.3.5      | Physicochemical characterization of extracted melanin .....  | 168        |
| 5.3.6      | Antioxidant assays .....   | 174        |
| 5.3.7      | Biocompatibility and cytotoxicity of melanin in human HEp-2 cancer cells .....                             | 178        |
| 5.3.8      | Phototoxicity assay of melanin (M8) .....  | 182        |
| <b>5.4</b> | <b>Discussion .....</b>  | <b>184</b> |
| <b>6</b>   | <b>Chitinolytic Streptomycetes as Biocontrol Agents .....</b>  | <b>195</b> |
| <b>6.1</b> | <b>Introduction .....</b>  | <b>195</b> |
| 6.1.1      | Antifungal agents .....  | 196        |
| 6.1.2      | Chitinolytic actinomycetes as biocontrol agents.....   | 199        |
| <b>6.2</b> | <b>Materials and methods.....</b>  | <b>201</b> |
| 6.2.1      | Chitinolytic marine actinomycetes as antifungal and insecticidal agents .....                              | 201        |
| 6.2.2      | Characterization of actinomycete isolates exhibiting fungicidal (J18) and insecticidal (M32) activity..... | 206        |
| 6.2.3      | Statistical analysis .....   | 207        |
| <b>6.3</b> | <b>Results .....</b>   | <b>207</b> |
| 6.3.1      | <i>In vitro</i> antagonism against pathogenic fungi .....  | 207        |
| 6.3.2      | Chitinolytic marine actinomycetes ( <i>Streptomyces</i> sp.) as biocontrol agents of insect pests .....    | 209        |
| 6.3.3      | Characterization of actinomycete isolates with potent fungicidal (J18) and insecticidal activity(M32)..... | 214        |
| <b>6.4</b> | <b>Discussion .....</b>  | <b>220</b> |
| <b>7</b>   | <b>Summary and Conclusion .....</b>  | <b>227</b> |
|            | <b>Bibliography .....</b>  | <b>235</b> |
|            | <b>Appendices.....</b>   | <b>273</b> |

---



# 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 Nocardiosporeaceae 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 *Streptomyces*. 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 *Streptomyces*. 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 *Actinomycetales*. 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 (ivermectin), 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 *Nocardiosis* have been reported from marine sediment samples of Indian coast. i.e., a protease-producing, crude oil degrading marine *Nocardiosis* sp. NCIM 5124 (Dixit and Pant, 2000) and bio surfactant producing marine *Nocardiosis* sp. (Khopade et al., 2012; Selvin et al., 2009) which is a clear proof of existence of potential *Nocardiosis* sp. along Indian coast. A sponge associated actinomycete, *Nocardiosis dasonvillei* 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 (Trincon, 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-Streptomyces* 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 *Nocardiodes* (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 Euzéby (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 Nocardiosis**

The genus *Nocardiosis* 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* was 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 *Nocardiosis*, 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  $\alpha$ -amylase from marine *Streptomyces* sp. D1 has also been reported with  $\alpha$  1–4,  $\alpha$ -1–6 activity, wide range of pH stability, and potential widespread application in the detergent industry (Chakraborty et al., 2009). Production of  $\alpha$ -amylase by *Nocardioopsis* sp., an endophytic actinobacterium was studied from yam bean (Stamford et al., 2001). An actinobacterium strain AE-19 producing  $\alpha$  -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  $\alpha$ -amylase (against surfactants, oxidants and detergents) (Chakraborty et al., 2011). Detergent stable and calcium ion independent  $\alpha$  -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 °C, 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, hemicellulases 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. (Tsujiho 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 reisolations 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<sub>2</sub>HPO<sub>4</sub> 0.7 g; KH<sub>2</sub>PO<sub>4</sub> 0.3 g; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g; FeSO<sub>4</sub>.7H<sub>2</sub>O

0.01 g; ZnSO<sub>4</sub> 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<sub>2</sub>HPO<sub>4</sub> 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<sub>2</sub>HPO<sub>4</sub> 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;  $\text{KH}_2\text{PO}_4$  9.1 g;  $\text{Na}_2\text{HPO}_4$  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 ( $\text{NO}_3$ ) to nitrite ( $\text{NO}_2$ ) 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 ( $\alpha$ -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;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.02 g; NaCl 15.0 g;  $(\text{NH}_4)_2\text{HPO}_4$  1.0 g;  $\text{K}_2\text{HPO}_4$  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;  $\text{K}_2\text{HPO}_4$  0.5 g;

MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g; FeSO<sub>4</sub>.7H<sub>2</sub>O 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 black 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<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 1 g; KCl 0.02 g; MgSO<sub>4</sub> .7H<sub>2</sub>O 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  $\mu$ l) 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  $\pm$  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<sub>2</sub>.2H<sub>2</sub>O 0.02g; NaCl 2g; FeCl<sub>3</sub>.6H<sub>2</sub>O 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 ( $28 \pm 2^\circ\text{C}$ ) 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;  $\text{NaNO}_3$  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 ( $28 \pm 2^\circ\text{C}$ ) 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  $28 \pm 2^\circ\text{C}$  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 phenolphthalein disulfate (PDS) was used for the production of aryl sulfatase. The plates were spot inoculated and incubated at room temperature ( $28 \pm 2^\circ\text{C}$ ) 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% phenolphthalein diphosphate. Incubated at room temperature ( $28 \pm 2^\circ\text{C}$ ) 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;  $\text{Na}_2\text{HPO}_4$  0.45 g;  $\text{KH}_2\text{PO}_4$  0.1 g;  $\text{MgSO}_4$  0.002 g;  $\text{CaCl}_2$  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 ( $28 \pm 2^\circ\text{C}$ ) 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;  $\text{K}_2\text{HPO}_4$  0.07g;  $\text{KH}_2\text{PO}_4$  0.03g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.05g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.001g;  $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$  0.0001g;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.0001g; agar 2g; seawater 100 ml pH 7.0). Incubation of the plates was done at  $28^\circ\text{C}$  for 7-14 days and the zone of clearance was recorded.



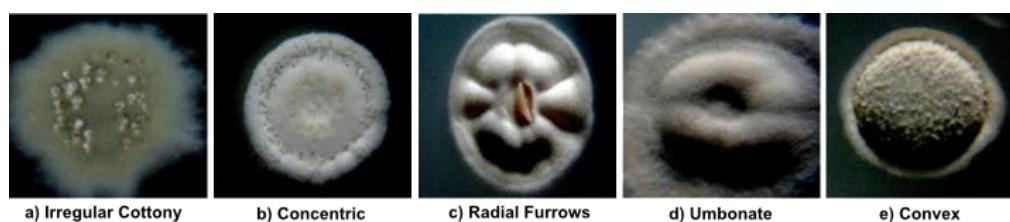
#### 2.2.7.8.1 Preparation of colloidal chitin

Purified chitin (15 g) was dissolved in 50% H<sub>2</sub>SO<sub>4</sub> (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.

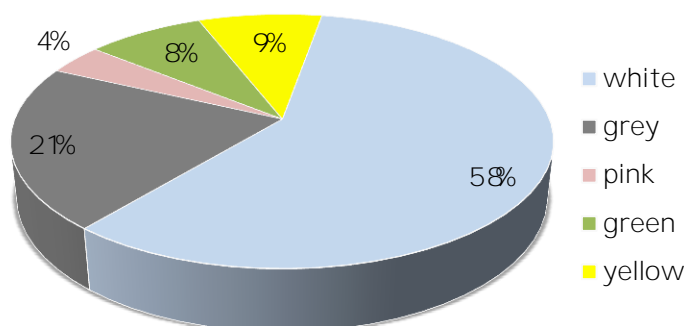
## 2.3 Results

### 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.

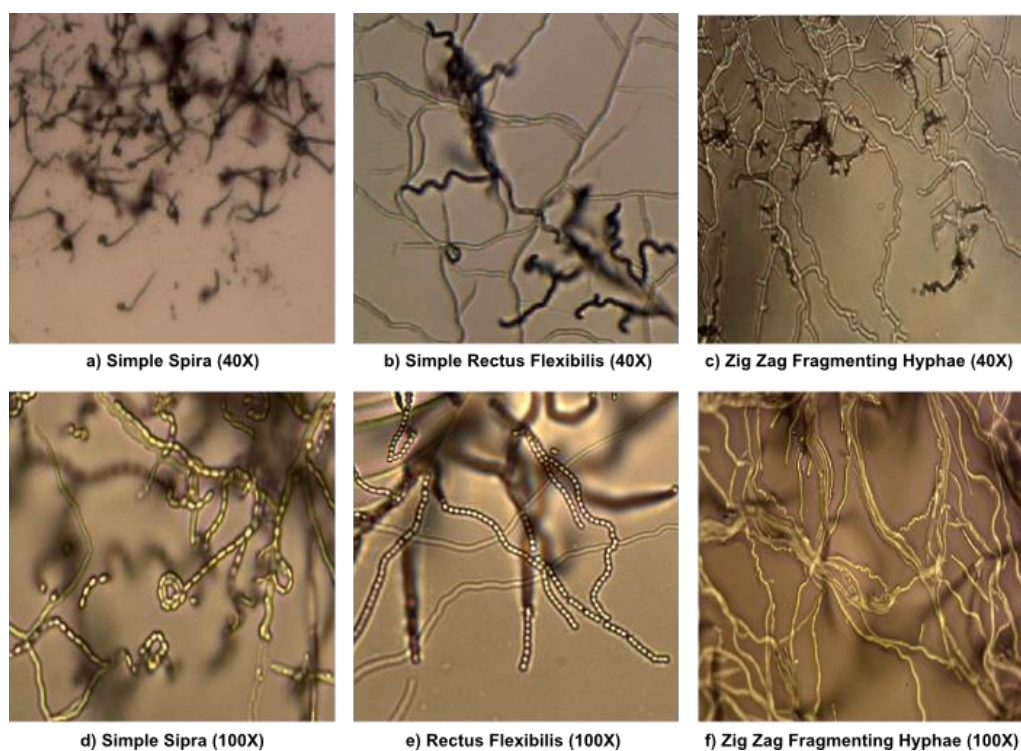


Fig. 2.3 Microscopic appearance of spore chain morphology of actinomycete isolates

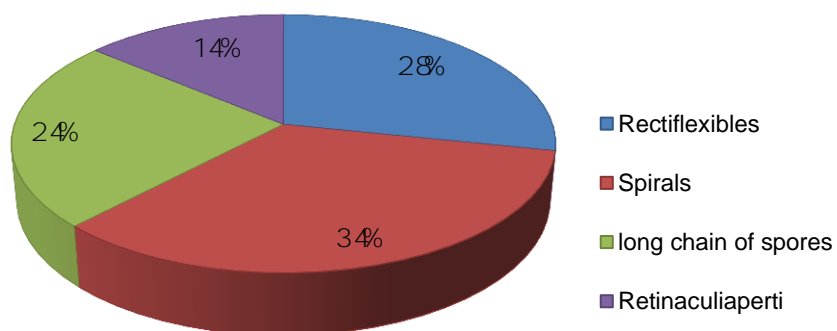
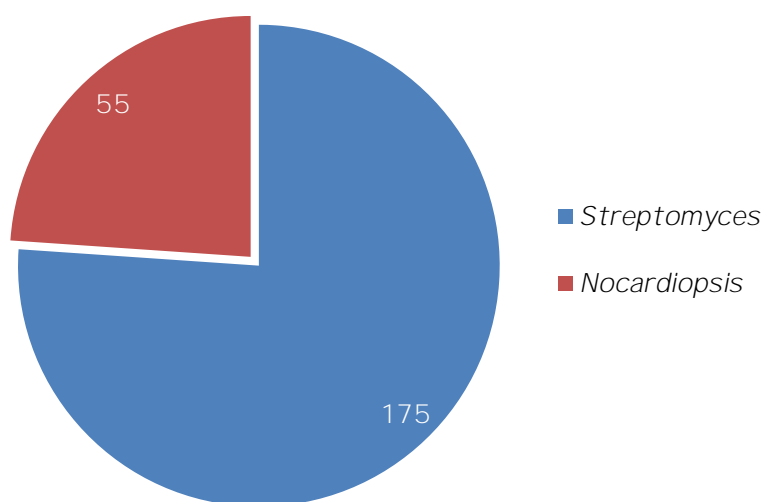


Fig. 2.4 Relative spore chain morphology of actinomycete isolates

### 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 *Nocardiosis* (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 *Nocardiosis* (Fig. 2.7).

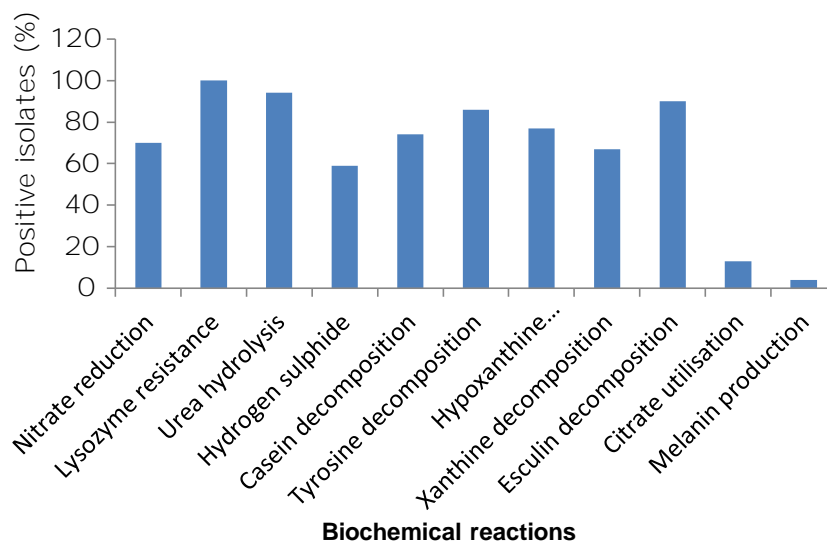


Fig. 2.6 Biochemical profile of actinomycete isolates

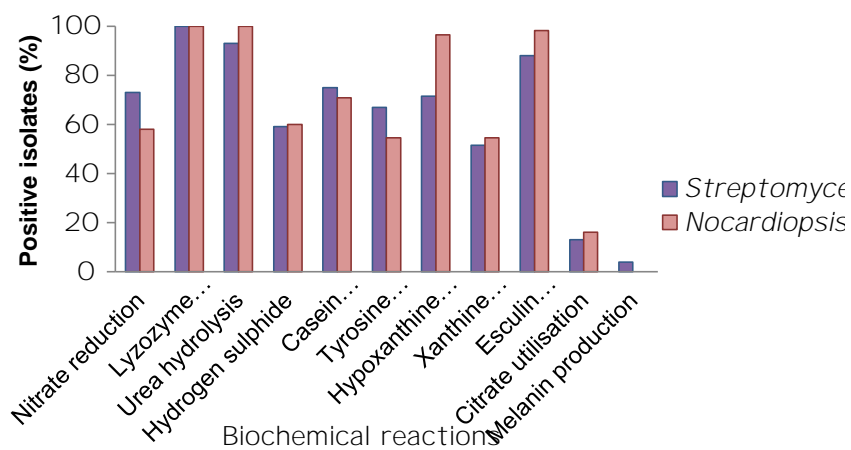


Fig. 2.7 Biochemical profile of *Streptomyces* and *Nocardiosis* isolates

### 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).

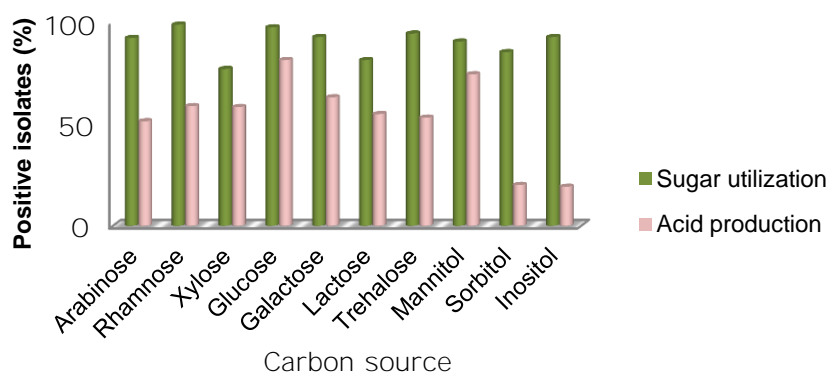


Fig. 2.8 Carbohydrate utilization profile of actinomycete isolates

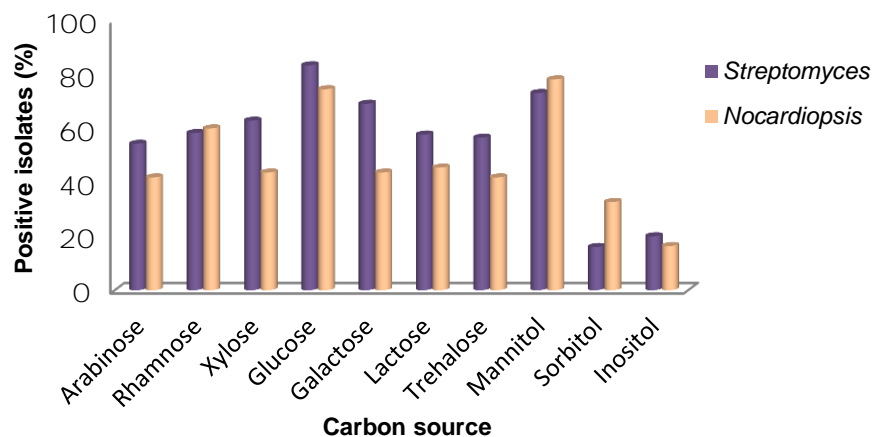


Fig. 2.9 Acid production ability of *Streptomyces* and *Nocardiosis* isolates

### 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).

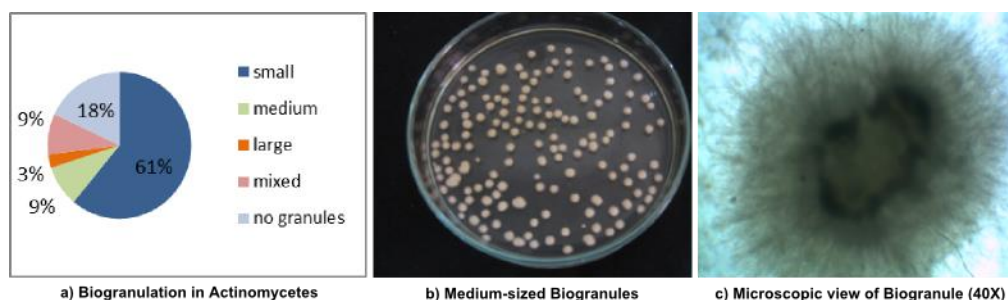


Fig. 2.10 Biogranulation in marine actinomycetes



### 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 *Pseudomonas 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.

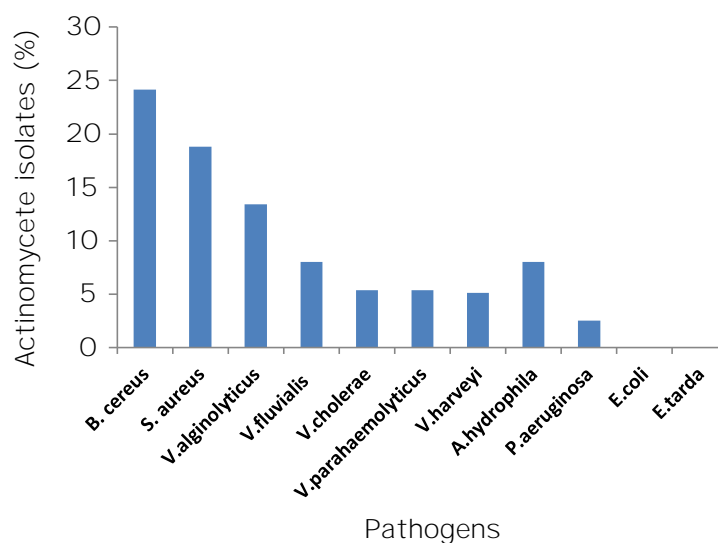
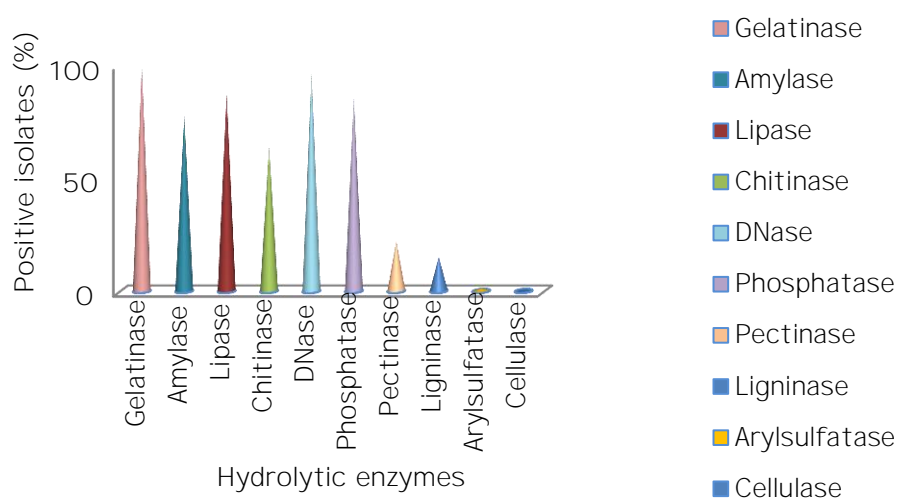


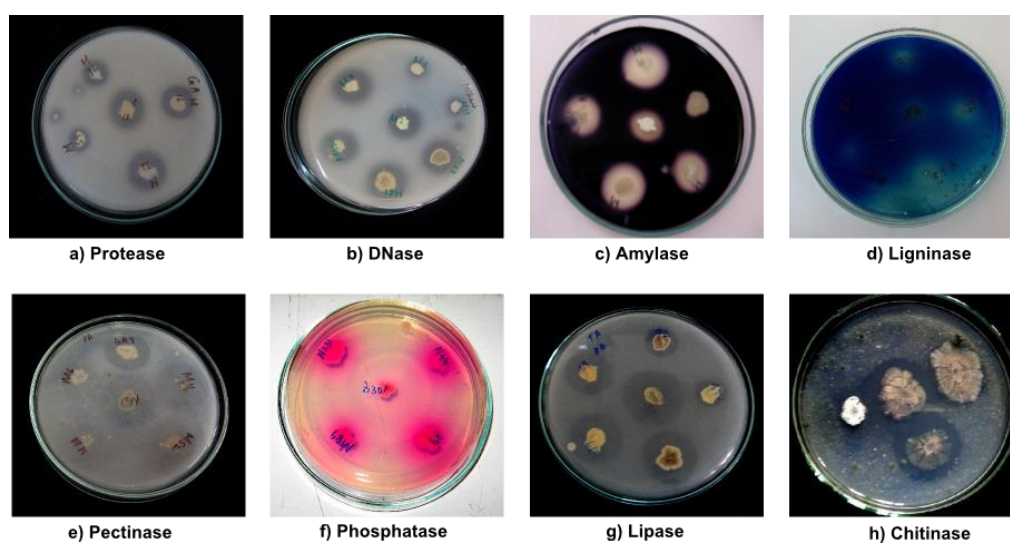
Fig. 2.11 Percentage of actinomycete isolates with antibacterial activity

### 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 rectiflexibles 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 *Nocardioopsis*. 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 its 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 Venezuelan 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. aureofaciens* (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  $\geq 70\%$  DNA-DNA relatedness and  $\leq 50^\circ\text{C}$  difference in the melting temperature ( $\Delta T_m$ ) 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 fingerprint 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 (*Bgl*II, *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 *MseI* 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, *Sau3AI*, *AsnI*, *KpnI* and *SphI* 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, *Sau3AI*, *AsnI*, *KpnI* and *SphI* was evaluated and found cost effective (Muharram et al., 2010).

### 3.1.2 Nucleic acid sequence comparisons

Zuckerkanndl 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 ( $\alpha$  and  $\beta$  regions) and species specific ( $\gamma$  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 ( $\alpha$ ,  $\beta$  and  $\gamma$ ) 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  $\gamma$  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 Micromonosporaceae, Nocardiaceae, Nocardiosporaceae, 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 *Micromonospora*, *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). Tauqueer 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 been 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.

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

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<sub>2</sub>, 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., *Sau3A1*, (G<sup>1</sup>ATC) *HinfI* (GANT<sup>1</sup>C) and *TaqI* (T<sup>1</sup>CGA) in separate reactions. The digestions were performed for 3-4 hours at an incubation temperature of 37°C for *Sau3A1* and *HinfI* and at 65°C for *TaqI* 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<sub>2</sub>, 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](http://www.ncbi.nlm.nih.gov)).

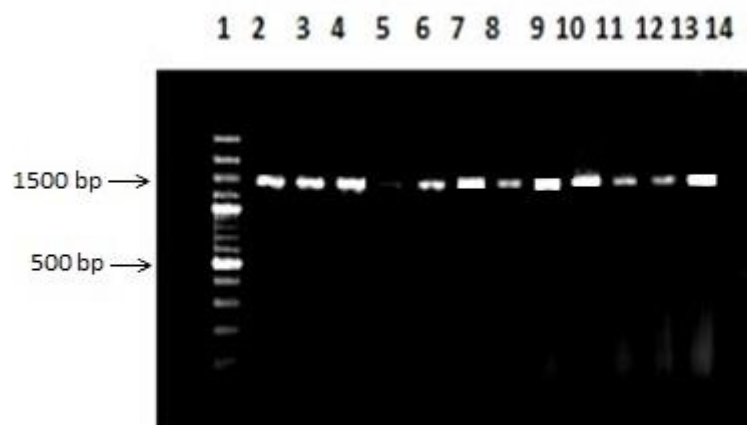
### **3.2.6 Nucleotide Sequence Accession Numbers**

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**

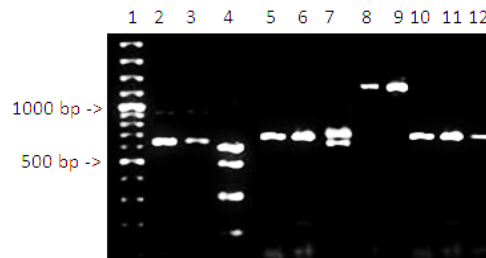
16S rRNA gene amplification using a set of universal eubacterial primers: 27F and 1492R yielded a single amplicon of approximately 1500 bp for all the marine actinomycete isolates (Fig. 3.1).



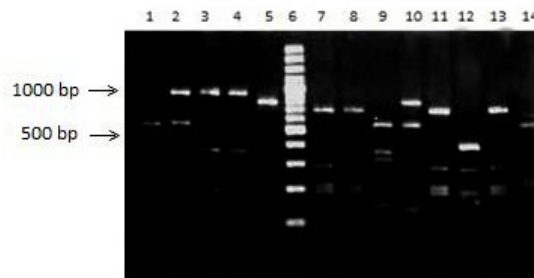
**Fig. 3.1** Amplified 16S ribosomal DNA of actinomycete isolates run on 1% agarose gel. Lane 1(100 bp ladder)

### 3.3.2 ARDRA analysis

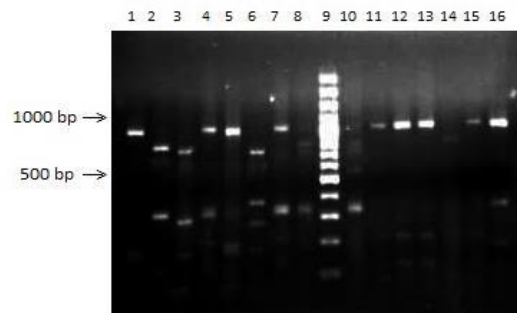
The 16S rDNA amplicons of actinomycete isolates subjected to ARDRA using *Sau3A1*, *Hinf1* and *Taq1* resulted in patterns. A total of eight polymorphic patterns were obtained for *Sau3A1* (Fig. 3.2), ten patterns for *Taq1* (Fig. 3.3) and only six patterns were generated by *Hinf1* digestions (Fig. 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 *Sau3A1* restriction on 16S rDNA amplicons of marine actinomycete isolates



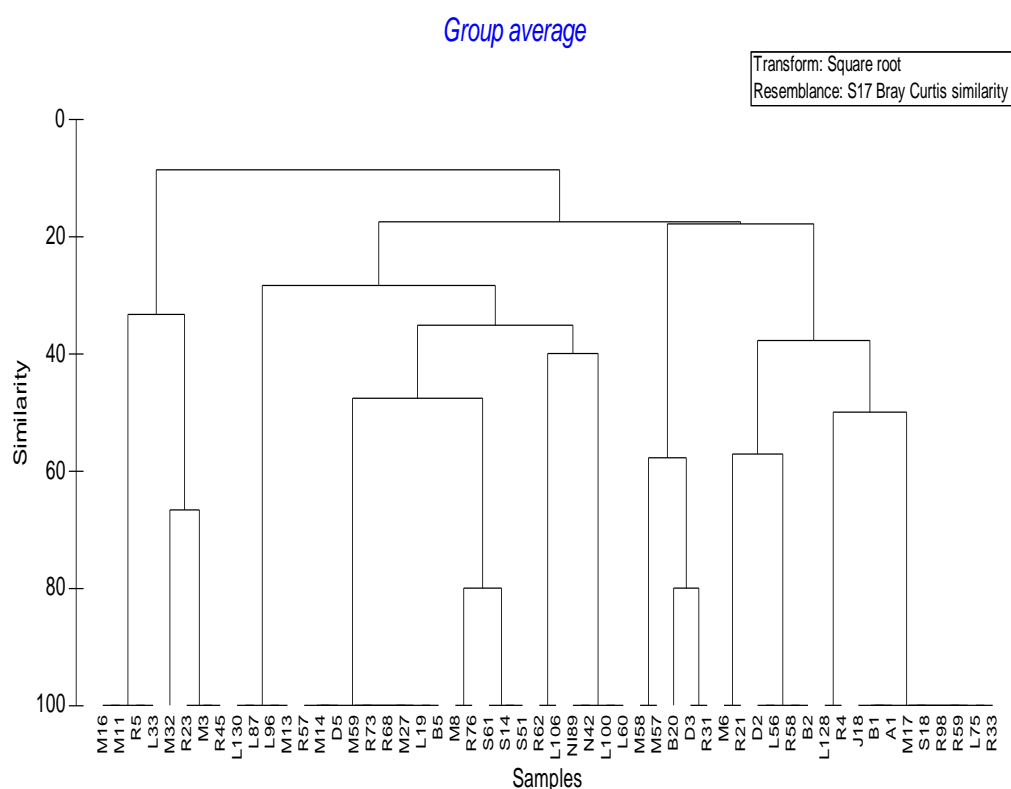
**Fig. 3.3** ARDRA patterns of *Taq1* restriction on 16S rDNA amplicons of marine actinomycete isolates (Lane 6 (100 bp ladder), 1-5 & 7-14 samples)



**Fig. 3.4** ARDRA patterns of *Hinf1* restriction on 16S rDNA amplicons of marine actinomycete isolates (Lane 9(100 bp ladder), 1-8 and 11-16 samples)

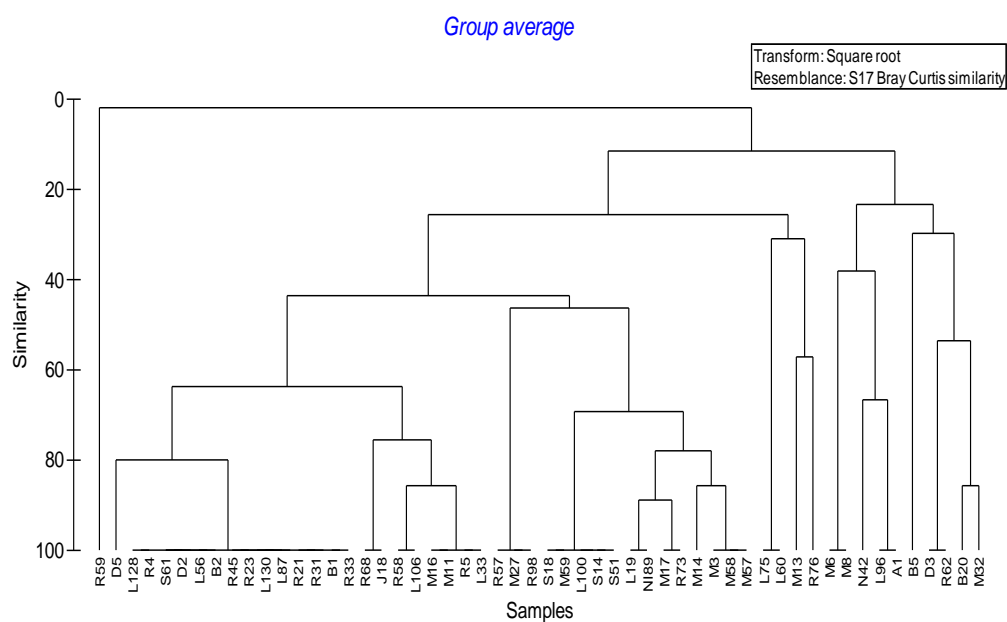
### 3.3.3 Gene sequence analysis and species identification

Dendrogram constructed based on ARDRA patterns obtained as a result of restriction digestions of 16S rDNA amplicons of marine actinomycete isolates using *Sau3A1*, (Fig. 3.5) *Hinf1* (Fig. 3.6) and *Taq1* (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.

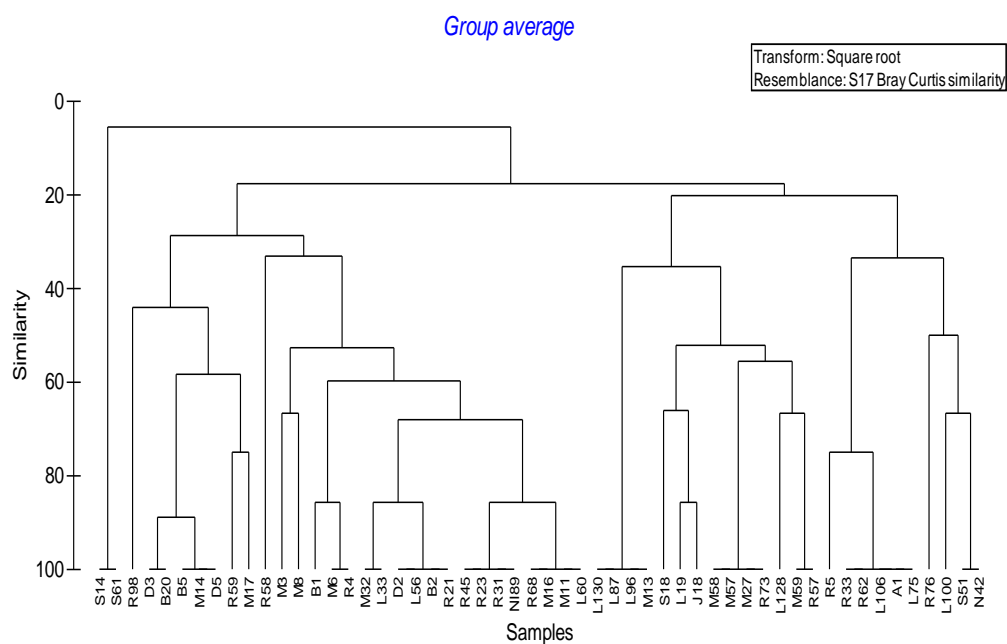


**Fig. 3.5 Dendrogram of marine actinomycete isolates constructed based on ARDRA patterns developed by *Sau3A1***





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



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

### 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 |
|-------------|-------------------------------|-------------------|------------------|---|-------------------|
|             | <i>Sau</i> 3A1(bp)            | <i>Hinf</i> 1(bp) | <i>Taq</i> 1(bp) |   |                   |
| M58         | 650+600+150                   | 800+325+175       | 550+350+325      | <i>S. thermolineatus</i>                          | <b>KC551929</b>   |
| M14         | 650+600+175                   | 850+325+225       | 400+350+325+150  | <i>S. acrimycini</i>                              | <b>KC570320</b>   |
| S14         | 1300+175                      | 800+325           | 900+325+200      | <i>Nocardiopsis alba</i> subsp.<br><i>prasina</i> | <b>KF713523</b>   |

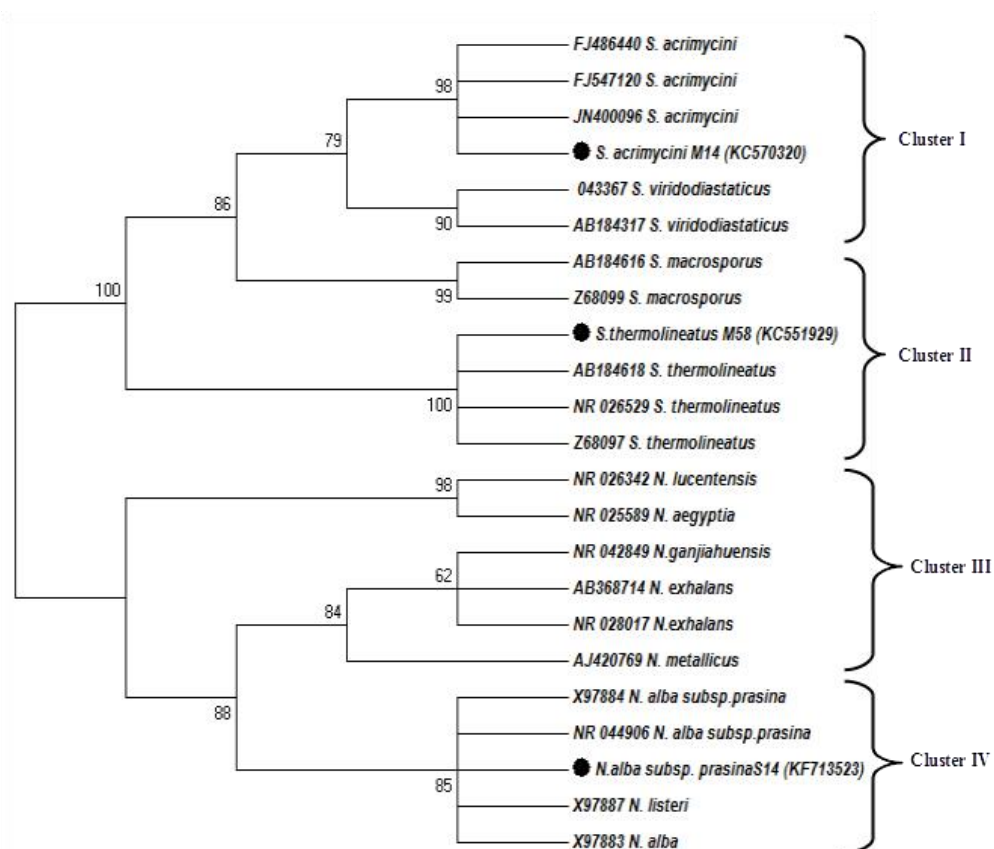
**Table 3.2 NCBI BLAST results of actinomycetes with green spore mass**

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



**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.thermolineatus*. 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

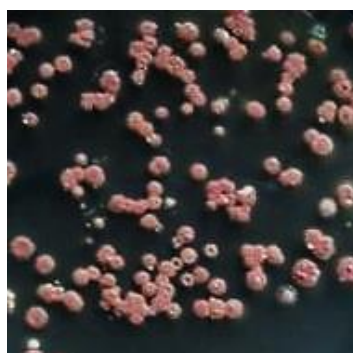
The ARDRA profile of representative actinomycetes with pink red spore mass (Fig. 3.10) and the species identity is given (Table 3.3). Isolate no. N42 and N189 on BLAST results revealed 99% identity to reference strain *Nocardiopsis listeri* (**NR026341**). 16S rDNA amplicon of M16 showed 100 % similarity 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**

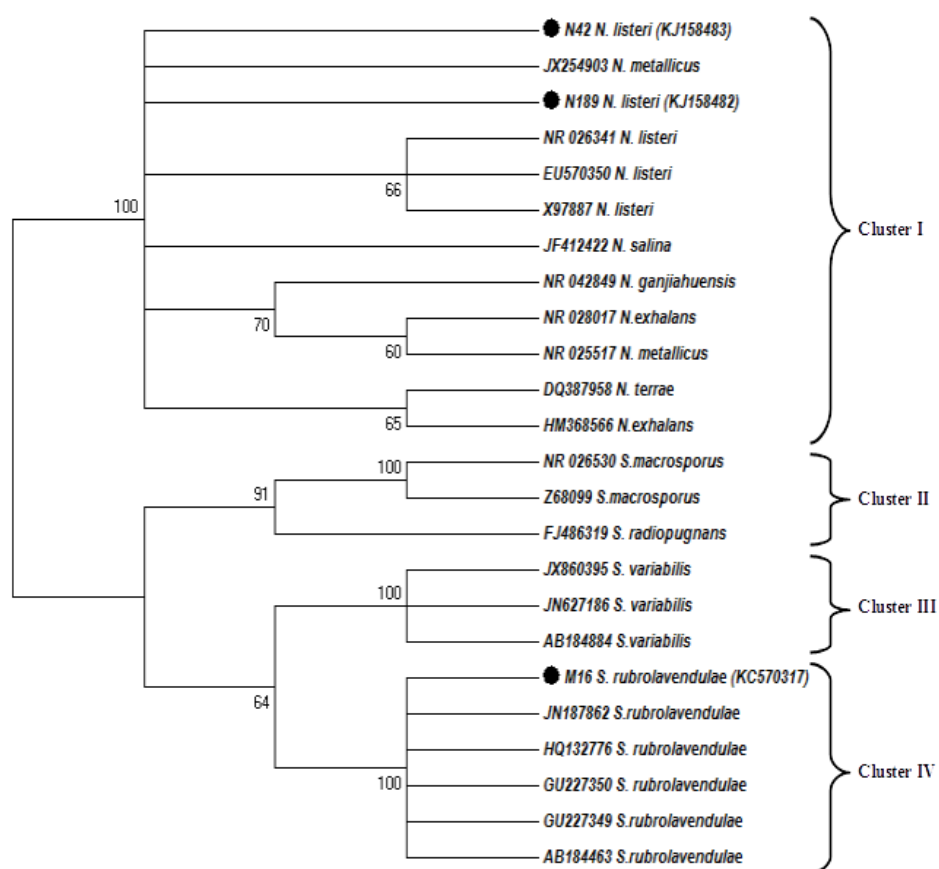
| Isolate no: | <i>In vitro</i> Restriction Fragments |                   |                   | Species identity         | GenBank Accession        |
|-------------|---------------------------------------|-------------------|-------------------|--------------------------|--------------------------|
|             | <i>Sau3A1</i> (bp)                    | <i>Hinf1</i> (bp) | <i>Taq1</i> ( bp) |                          |                          |
| M16         | 650+600+175                           | 800+300+200+175   | 600+350+200       | <i>S.rubrolavendulae</i> | <a href="#">KC570317</a> |
| N189        | 1300+175                              | 800+325+200+175   | 900+350+200       | <i>N. listeri</i>        | <a href="#">KJ158482</a> |
| N42         | 1300+175                              | 800+350+150       | 850+325+200       | <i>N. listeri</i>        | <a href="#">KJ158483</a> |

**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                | <i>Streptomyces rubrolavendulae</i> |
| M189        | 1308      | 1308        | 100%         | 0.0     | 99%            | NR026341                | <i>Nocardiopsis listeri</i>         |
| N42         | 1423      | 1423        | 100%         | 0.0     | 99%            | NR026341                | <i>Nocardiopsis listeri</i>         |



**Fig. 3.10 Actinomycete with pink spore mass**



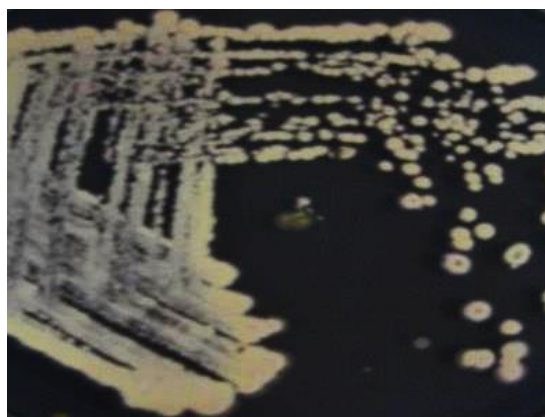
**Fig. 3.11** A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the pink spore mass actinomycetes with other actinomycetes. Values at the node indicate the percentage with which the particular node occurred in 1000 trees generated by 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 *Nocardiosis* sp. and the other three of *Streptomyces* species. Ribotypes N189 (GenBank: **KJ158482**) and N42 (GenBank: **KJ158483**) are with the *Nocardiosis 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: | <i>In vitro</i> Restriction Fragments |                   |                  | Species identity                | GenBank Accession        |
|-------------|---------------------------------------|-------------------|------------------|---------------------------------|--------------------------|
|             | <i>Sau</i> 3A1(bp)                    | <i>Hinf</i> 1(bp) | <i>Taq</i> 1(bp) |                                 |                          |
| D2          | 650+350+300                           | 1100+325          | 900+350+175      | <i>S.chungwhensis</i>           | <a href="#">KF713524</a> |
| M3          | 650+350+300                           | 800+325+175       | 900+350+175      | <i>S.chungwhensis</i>           | <a href="#">KC570325</a> |
| L130        | 1350+150                              | 800+225           | 850+300+150      | <i>Nocardiopsis alkaliphila</i> | <a href="#">KF713525</a> |

**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%       | <b>JX486779</b>         | <i>Streptomyces chungwhensis</i> |
| M3         | 1975      | 1975        | 100%        | 0.0     | 99%       | <b>JX486779</b>         | <i>Streptomyces chungwhensis</i> |
| L130       | 1303      | 1303        | 100%        | 0.0     | 99%       | <b>NR042798</b>         | <i>Nocardiopsis alkaliphila</i>  |

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). Cluster I 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.



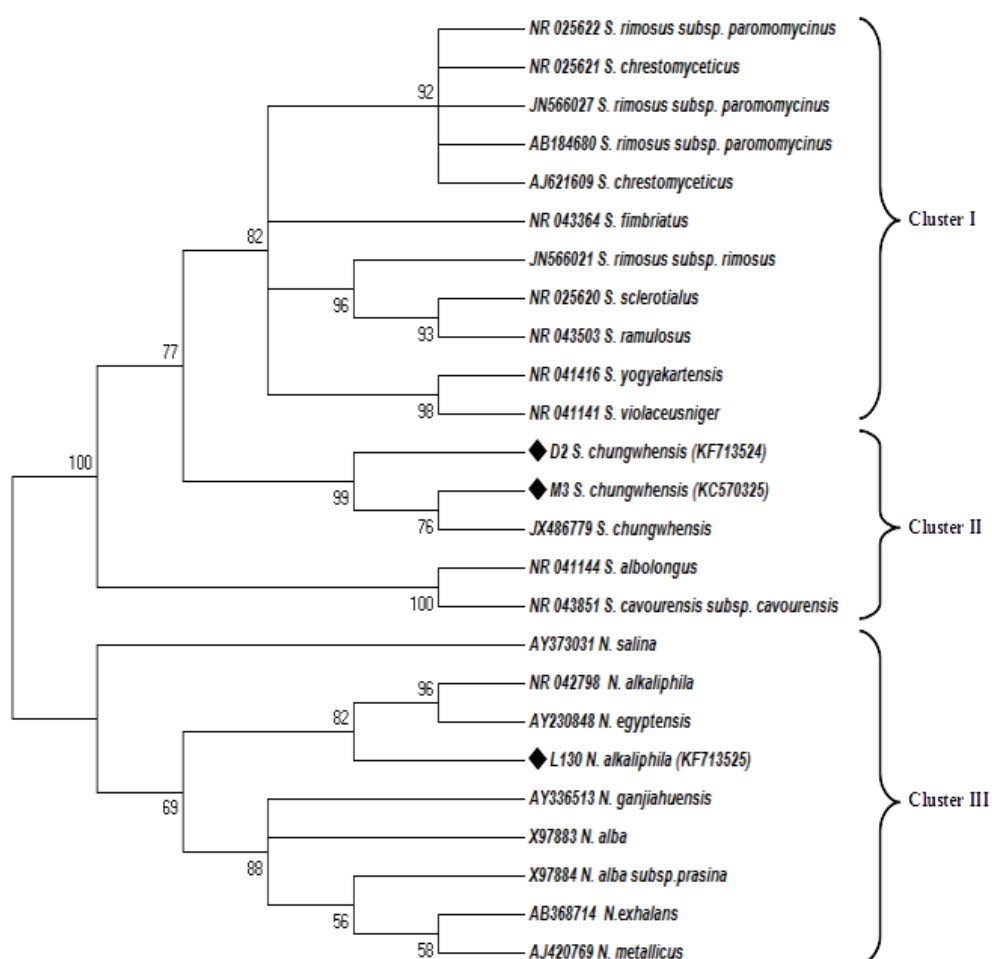


Fig. 3.13 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the yellow spore series actinomycetes with related *Streptomyces* and *Nocardiopsis* sp. Values at the node indicates the percentage with which the particular node occurred in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

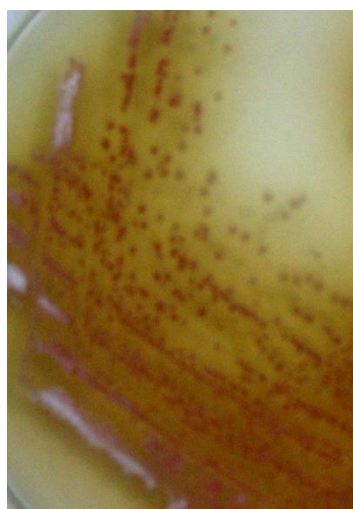
with diffusible pigment production, their amplicons on restriction exhibited different profiles. Both the strains on BLAST analysis showed 99% similarity and 100% query coverage to its nearest match, *Streptomyces bikiniensis* isolated from Pacific and Caribbean waters of Costa Rica and also to type 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 *HinfI* restriction profile and slightly different *Sau3A1* and *TaqI* 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: | <i>In vitro</i> Restriction Fragments |                   |                     | Species identity                                  | GenBank Accession      |
|-------------|---------------------------------------|-------------------|---------------------|---|------------------------|
|             | <i>Sau3A1</i> ( bp)                   | <i>HinfI</i> (bp) | <i>TaqI</i> ( bp)   |   |                        |
| M6          | 600+550+175                           | 800+300+250+150   | 900+350+150         | <i>S. bikiniensis</i>                             | <b><u>KC570315</u></b> |
| M8          | 625+600+175                           | 800+350+150       | 900+350+225         | <i>S. bikiniensis</i>                             | <b><u>JX657681</u></b> |
| D3          | 700+650+150                           | 825+350+200       | 400+350+325+150     | <i>S. griseoaurantiacus</i>                       | <b><u>KJ158466</u></b> |
| B5          | 650+600+175                           | 800+200+100       | 400+350+325+150+100 | <i>S. tendae</i>                                  | <b><u>KF713526</u></b> |
| B20         | 700+650+150                           | 825+350+100       | 400+350+325+150     | <i>S. diastaticus</i><br>subsp. <i>ardesiacus</i> | <b><u>KJ158467</u></b> |
| B1          | 650+600                               | 1100+325          | 900+350+225         | <i>S. rubrogriseus</i>                            | <b><u>KJ158468</u></b> |
| R21         | 600+550+175                           | 1100+325          | 900+350+175         | <i>S. rubrogriseus</i>                            | <b><u>KJ158469</u></b> |

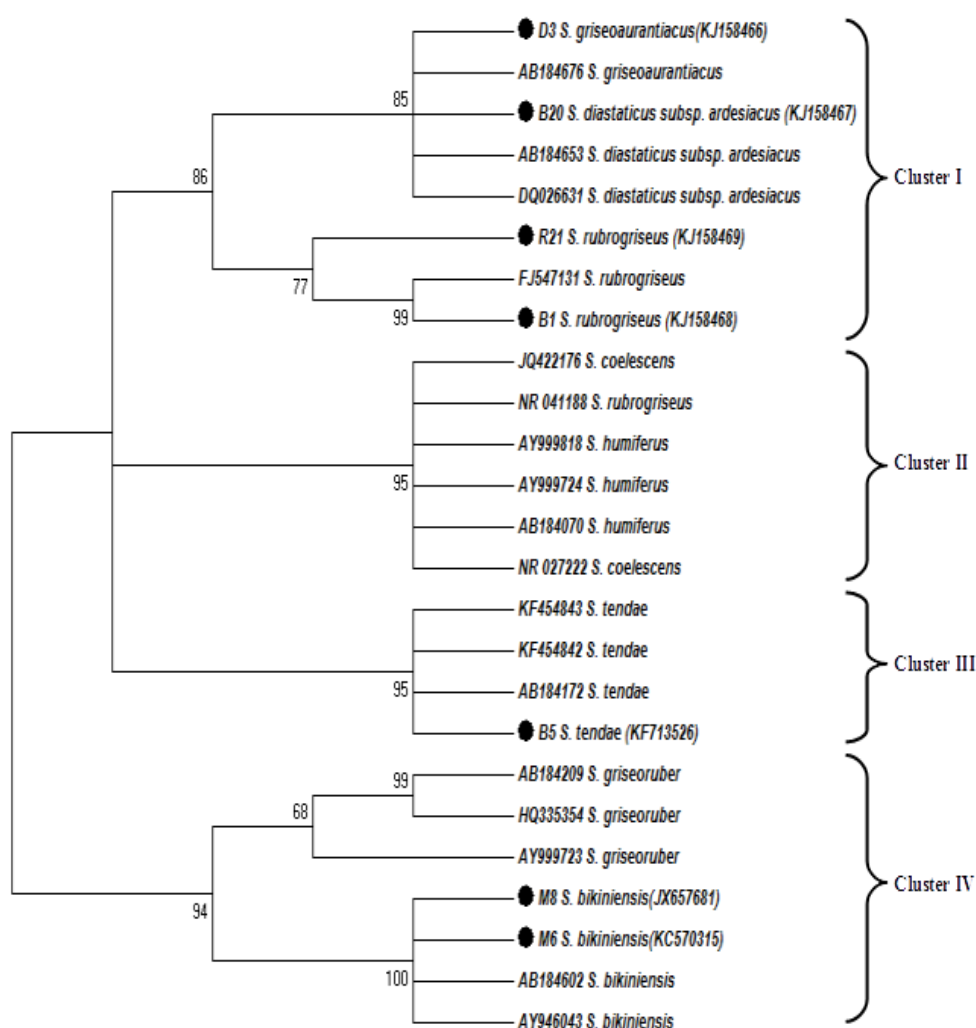
**Table 3.8 NCBI BLAST results of actinomycete with Grey spore mass and red substrate mycelium**

| 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                | <i>Streptomyces griseoaurantiacus</i>                    |
| M6          | 1319        | 1319      | 100%         | 0.0     | 99%            | AB184602                | <i>Streptomyces bikiniensis</i>                          |
| M8          | 1940        | 1940      | 100%         | 0.0     | 99%            | AB184602                | <i>Streptomyces bikiniensis</i>                          |
| BA5         | 817         | 817       | 100%         | 0.0     | 99%            | AB184172                | <i>Streptomyces tendae</i>                               |
| B20         | 1953        | 1953      | 100%         | 0.0     | 99%            | AB184653                | <i>Streptomyces diastaticus</i> subsp. <i>ardesiacus</i> |
| R21         | 1740        | 1740      | 100%         | 0.0     | 99%            | FJ547131                | <i>Streptomyces rubrogriseus</i>                         |
| B1          | 1884        | 1884      | 100%         | 0.0     | 99%            | FJ547131                | <i>Streptomyces rubrogriseus</i>                         |



**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. coelestis* 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 IV 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**

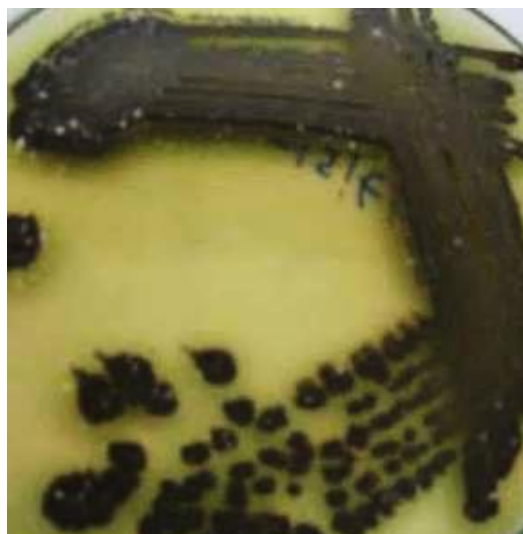
| Isolate no: | In vitro Restriction fragments |                 |                  | Species identity           | GenBank accession      |
|-------------|--------------------------------|-----------------|------------------|----------------------------|------------------------|
|             | Sau3A1(bp)                     | Hinf1(bp)       | Taq1(bp)         |                            |                        |
| M27         | 650+600+175                    | 850+325+225     | 550+350+325      | <i>S. radiopugnans</i>     | <b><u>KC570323</u></b> |
| R94         | 650+600+175                    | 1100+325+100    | 900+350+150      | <i>S. variabilis</i>       | <b><u>KF713527</u></b> |
| B2          | 650+600                        | 800+325+175     | 900+350+175      | <i>S. flavomacrosporus</i> | <b><u>KF713528</u></b> |
| R98         | 600+550                        | 850+325+225     | 400+375+ 350+300 | <i>S. variabilis</i>       | <b><u>KF713529</u></b> |
| R59         | 600+550                        | 800+300         | 400+325+375+200  | <i>S. violorubens</i>      | <b><u>KF713530</u></b> |
| M17         | 600+550                        | 800+325+200+175 | 400+375+350+325  | <i>S. variabilis</i>       | <b><u>KC570324</u></b> |
| M13         | 625+550+175                    | 800+275+300     | 550+350          | <i>S. radiopugnans</i>     | <b><u>KC570319</u></b> |
| M59         | 650+600+175                    | 800+325         | 550+375+325      | <i>S. radiopugnans</i>     | <b><u>KC570318</u></b> |

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. radiopugnans* (**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 violorubens* (**AB184464**). Amplicon of isolate B2 on BLAST analysis revealed 99% similarity to *S. flavomacrosporus* (**JX860361**), isolated from rhizospheric soils in Africa (Table 3.10).

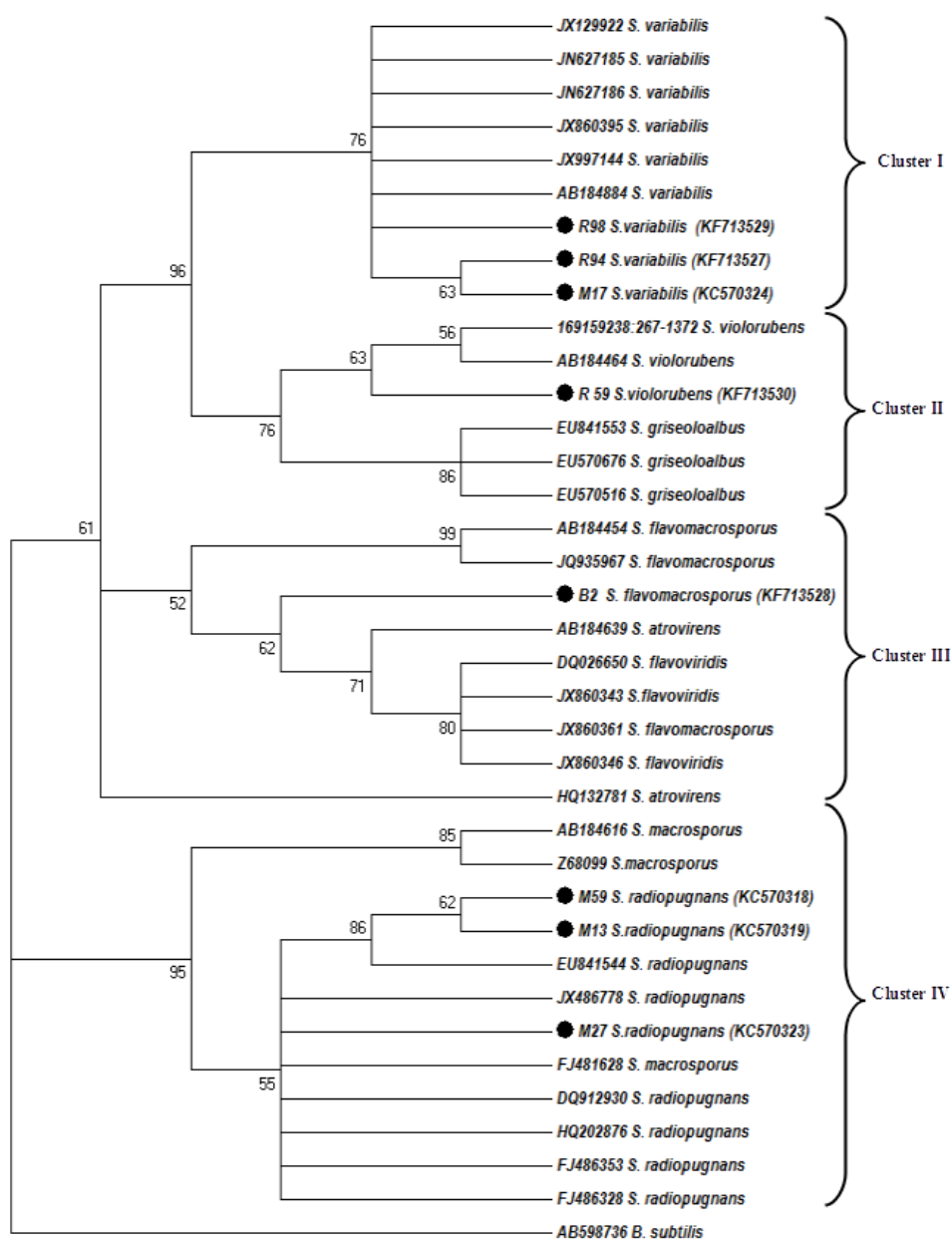
**Table 3.10 NCBI BLAST results of actinomycetes with grey spore mass**

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



**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. radiopugnans*. 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. griseoalbulus*. 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.

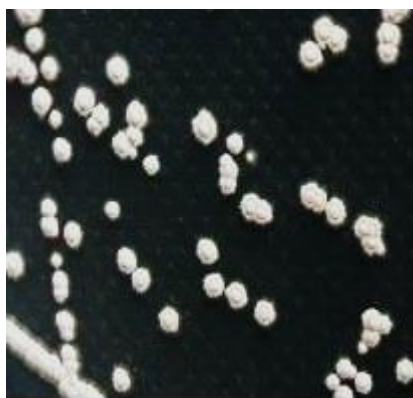


**Fig. 3.17** A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the grey spore series actinomycetes with other *Streptomyces*. Values at the node indicates the percentage with which the particular node occurred in 1000 trees generated 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 *Sau3A1* and *Hinf1* whereas *Taq1* 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: | <i>In vitro</i> Restriction fragments |                 |                 | Species Identity            | GenBank Accession        |
|-------------|---------------------------------------|-----------------|-----------------|-----------------------------|--------------------------|
|             | <i>Sau3A1</i>                         | <i>Hinf1</i>    | <i>Taq1</i>     |                             |                          |
| M31         | 650+600+225                           | 825+350+150+100 | 900+350+200     | <i>S. radiopugnans</i>      | <a href="#">KC570321</a> |
| R33         | 650+600+200                           | 1100+325        | 550+325+250+225 | <i>S. champavatii</i>       | <a href="#">KJ158471</a> |
| R73         | 650+600+175                           | 800+325+200+150 | 550+350+325     | <i>S. exfoliatus</i>        | <a href="#">KJ158473</a> |
| S18         | 600+175                               | 800+325         | 550+350+300     | <i>S. albidoflavus</i>      | <a href="#">KJ158474</a> |
| L128        | 700+600                               | 1100+325        | 600+550+325     | <i>S. luteogriseus</i>      | <a href="#">KJ158476</a> |
| R57         | 650+600+175                           | 800+325+200+150 | 550+375+325     | <i>S. exfoliatus</i>        | <a href="#">KJ158479</a> |
| R62         | 700+650+175                           | 825+350+200     | 850+325+250     | <i>S. minutiscleroticus</i> | <a href="#">KJ158480</a> |
| L33         | 600+250+225                           | 1100+200+175    | 900+350+200     | <i>Streptomyces</i> sp.     | <a href="#">KJ158481</a> |
| B4          | 1300+175                              | 1100+325        | 850+325+200     | <i>Nocardiopsis alba</i>    | <a href="#">KF713531</a> |

**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                | <i>Streptomyces radiopugnans</i>      |
| R33        | 874       | 874         | 100%        | 0.0     | 99%       | JN652257                | <i>Streptomyces champavatii</i>       |
| R73        | 1947      | 1947        | 100%        | 0.0     | 100%      | NR041229                | <i>Streptomyces exfoliatus</i>        |
| S18        | 2010      | 2010        | 100%        | 0.0     | 99%       | NR041095                | <i>Streptomyces albidoflavus</i>      |
| L128       | 1502      | 1502        | 100%        | 0.0     | 99%       | NR041128                | <i>Streptomyces luteogriseus</i>      |
| R57        | 1901      | 1901        | 100%        | 0.0     | 99%       | NR041229                | <i>Streptomyces exfoliatus</i>        |
| R62        | 863       | 863         | 100%        | 0.0     | 99%       | NR044149                | <i>Streptomyces minutiscleroticus</i> |
| L33        | 795       | 795         | 100%        | 0.0     | 99%       | NR044582                | <i>Streptomyces</i> sp.               |
| B4         | 719       | 719         | 100%        | 0.0     | 99%       | KC798063                | <i>Nocardiopsis alba</i>              |

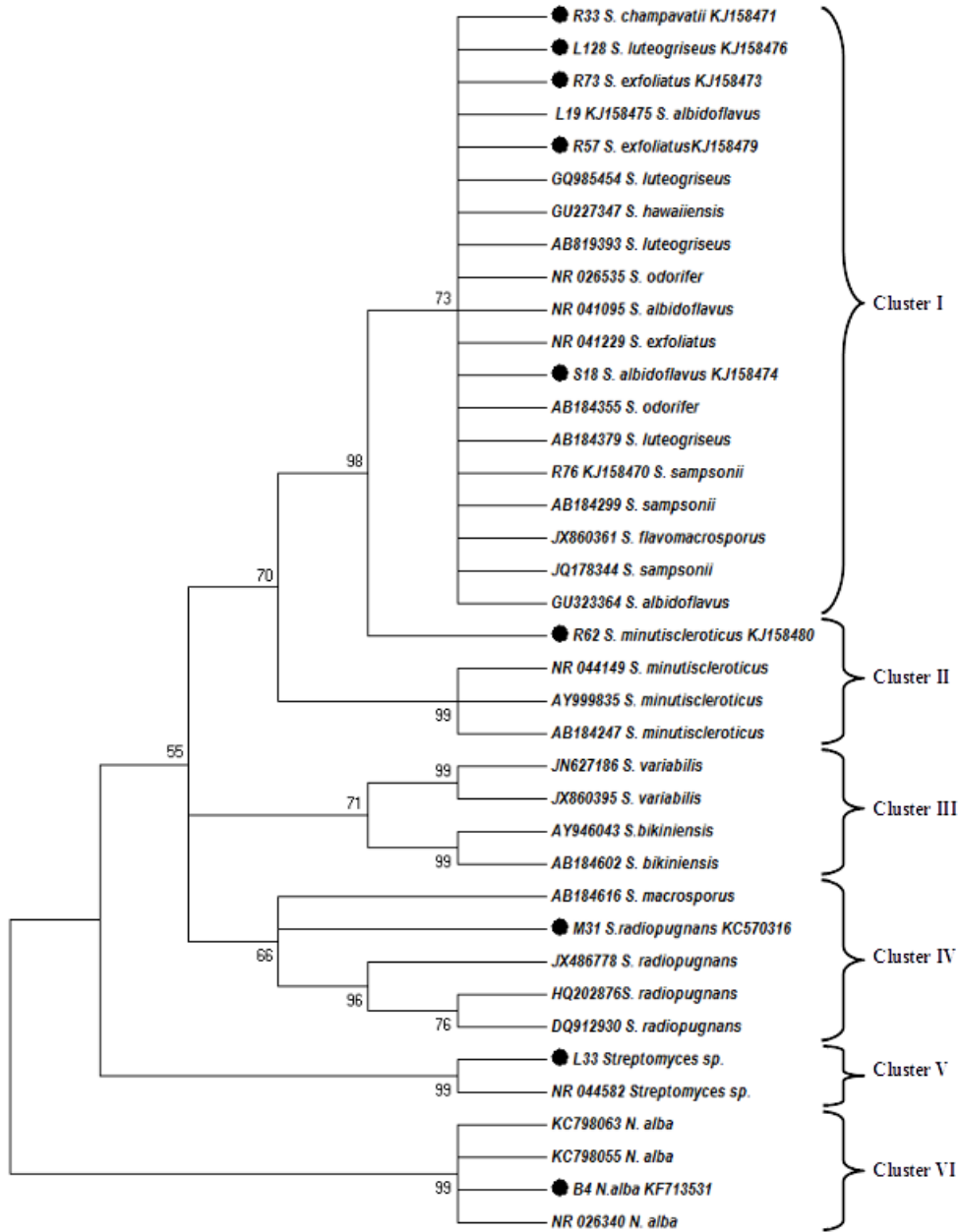
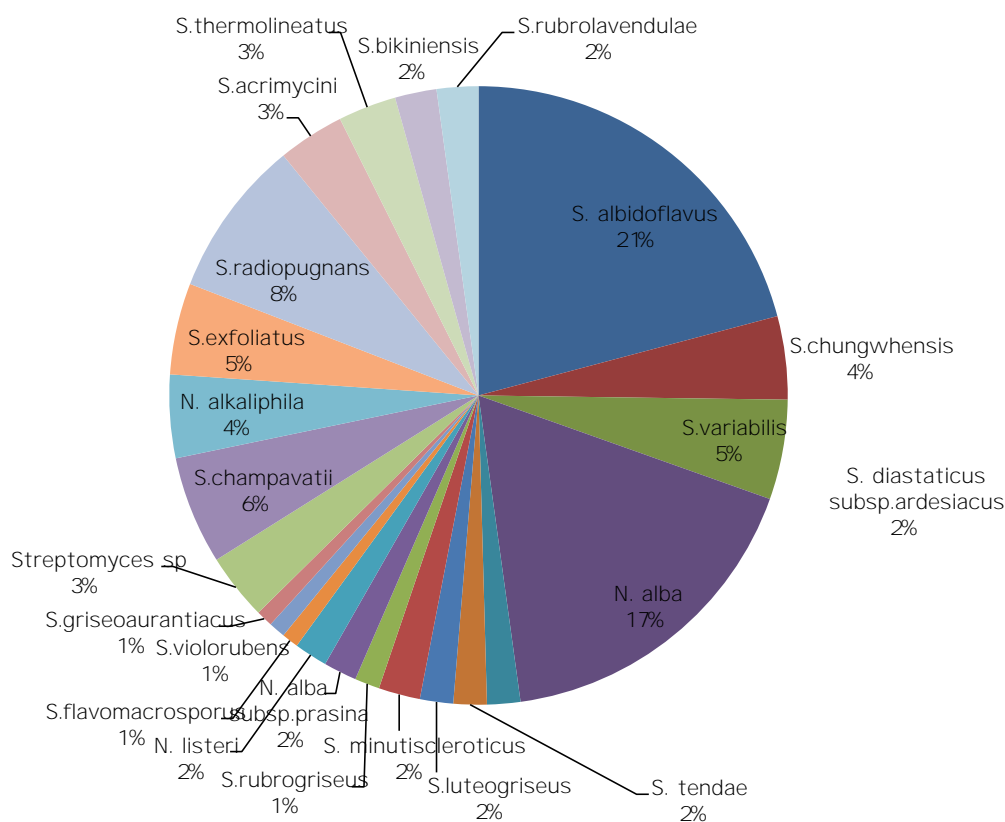


Fig. 3.19 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between the white spore series actinomycetes with other actinomycetes. Values at the node indicates the percentage with which the particular node occurred in 1000 trees generated by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

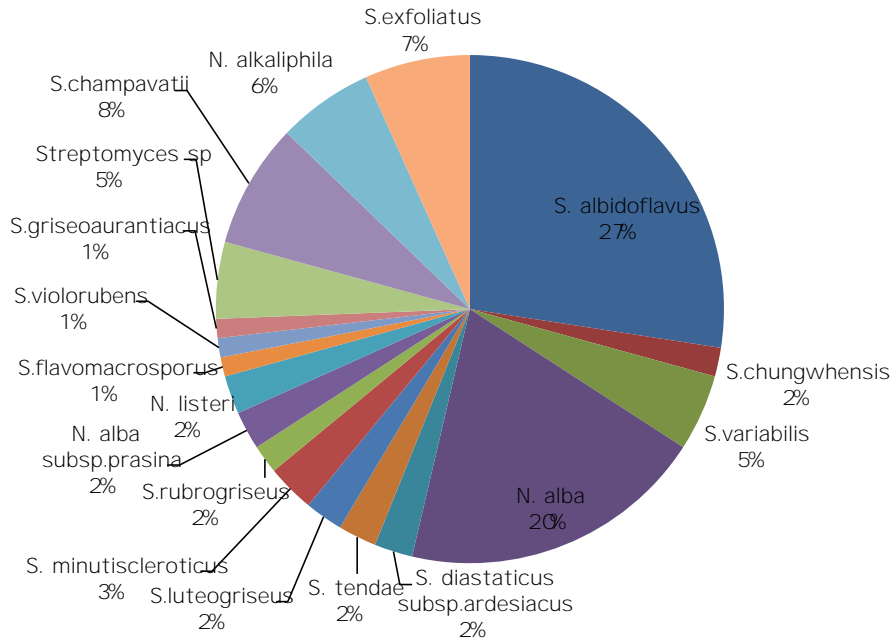
Of the total 230 marine actinomycete isolates, the most abundant species in marine sediment was identified as *Streptomyces albidoflavus* (20.42%), followed by *Nocardiopsis alba* (17.82%), *Streptomyces radiopugnans* (8.26%), *Streptomyces champavatii* (5.65%), *Streptomyces variabilis* (5.22%), *Streptomyces 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%),

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

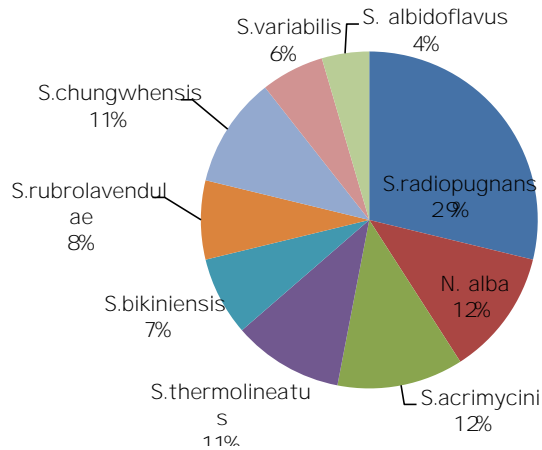
Approximately 3.47% actinomycete isolates were not assigned to any species and were recorded as *Streptomyces* sp. (Fig. 3.20). Genus *Nocardioopsis* was represented by *Nocardioopsis alba* (17.82%) as the prominent species followed by *Nocardioopsis alkaliphila* (4.34%), *Nocardioopsis alba* subsp *prasina* (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. *Nocardioopsis 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 *Nocardioopsis 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 (Vanechoutte et al., 1995). In this study we employed, a combination of *Sau3A1*, *Hinf1* and *Taq1* 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 *Sau3AI* 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 rRNA gene 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 *Sau3A1*. *Sau3A1* 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 *Sau3A1* recognition site or destroyed a genus characteristic *Sau3A1* 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.

*TaqI* 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 *TaqI* which produced clear ARDRA

patterns, capable of distinguishing species members in each of the 3 genera viz., *Bacillus*, *Paenibacillus* and *Brevibacillus*. *TaqI* is an efficient restriction endonuclease for actinomycete analysis (Jing et al., 2008; Steingrube et al., 1997). In the present study, *HinfI* 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 *Nocardioopsis 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. *Sau3A1* profile was almost similar for the *Streptomyces* sp., whereas

*Nocardiopsis* sp. had a different profile with the largest fragment size of 1300 bp. *Hinf*I 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*I 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*I and *Taq*I 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* subsp. *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 rubrogriseus*. 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*I, slightly different in the case of *Taq*I 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. bikiniensis*. 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 *Nocardiosis 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 Kampfner 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 *streptomyces* 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, it 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 *Streptomyces* 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 *Streptomyces* 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 *Sau3A1*, *Hinf1* and *Taq1* 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 *Taq1* and *Sau3A1* compared to *Hinf1* 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 *Nocardiopsis*.

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 *Streptomyces* 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 *Pseudomonas 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), *Pseudomonas* (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. aureofasciculus*, *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.

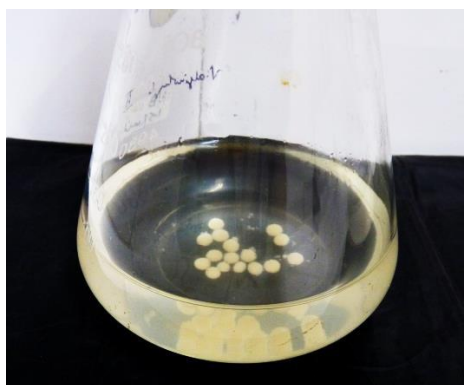
#### **4.2.5 Application potential of biogranule in aquaculture**

##### **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 log<sub>10</sub> 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).

$$\begin{aligned} & \text{ATP Concentration per wet weight of biogranule}(fg/g) \\ & = \text{Relative Light Units (RLU)} \times \text{Factor Value} \\ & \times \text{correctionfactor} \times \text{Dilution Factor/wet weight} \end{aligned}$$

#### **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.

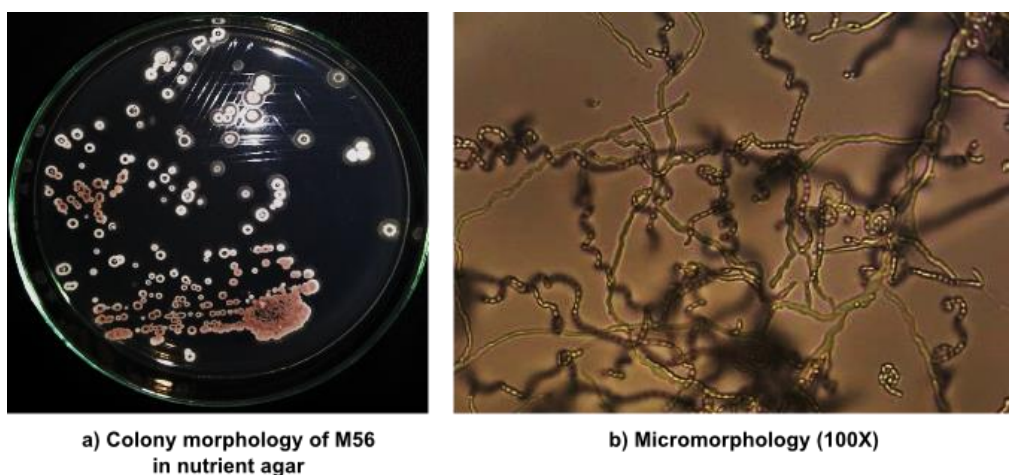
## 4.3 Results

### 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*.



**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                |
|-----------------------------------|--|-----------------------|
| <b>Morphological</b>              | Spore chain morphology                               | Spirals               |
|                                   | Spore mass colour                                    | Pink red              |
|                                   | Aerial mycelium                                      | Pink red              |
|                                   | Substrate mycelium                                   | Off white             |
|                                   | Diffusible pigment production                        | -                     |
| <b>Biochemical reactions</b>      | 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 <sub>2</sub> S production                          | -                     |
|                                   | Urea Hydrolysis                                      | +                     |
|                                   | Citrate utilization                                  | -                     |
|                                   | <b>Acid Production from Carbohydrates</b>            | Lactose               |
| Mannitol                          |  | -                     |
| Glucose                           |  | +                     |
| Arabinose                         |  | -                     |
| Rhamnose                          |  | -                     |
| Galactose                         |  | -                     |
| Inositol                          |  | -                     |
| Trehalose                         |  | +                     |
| Sorbitol                          |  | -                     |
| Xylose                            |  | +                     |
| <b>Resistance to Antibiotics</b>  |  | 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)                                   | -                     |
| <b>Hydrolytic Enzyme Activity</b> | Protease   | +                     |
|                                   | Amylase  | +                     |
|                                   | Lipase   | +                     |
|                                   | DNase  | +                     |
|                                   | Phosphatase  | +                     |
|                                   | Pectinase  | -                     |
|                                   | Ligninase  | -                     |
|                                   | Cellulase  | -                     |
|                                   | Aryl sulphatase                                      | -                     |
|                                   | Chitinase  | -                     |



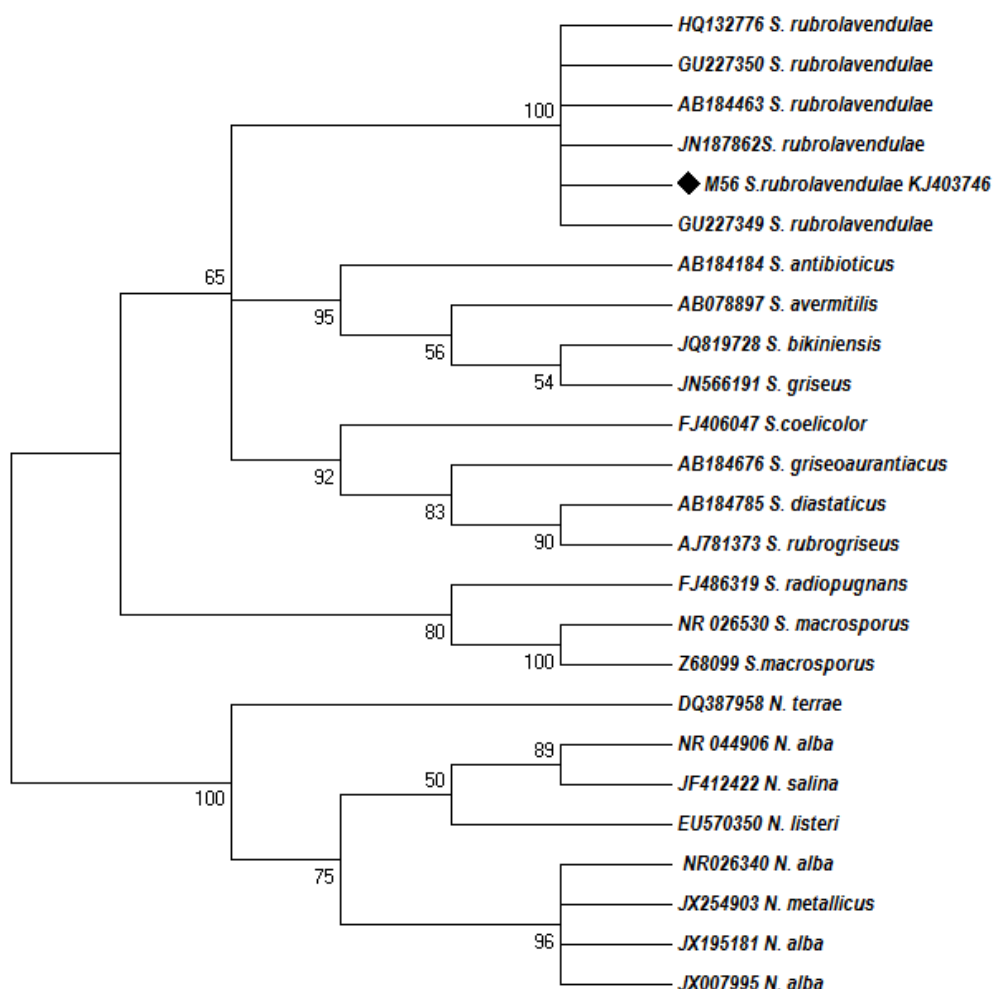


Fig. 4.3 A bootstrapped Neighbor joining phylogenetic tree obtained using MEGA version 5.0 illustrating relationships between *S. rubrolavendulae* (M56) and other related species. Values at the node indicate the percentage with which the particular node occurred in 1000 trees generated by bootstrapping 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                           | A2                          | A3                    | A4               | A5           | A6                         | A7                              | A8                           | A9                             | A10                             | A11                               | A12                                       |
| Water                        | $\alpha$ -Cyclodextrin      | $\beta$ -Cyclodextrin | Dextrin          | Glycogen     | Inulin                     | Mannan                          | Tween 40                     | Tween 80                       | N-Acetyl-D-Glucosamine          | N-Acetyl- $\alpha$ -D-Mannosamine | Amygdalin                                 |
| B1                           | B2                          | B3                    | B4               | B5           | B6                         | B7                              | B8                           | B9                             | B10                             | B11                               | B12                                       |
| L-Arabinose                  | D-Arabitrol                 | Arbutin               | D-Cellobiose     | D-Fructose   | L-Fucose                   | D-Galactose                     | D-Galacturonic Acid          | Gentiobiose                    | D-Gluconic Acid                 | $\alpha$ -D-Glucose               | m-Inositol                                |
| C1                           | C2                          | C3                    | C4               | C5           | C6                         | C7                              | C8                           | C9                             | C10                             | C11                               | C12                                       |
| $\alpha$ -D-Lactose          | Lactulose                   | Maltose               | Maltotriose      | D-Mannitol   | D-Mannose                  | D-Melezitose                    | D-Melibiose                  | $\alpha$ -Methyl-D-Galactoside | $\beta$ -Methyl-D-Galactoside   | 3-Methyl-D-Glucose                | $\alpha$ -Methyl-D-Glucoside              |
| D1                           | D2                          | D3                    | D4               | D5           | D6                         | D7                              | D8                           | D9                             | D10                             | D11                               | D12                                       |
| $\alpha$ -Methyl-D-Glucoside | $\beta$ -Methyl-D-Mannoside | Palatinose            | D-Fsucose        | D-Raffinose  | L-Rhamnose                 | D-Ribose                        | Salicin                      | Sedoheptuloaan                 | D-Sorbitol                      | Stachyose                         | Sucrose                                   |
| E1                           | E2                          | E3                    | E4               | E5           | E6                         | E7                              | E8                           | E9                             | E10                             | E11                               | E12                                       |
| D-Tagatose                   | D-Trehalose                 | Turanose              | Xylitol          | D-Xylose     | Acetic Acid                | $\alpha$ -Hydroxybutyric Acid   | $\beta$ -Hydroxybutyric Acid | $\gamma$ -Hydroxybutyric Acid  | p-Hydroxy-phenylacetic Acid     | $\alpha$ -Ketoglutaric Acid       | $\alpha$ -Ketovaleric Acid                |
| F1                           | F2                          | F3                    | F4               | F5           | F6                         | F7                              | F8                           | F9                             | F10                             | F11                               | F12                                       |
| Lactamide                    | D-Lactic Acid Methyl Ester  | L-Lactic Acid         | D-Malic Acid     | L-Malic Acid | Pyruvic Acid Methyl Ester  | Succinic Acid Mono-Methyl Ester | Propionic Acid               | Pyruvic Acid                   | Succinamic Acid                 | Succinic Acid                     | N-Acetyl-L-Glutamic Acid                  |
| G1                           | G2                          | G3                    | G4               | G5           | G6                         | G7                              | G8                           | G9                             | G10                             | G11                               | G12                                       |
| L-Alaninamide                | D-Alanine                   | L-Alanine             | L-Alanyl-Glycine | L-Asparagine | L-Glutamic Acid            | Glycyl-L-Glutamic Acid          | L-Pyroglutamic Acid          | L-Serine                       | Putrescine                      | 2,3-Butanediol                    | Glycerol                                  |
| H1                           | H2                          | H3                    | H4               | H5           | H6                         | H7                              | H8                           | H9                             | H10                             | H11                               | H12                                       |
| Adenosine                    | 2-Deoxy Adenosine           | Inosine               | Thymidine        | Uridine      | Adenosine-5'-Monophosphate | Thymidine-5'-Monophosphate      | Uridine-5'-Monophosphate     | D-Fructose-6-Phosphate         | $\alpha$ -D-Glucose-1-Phosphate | D-Glucose-6-Phosphate             | D-L-Glycerol $\alpha$ -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 |
|----------------------------------|--------|---------------------------|--------|-----------------------------------|--------|
| $\alpha$ - Cyclodextrin          | -      | L-Arabinose               | +      | $\alpha$ - D-Lactose              | +      |
| $\beta$ -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               | -      | $\alpha$ -Methyl-D-Galactoside    | -      |
| N-acetyl- $\beta$ -D-Mannosamine | +      | D- Gluconic acid          | +      | $\beta$ -Methyl-D-Galactoside     | +      |
| Amygdalin                        | -      | $\alpha$ -D- Glucose      | +      | 3-Methyl-D- Glucose               | -      |
| D-Tagatose                       |        | m-Inositol                | +      | $\alpha$ -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                           | -      |
| $\alpha$ -Hydroxybutyric acid    | -      | L-Glutamic acid           | +      | Adenosine-5 Monophosphate         | -      |
| B- Hydroxybutyricacid            | -      | Glycyl-L-Glutamic acid    | -      | Thymidine-5-Monophosphate         | -      |
| $\gamma$ -Hydroxybutyricacid     | -      | L-Pyrogutamic acid        | -      | Uridine-5-Monophosphate           | +      |
| p-Hydroxyphenyl aceticacid       | -      | L-Serine                  | +      | D-Fructose-6-Phosphate            | +      |
| $\alpha$ -Ketoglutaricacid       | +      | Putrescine                | +      | D-Glucose-1- Phosphate            | -      |
| $\alpha$ -Ketovalericacid        | +      | 2,3-Butanediol            | -      | D-Glucose-6 -phosphate            | +      |
| B-methylD glucoside              | -      | Glycerol                  | +      | D-L- $\alpha$ -glycerol Phosphate | +      |
| $\alpha$ -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,  $\alpha$ -D lactose, maltose, mannitol, sorbitol, inositol, adenosine, deoxy adenosine, D-fructose-6-phosphate, D glucose-6-phosphate,  $\beta$ -cyclodextrin, N-acetyl-D-glucosamine, N-acetyl-D mannosamine, D-L- $\alpha$ -glycerol phosphate, uridine-5-monophosphate, amino acids such as L-glutamic acid, L-alanine, L-serine, L-asparagine, putrescine, carboxylic acids such as  $\alpha$ -ketoglutaric acid,  $\alpha$ -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  $72 \times 10^6$  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) |
|---|------------------------------|----------------------------|---|
| <i>Streptomyces rubrolavendulae</i> M56 | 0.03                         | $3834 \pm 1.34$            | $72 \times 10^6$                                |

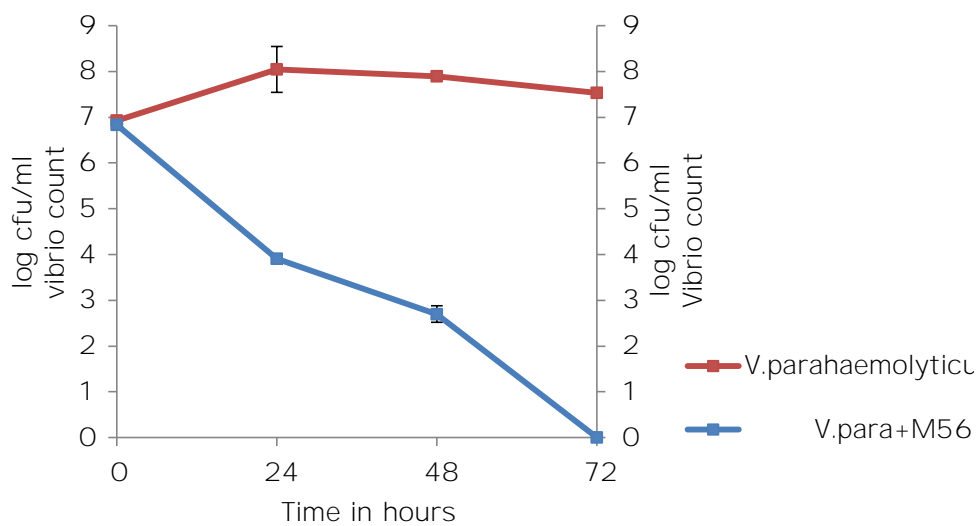
\*ATP concentration per wet weight of biogranule = Relative light Units (RLU) x factor value x dilution factor x correction factor/wet weight

Factor value (calculated from standards) =  $4.5 \times 10^{-6}$

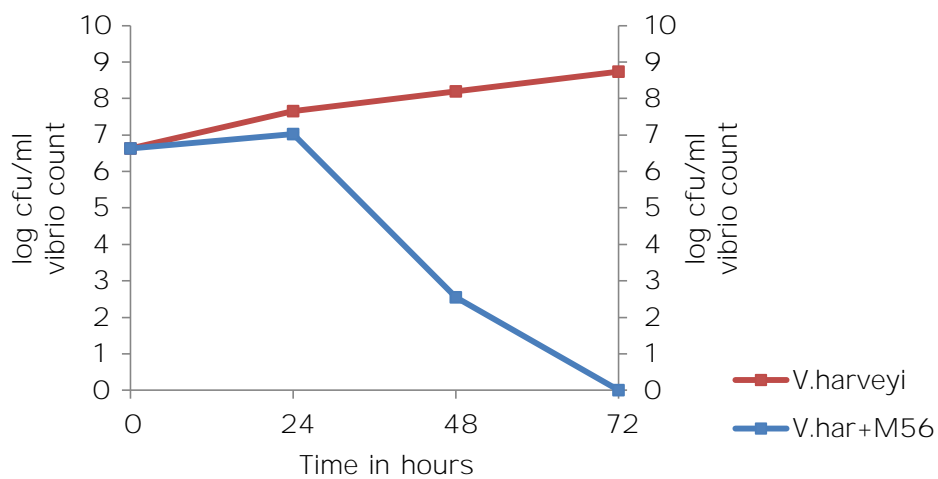
#### 4.3.2 *In vitro* exclusion of *Vibrio* spp. by actinomycete biogranule

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  $10^5$  cfu/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)**

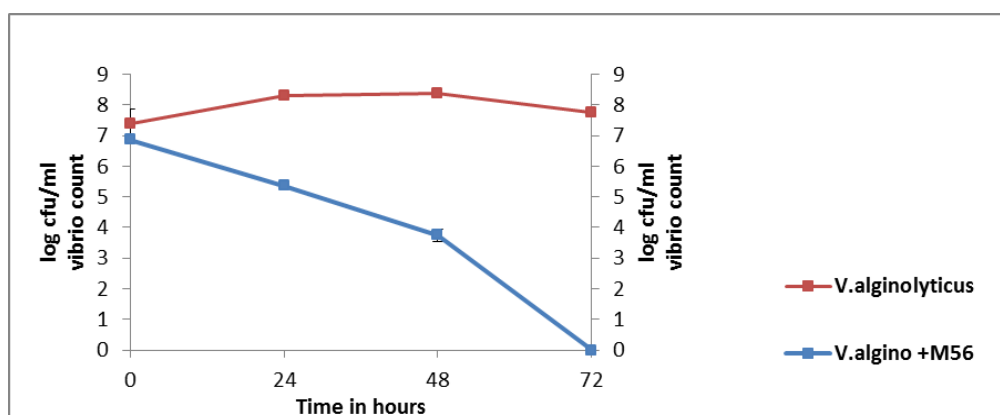


Fig. 4.6 Inhibition of *Vibrio alginolyticus* by actinomycete biogranule (M56)

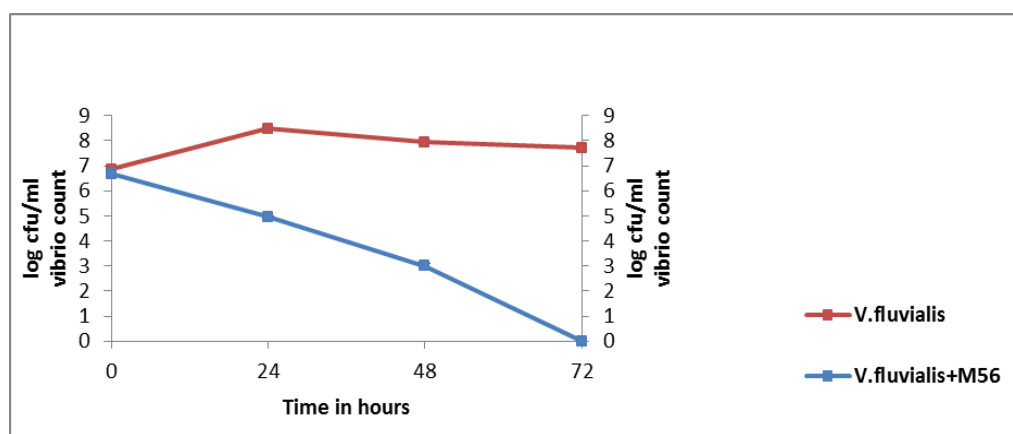


Fig. 4.7 Inhibition of *Vibrio fluvialis* by actinomycete biogranule (M56)

#### 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,  $\beta$ -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  $72 \times 10^6$  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  $7.2 \times 10^7$  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  $72 \times 10^6$  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 *Pseudomonas* 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 parallelly 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 cysteinylolation, 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 pyromelanogenetic and eu/pheomelanogenetic pathways, sometimes simultaneously active in one organism. Free living strains of *Vibrio cholerae* are usually amelanotic or they produce pyromelanin (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 salinestrus* 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<sup>+3</sup> 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<sub>3</sub> 2.0 g; K<sub>2</sub>HPO<sub>4</sub> 1.0 g; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g; KCl 0.5 g; FeSO<sub>4</sub>.7H<sub>2</sub>O 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](http://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<sup>TM</sup> 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<sub>2</sub>O at 80°C on a shaker. M8 was dissolved in 1M NaOD in D<sub>2</sub>O 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  $\text{cm}^{-1}$  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-azinobis(3-ethyl benzothiazoline-6-sulfonic acid) 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<sub>4</sub> (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.

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

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-sulfonic acid (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<sup>+</sup>) 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 (ABTS<sup>+</sup>) 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 (ABTS<sup>+</sup>) 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<sup>-1</sup>), 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:

$$I\% = \left[ 1 - \frac{A_i - A_j}{A_c} \right] \times 100\%$$

Where,

*A<sub>c</sub>* is the absorbance of DPPH solution without sample (2 ml DPPH + 3 ml 95% ethanol),

*A<sub>i</sub>* is the absorbance of the test sample mixed with DPPH solution (1 ml sample + 2 ml DPPH + 2 ml 95% ethanol) and

*A<sub>j</sub>* 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<sub>50</sub>) 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), lysosomal 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] XTT assay**

For the above sequential assays,  $\sim 1 \times 10^6$  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 °C 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.

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

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 37°C. 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.



$$\text{Percentage of viable cells} = 100 - \left( \frac{A_c}{A_o} \times 100 \right)$$

Where,

*A<sub>c</sub>* is the average absorbance of accumulated neutral red in cells at a particular concentration of melanin, and *A<sub>o</sub>* 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 4°C. 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).

$$\text{Percentage of viable cells} = 100 - \left( \frac{A_c}{A_o} \times 100 \right)$$

Where,

*A<sub>c</sub>* is the average absorbance of cells at a particular concentration of melanin and *A<sub>o</sub>* 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 ( $2 \text{ mg ml}^{-1}$ ) in  $0.05 \text{ mol l}^{-1}$  Tris HCl buffer (pH 7.6) was added to the melanin exposed cell lines and incubated for 1 hour at  $10^\circ\text{C}$ . The samples were then centrifuged at 5000 rpm for 5 minutes, discarded the supernatant and quenched the reaction by adding  $500 \mu\text{l}$  absolute methanol. Following incubation for 10 minutes, the insoluble formazan residue was fixed by rinsing thrice with  $500 \mu\text{l}$  of 50% methanol. The formazan residue was solubilized with  $120 \mu\text{l}$  of 2 M KOH and  $140 \mu\text{l}$  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  $240 \mu\text{l}$  sodium pyrophosphate buffer (pH 8.3),  $20 \mu\text{l}$  phenazine methosulphonate,  $80 \mu\text{l}$  nitro blue tetrazolium dye,  $60 \mu\text{l}$  NADH,  $240 \mu\text{l}$  distilled water and  $60 \mu\text{l}$  of melanin treated HEp-2 cell lines. Tubes were incubated at  $30^\circ\text{C}$  for 2.5 minutes and reaction was stopped by the addition of  $200 \mu\text{l}$  glacial acetic acid. Reaction mixture shaken vigorously with  $800 \mu\text{l}$  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,  $200 \mu\text{g ml}^{-1}$  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 ( $50 \mu\text{g ml}^{-1}$ ) was used as positive control.

### 5.2.10 Statistical analysis

The data of all experiments were recorded as mean  $\pm$  SD and were analyzed with SPSS (version 17.0 for Windows, SPSS Inc.). The  $\text{IC}_{50}$  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.

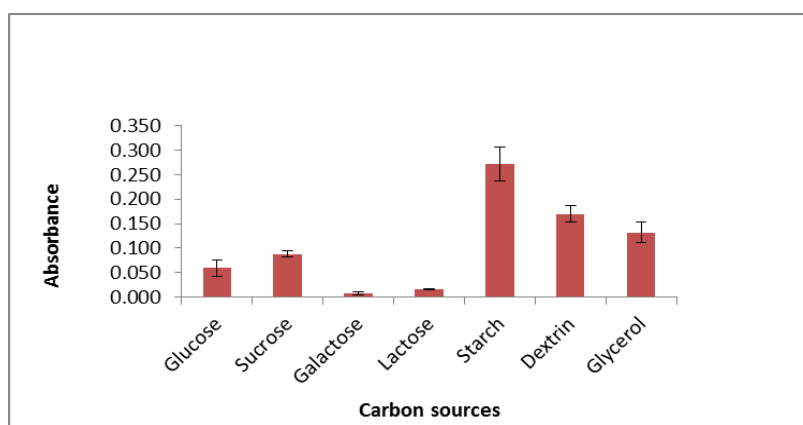
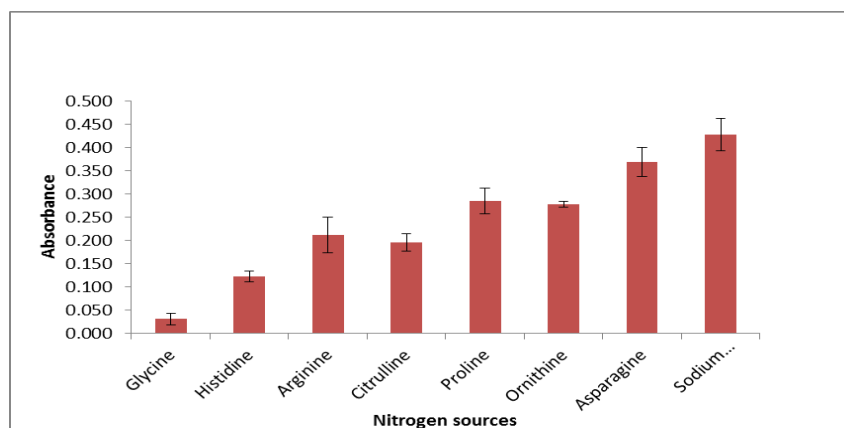


Fig. 5.1 Effect of different carbon sources on melanin production by M8



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

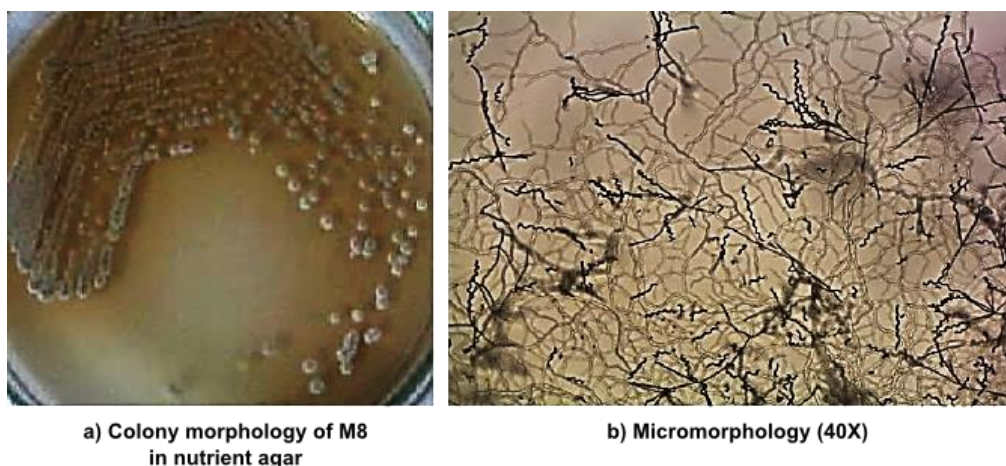
### 5.3.2 Effect of nitrogen source on melanin production

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 rectiflexibles. 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  $\mu$ g). 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)**

|                                    | <b>Characteristic</b>                                | <b>Result</b>  |
|------------------------------------|--|----------------|
| Morphology                         | Spore chain morphology                               | Rectiflexibles |
|                                    | 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 <sub>2</sub> S 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).

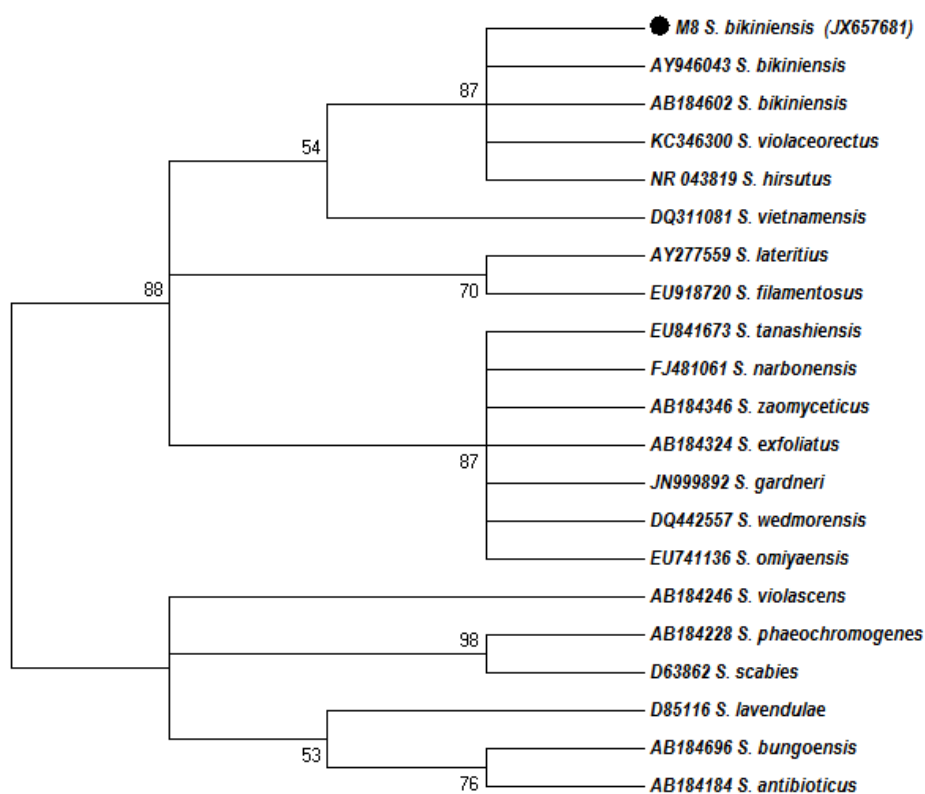
#### 5.3.3.3 *Sequence Accession Number*

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.,  $\beta$ -cyclodextrin, dextrin, glycogen, mannan, N-acetyl-D-glucosamine, amygdalin, tagatose, trehalose, turanose, xylose, palatinose, piscose, raffinose, ribose, arabinose, cellobiose, fructose, galactose, gentiobiose,  $\alpha$ -D-glucose,  $\alpha$ -D-lactose, maltotriose, mannose, melezitose, melibiose, 3-methyl-D-glucose,  $\alpha$ -D-glucose-1 phosphate, 2-deoxy adenosine, inosine, sugar alcohols such as xylitol, glycerol, arabitol, sorbitol, mannitol, D-L- $\alpha$ -glycerol phosphate, and

carboxylic acids viz., acetic acid,  $\alpha$ -hydroxybutyric acid,  $\beta$ -hydroxybutyric acid,  $\gamma$ -hydroxybutyric acid,  $\alpha$ -ketoglutaric acid, p-hydroxyphenylacetic acid, 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

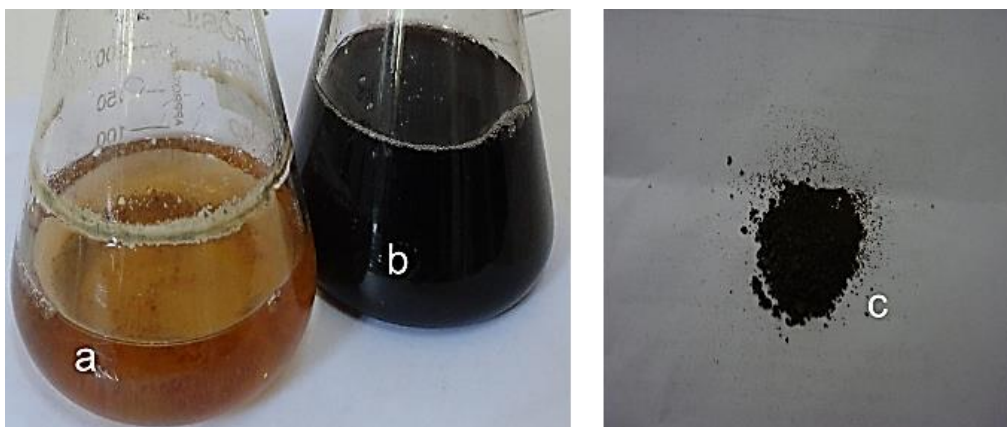


**Table 5.2 Carbon utilization profile of *S. bikiniensis* (M8) (Acc.no: JX657681)**

| Carbon source                    | Result | Carbon source              | Result | Carbon source                    | Result |
|----------------------------------|--------|----------------------------|--------|----------------------------------|--------|
| $\alpha$ -Cyclodextrin           | -      | L-Arabinose                | +      | $\alpha$ -D-Lactose              | +      |
| $\beta$ -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                | +      | $\alpha$ -Methyl-D-galactoside   | -      |
| N-Acetyl- $\beta$ -D-Mannosamine | -      | D- Gluconic acid           | +      | $\beta$ -Methyl-Dgalactoside     | +      |
| Amygdalin                        | +      | $\alpha$ -D-Glucose        | +      | 3-Methyl-D-glucose               | +      |
| D-Tagatose                       | +      | m-Inositol                 | -      | $\alpha$ -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                          | +      |
| $\alpha$ -Hydroxybutyricacid     | +      | L-glutamic acid            | +      | Adenosine- 5- monophosphate      | -      |
| $\beta$ - Hydroxybutyricacid     | +      | Glycyl-L-glutamic acid     | +      | Thymidine-5-monophosphate        | +      |
| $\gamma$ - -Hydroxybutyricacid   | +      | L-Pyroglutamic acid        | +      | Uridine-5-monophosphate          | +      |
| p-Hydroxyphenylaceticacid        | +      | L-Serine                   | +      | D-Fructose- 6-Phosphate          | -      |
| $\alpha$ -Ketoglutaricacid       | +      | Putrescine                 | -      | $\alpha$ -D-glucose- 1-phosphate | +      |
| $\alpha$ -ketovalericacid        | -      | 2,3-Butanediol             | -      | D- Glucose- 6- phosphate         | -      |
| $\beta$ -Methyl-D-Glucoside      | -      | Glycerol                   | +      | D-L- $\alpha$ -Glycerolphosphate | +      |
| $\alpha$ -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)

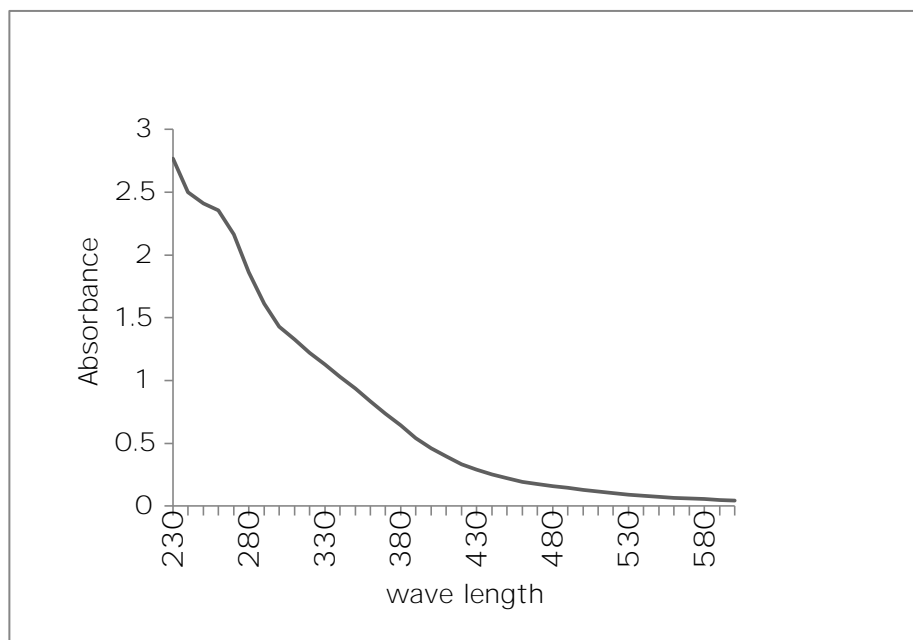
### 5.3.5 Physicochemical characterization of extracted melanin

#### 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.

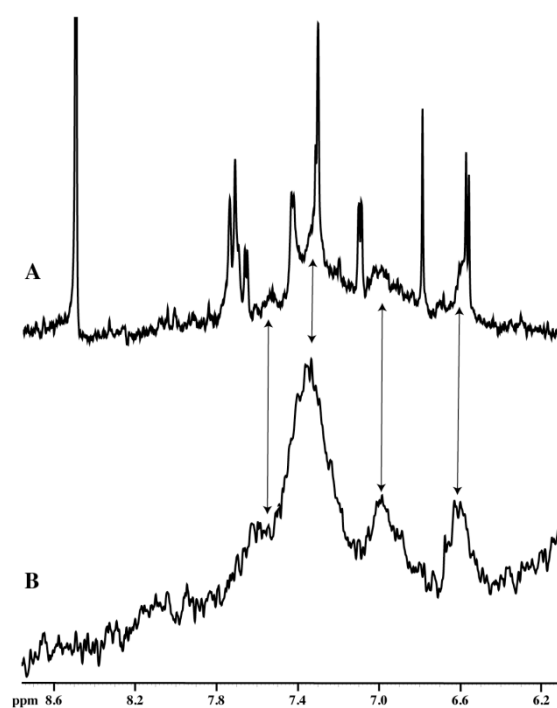


**Fig. 5.6 UV-VIS Spectra of M8 melanin**

### 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<sup>-1</sup> (CH<sub>2</sub>-CH<sub>3</sub> bending) characteristic of melanin pigment. Phenolic COH stretching at 1233.9 cm<sup>-1</sup> relates to phenolic compounds. On the basis of the above results it was concluded that the pigment was eumelanin.

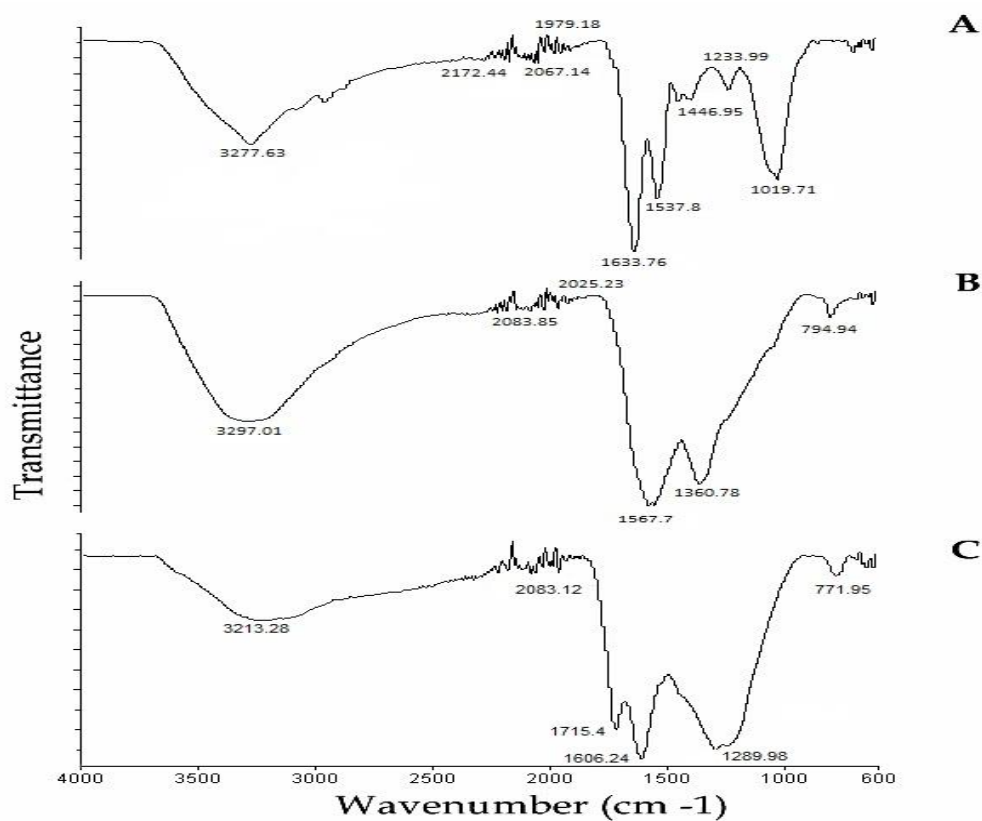


Fig. 5.8 FTIR Spectra analysis A) M8 melanin B) Sepia melanin (Sigma) C) Synthetic melanin

### 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).

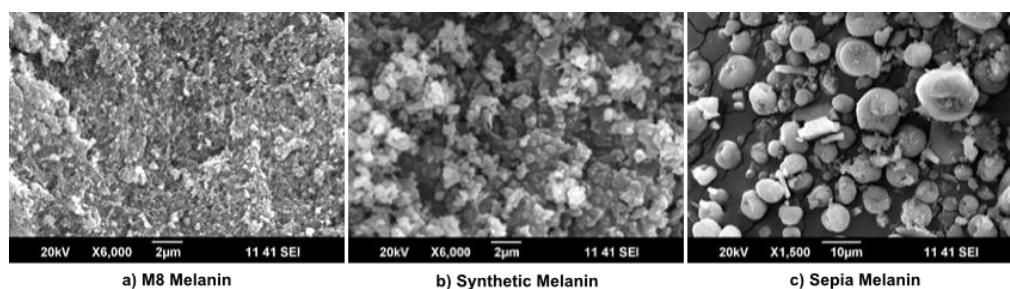


Fig. 5.9 Scanning electron microscope imaging of melanin samples

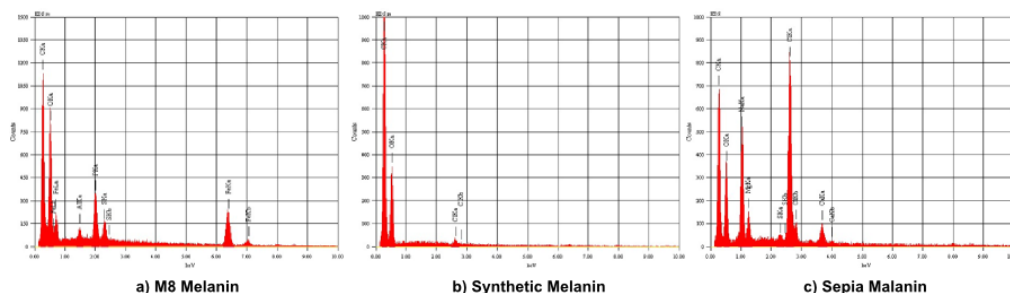


Fig. 5.10 Energy dispersive spectra of melanin samples

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          |
| <b>Total</b> |       | <b>99.99</b> | <b>100.01</b> | <b>100.00</b>     | <b>100.00</b> | <b>100.01</b> | <b>100.01</b> |

### 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  $25 \mu\text{g ml}^{-1}$ , the inhibition was  $22.67 \pm 4.72\%$ , and for an increase in concentration of melanin from  $50 - 150 \mu\text{g ml}^{-1}$  inhibition of lipid peroxidation increased as  $37.90 \pm 2.47\%$ , to  $65.29 \pm 2.15\%$ . At a concentration of  $5.5 \mu\text{g ml}^{-1}$ , positive control butylated hydroxyl toluene (BHT) exhibited  $70.84 \pm 0.11\%$  inhibition on lipid peroxidation. The inhibitory concentration ( $\text{IC}_{50}$ ) of extracted crude M8 melanin on lipid peroxidation was calculated as  $73.70 \pm 12.6 \mu\text{g ml}^{-1}$ .

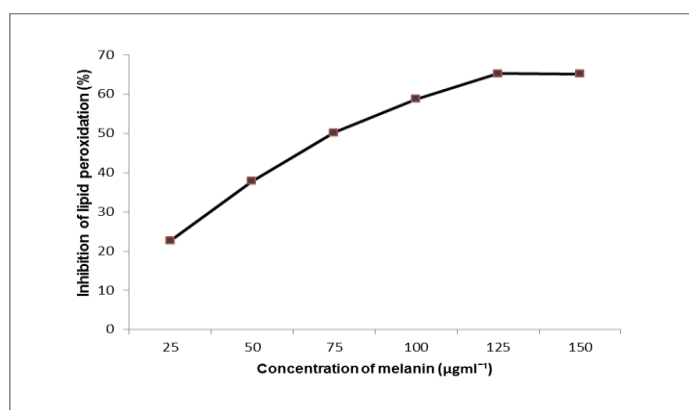


Fig. 5.11 Lipid peroxidation inhibition of crude M8 melanin

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

| Concentration( $\mu\text{g/ml}$ ) Melanin | Inhibition% (I%)                                       |
|---|--|
| 25  | $22.67 \pm 4.72$                                       |
| 50  | $37.90 \pm 2.47$                                       |
| 75  | $50.24 \pm 2.75$                                       |
| 100                                       | $58.75 \pm 7.32$                                       |
| 125                                       | $65.29 \pm 2.15$                                       |
| 150                                       | $65.1 \pm 1.05$  |
| <b><math>\text{IC}_{50}</math></b>        | <b><math>73.70 \pm 12.6 \mu\text{g ml}^{-1}</math></b> |
| BHT( $5.5 \mu\text{g/ml}$ )               | $70.84 \pm 0.11$                                       |

**Table 5.7 ABTS scavenging assay of M8 melanin and Trolox**

| Concentration M8<br>( $\mu\text{g/ml}$ ) | Inhibition% (I%)   | Concentration Trolox<br>( $\mu\text{g/ml}$ ) | Inhibition% (I%)  |
|--|--|--|---|
| 4  | 17.66 $\pm$ 2.12   | 0.4  | 15.14 $\pm$ 0.13  |
| 8  | 26.26 $\pm$ 0.60   | 0.8  | 23.29 $\pm$ 0.23  |
| 16                                       | 34.55 $\pm$ 0.13   | 1.6  | 36.83 $\pm$ 0.35  |
| 24                                       | 47.26 $\pm$ 1.78   | 2.4  | 53.27 $\pm$ 0.13  |
| 32                                       | 59.28 $\pm$ 0.23   | 3.2  | 63.7 $\pm$ 0.23   |
| 40                                       | 61.95 $\pm$ 0.57   | 4  | 89.35 $\pm$ 1.32  |
| EC <sub>50</sub>                         | <b>25.01<math>\pm</math>5.08 <math>\mu\text{g/ml}</math></b> |  | <b>2.2<math>\pm</math>0.2 <math>\mu\text{g/ml}</math></b> |

**Table 5.8 DPPH Scavenging assay of M8 melanin and Trolox**

| Concentration M8<br>( $\mu\text{g/ml}$ ) | Inhibition% (I%)   | Concentration Trolox<br>( $\mu\text{g/ml}$ ) | Inhibition% (I%)  |
|--|--|--|---|
| 20                                       | 36.92 $\pm$ 1.36   | 0.5 $\mu\text{g}$                            | 8.30 $\pm$ 0.03   |
| 60                                       | 56.25 $\pm$ 1.18   | 1.0 $\mu\text{g}$                            | 17.26 $\pm$ 0.30  |
| 100                                      | 80.65 $\pm$ 0.21   | 1.5  | 31.55 $\pm$ 2.89  |
| 140                                      | 88.89 $\pm$ 0.3  | 2.0  | 47.22 $\pm$ 0.17  |
| 180                                      | 88.9 $\pm$ 0.1   | 4.0  | 68.65 $\pm$ 0.97  |
| -  | -  | 8.0  | 73.21 $\pm$ 0   |
| EC <sub>50</sub>                         | <b>35.49<math>\pm</math>6.19 <math>\mu\text{g/ml}</math></b> |  | <b>2.73<math>\pm</math>0.91 <math>\mu\text{g/ml}</math></b> |

### 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  $\mu\text{g/ml}$  was 17.66  $\pm$  2.12% which increased in a concentration dependent manner and attained an inhibition % of 61.95  $\pm$  0.57% at a concentration of 40  $\mu\text{g/ml}$  as shown (Table 5.7). The effective concentration (EC<sub>50</sub>) of M8 melanin which effects scavenging of 50% ABTS radicals was calculated as 25.01 $\pm$ 5.08  $\mu\text{g/ml}$ . The EC<sub>50</sub> of standard trolox on ABTS scavenging was calculated as 2.2  $\pm$  0.2  $\mu\text{g/ml}$ .

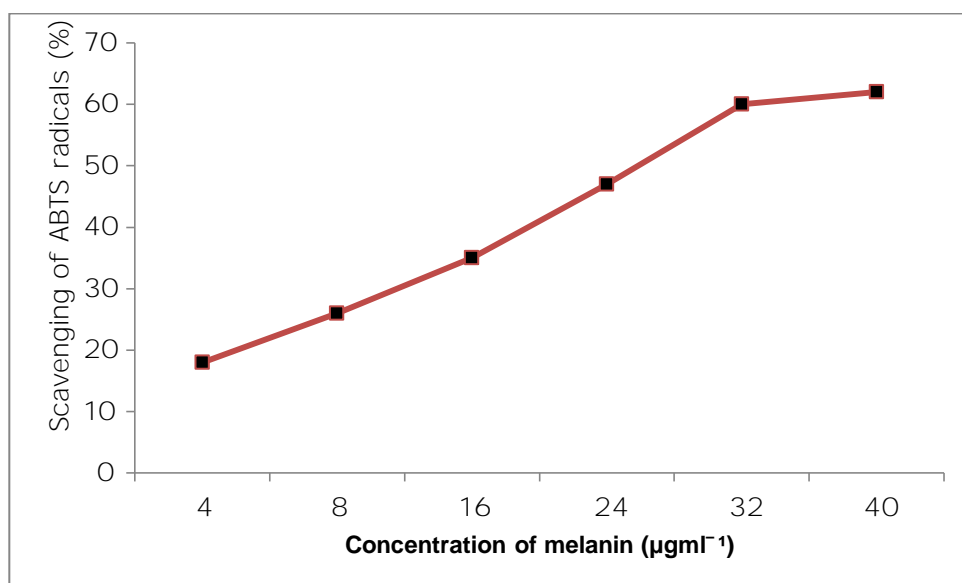
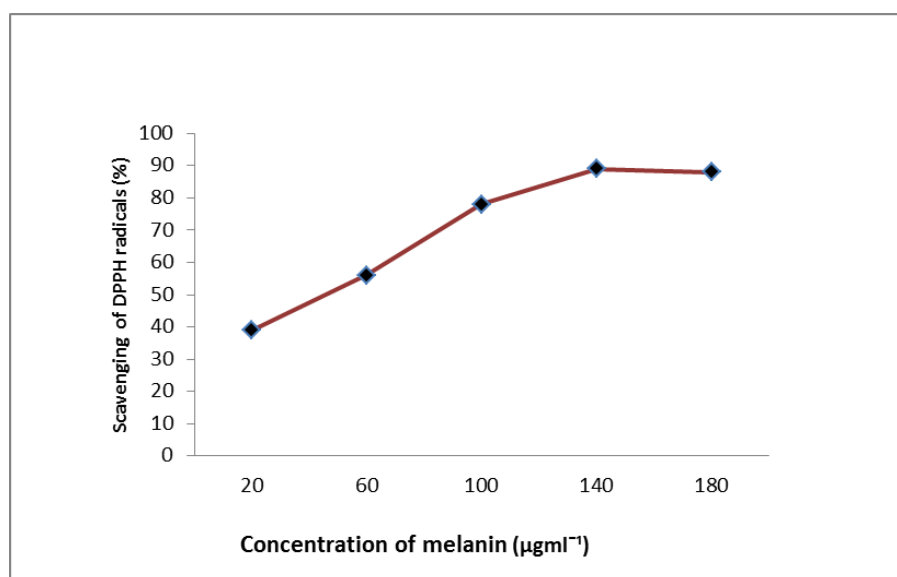


Fig. 5.12 Scavenging ability of M8 melanin on ABTS radicals

### 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  $\mu\text{g/ml}$ , the inhibition % was  $36.92 \pm 1.36$ , which increased in a concentration dependent manner to  $85.98 \pm 1.29\%$  at a concentration of 120  $\mu\text{g/ml}$ . The effective concentration ( $\text{EC}_{50}$ ) of M8 melanin which effects scavenging of 50% DPPH radicals was calculated as  $35.49 \pm 6.19 \mu\text{g/ml}$ . The  $\text{EC}_{50}$  of standard Trolox on DPPH scavenging was calculated as  $2.73 \pm 0.91 \mu\text{g/ml}$  (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.

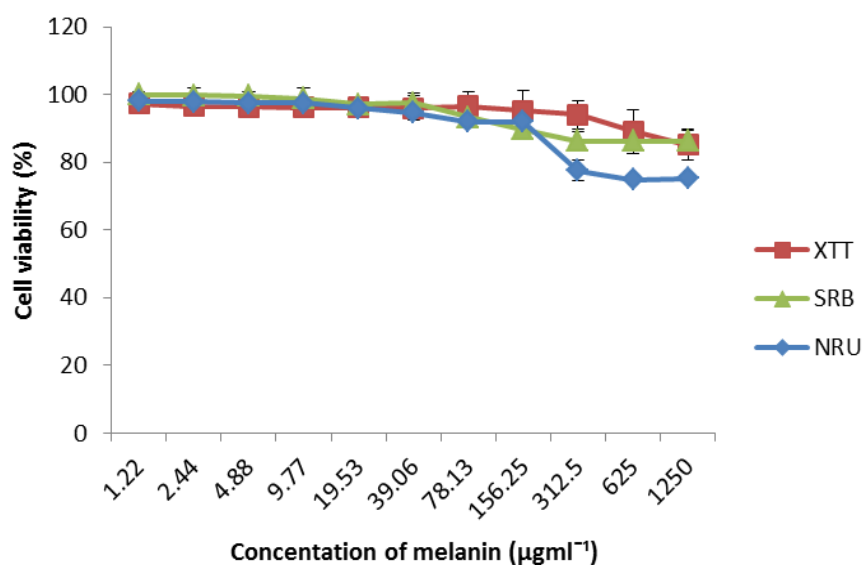
#### **5.3.7.1 XTT assay**

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  $156.25 \mu\text{g ml}^{-1}$ . At a concentration of  $156.25 \mu\text{g ml}^{-1}$  only <5% inhibition of

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

### 5.3.7.2 Neutral red uptake (NRU) assay

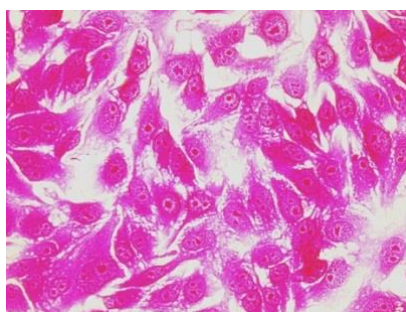
In the case of neutral red uptake assay, the viability was maintained up to a concentration of 156.25  $\mu\text{g ml}^{-1}$  and only a small percent loss of viability (8%) in terms of plasma membrane damage was observed. The viability in terms of neutral red uptake was found to be decreasing progressively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



**Fig. 5.14** Biocompatibility assay of melanin (3-bis [2- methoxy 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*

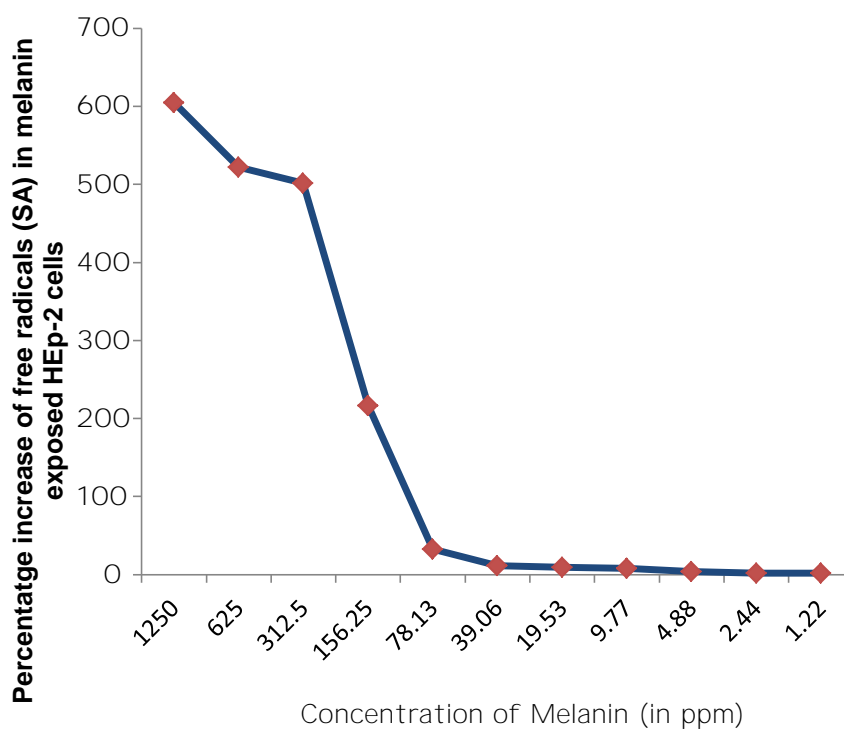
There was not much inhibition of protein synthesis up to a concentration of  $156.25 \mu\text{g ml}^{-1}$ . Approximately, 10% inhibition of protein synthesis was observed at  $156.25 \mu\text{g ml}^{-1}$  and reached a maximum of <15% inhibition of protein synthesis at  $1250 \mu\text{g ml}^{-1}$  concentration (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*

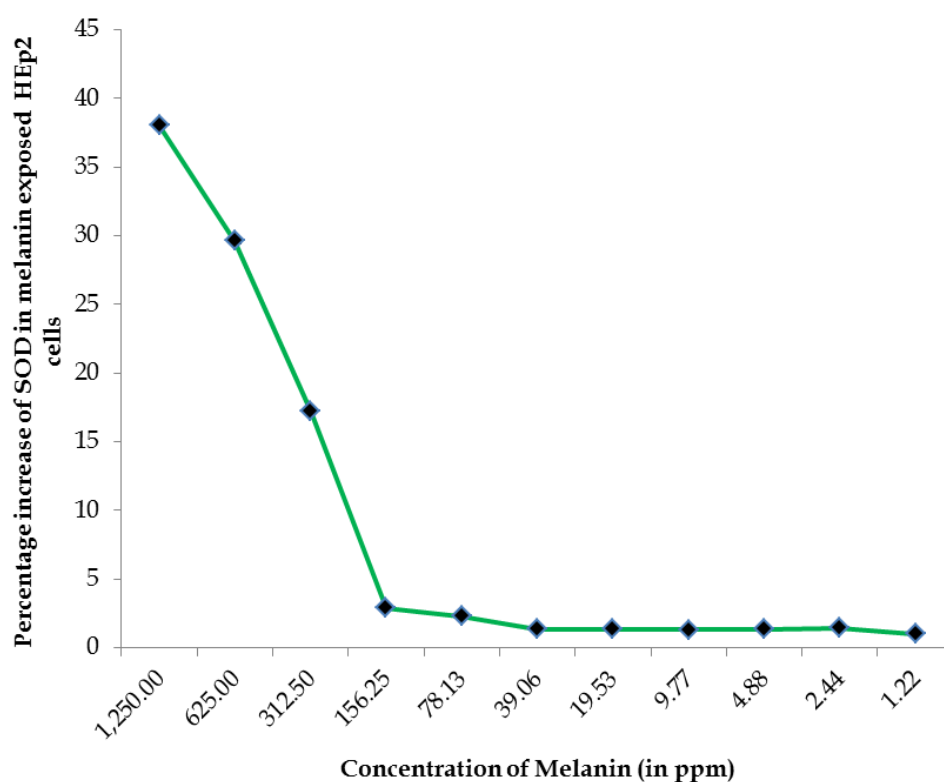
Exposure of HEp-2 cells to melanin resulted in the generation of free radicals in a dose dependent manner (Fig. 5.16). Basal production of free radicals by untreated cells was determined and this increased in the presence of M8 melanin to a maximum of ~600 % at the highest concentration of melanin (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



**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  $156.25 \mu\text{g ml}^{-1}$  of melanin and 38% when the melanin concentration was  $1250 \mu\text{g ml}^{-1}$ .

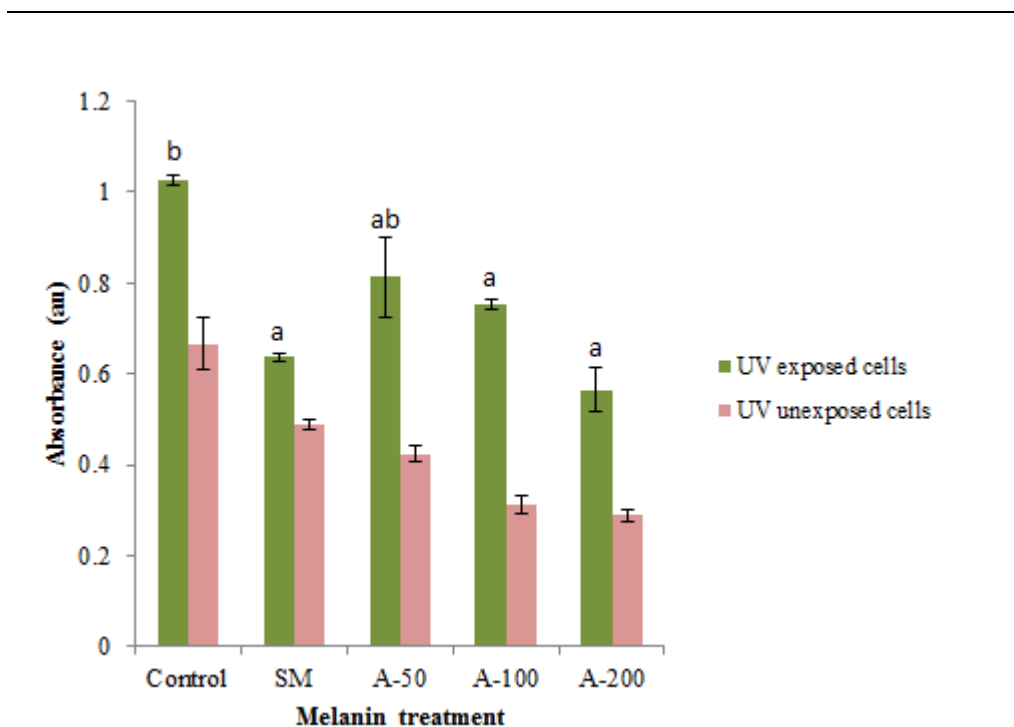


**Fig. 5.17** Melanin induced Super oxide dismutase (SOD) production in human HEp-2 cancer cell line. The Y axis shows the percentage increase of SOD based on scavenging property on free radicals (n=3)

### 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  $\mu\text{g ml}^{-1}$ , 100  $\mu\text{g ml}^{-1}$ ; and 200  $\mu\text{g ml}^{-1}$  respectively

There was not much significant variation in the ROS generated between synthetic melanin (50  $\mu\text{g ml}^{-1}$ ) treated HEP-2 cells and M8 melanin treated cells at concentrations 100  $\mu\text{g ml}^{-1}$  and 200  $\mu\text{g ml}^{-1}$  ( $p < 0.05$ ). 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  $\mu\text{g ml}^{-1}$  and the synthetic melanin used as positive control, scavenged 37.93% of the ROS at the same concentration. At 100  $\mu\text{g ml}^{-1}$  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  $\mu\text{g ml}^{-1}$  where M8

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

**Table 5.9 Phototoxicity assay of melanin (M8)**

| Melanin ( $\mu\text{g ml}^{-1}$ ) | 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 *Streptomyces*. 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 *Streptomyces*.

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<sup>-1</sup> which is comparable with that of yeast melanin synthesized by *Yarrowia lipolytica* (160 mg l<sup>-1</sup>) and tyrosine-mediated melanin production (130 mg l<sup>-1</sup>) 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  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  area of *Sepia* melanin; also spectra of *Sepia* presented a bending vibration in the 1400-1300  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  which also implies the presence of a substantial amount of aliphatic groups in the M8 melanin structure. Free carboxylic groups (1715.4  $\text{cm}^{-1}$ ) 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  $\sim 15$  nm (Simon et al., 2000). Structural order is also lacking in the case of melanin produced by *S. bikiniensis*.

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  $73.7 \pm 12.6 \mu\text{g ml}^{-1}$ , 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 ( $25.09 \pm 5.08 \mu\text{g ml}^{-1}$ ) was only 12 times lower than the standard trolox ( $2.2 \pm 0.2 \mu\text{g ml}^{-1}$ ). The ethyl acetate extract of *Streptomyces* sp. isolated from rhizosphere of *curcumae longae* exhibited an  $IC_{50}$  of  $172.43 \pm 22.19 \mu\text{g ml}^{-1}$  for ABTS, which was 220 times lower than the positive control (trolox  $0.76 \mu\text{g ml}^{-1}$ ). 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<sub>50</sub> of M8 melanin for DPPH scavenging was determined as  $35.49 \pm 6.19 \mu\text{g ml}^{-1}$ . 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  $100 \mu\text{g ml}^{-1}$  the inhibition percentage exhibited by M8 melanin was  $80.65 \pm 0.21\%$ . Recently, purified melanin extracted from marine actinobacteria, *Actinoalloteichus* sp. MA-32, exhibited DPPH radical-scavenging activity with an EC<sub>50</sub> value of  $85 \mu\text{g ml}^{-1}$  (Manivasagan et al., 2013). The EC<sub>50</sub> exhibited by purified melanin (ABM) pigment from *A. bridgeri* exhibited a significant DPPH radical scavenging activity of  $54.12 \mu\text{g ml}^{-1}$  (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<sub>50</sub> of  $21.50 \mu\text{g/ml}$  (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<sub>50</sub> of ethyl acetate extract of *Streptomyces* sp. isolated from rhizosphere of curcuma longae for DPPH radical scavenging was  $842.18 \pm 161.24 \mu\text{g/ml}$  which was 200 times lower than the trolox standard (Zhong et al., 2011). In the present study, the EC<sub>50</sub> of extracted M8 melanin on DPPH radicals was only 13 times lower than the trolox standard which is much

higher than the EC<sub>50</sub> 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<sup>-1</sup>. 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<sup>-1</sup> upto a concentration of 1250 µg ml<sup>-1</sup> 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<sup>-1</sup>. However a marked shift in elevated production of super oxide anions (~200) was observed at 156.25 µg ml<sup>-1</sup>, while the value was only 32% when the concentration was 78.13 µg ml<sup>-1</sup>.

Consequently, 3% increase of SOD was observed at 156.25 µg ml<sup>-1</sup>, which further increased to 38% when the melanin concentration reached the maximum of 1250 µg ml<sup>-1</sup>. 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<sub>50</sub> of M8 melanin for various antioxidant assays viz., lipid peroxidation inhibition (73.7 ± 12.6 µg ml<sup>-1</sup>), ABTS (25.01 ± 5.08 µg ml<sup>-1</sup>) and DPPH (35.49 ± 6.19 µg ml<sup>-1</sup>) are well below the concentration of melanin

156.25  $\mu\text{g ml}^{-1}$  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  $\mu\text{g ml}^{-1}$ . 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 Streptomyces 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 antifungal 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 hygrosopicus* 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 mosquito 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, *Spodoptera 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 *Streptomyces* 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**

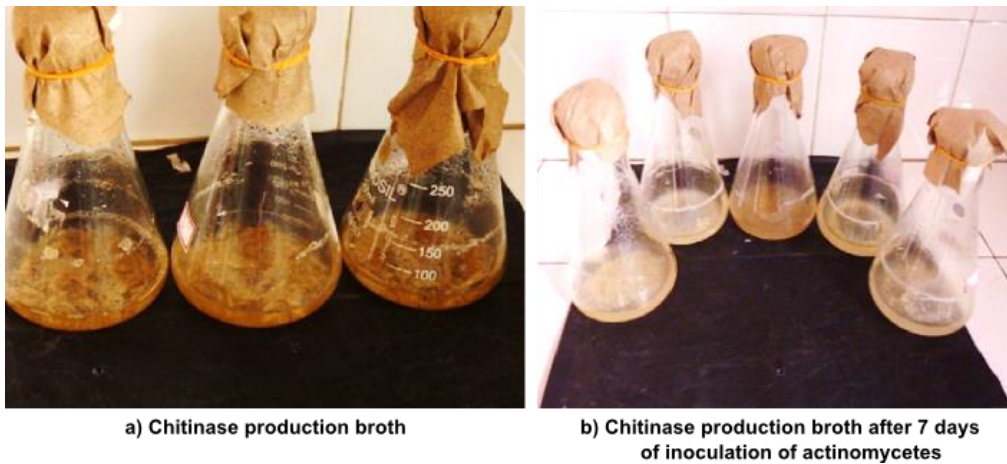
##### **Fungal pathogens**

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), *Paecilomyces liliacinum* (SF166), *Mucor* sp. (SF112), and *Scopulariopsis brevicaulis* (1469). The yeast cultures were obtained from IMTECH Chandigarh and the filamentous 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 (Tsuji 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.



**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  $25 \pm 2^\circ\text{C}$  (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 28°C 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

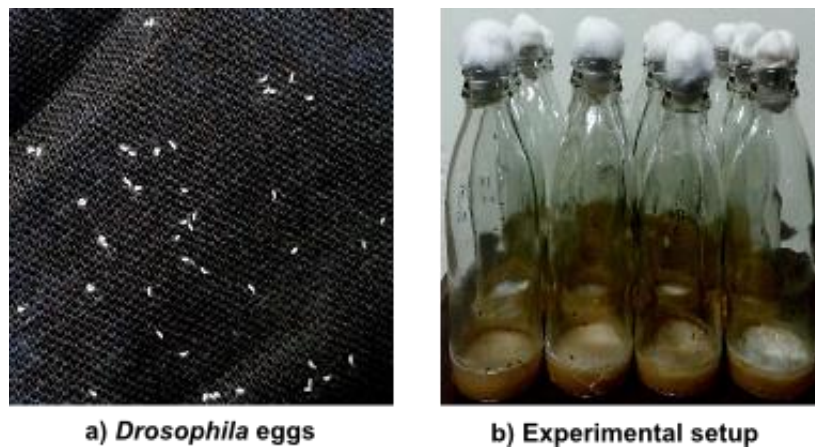
The three potential chitinolytic actinomycete isolates (J18, M32, L128) (clear zone >15 mm) in chitin agar plates, were tested for their ability to inhibit the growth and development of *Drosophila* under controlled laboratory conditions.

#### 6.2.1.2.4 Actinomycete preparation

Actinomycetes were cultured in colloidal chitin agar and incubated at 28°C 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 ( $3 \times 10^7$  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 ( $3 \times 10^6$  spores/ml) and 0.01 ( $3 \times 10^5$  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 25°C for approximately 9-12 days, until pupation. Successful pupations were counted at the end of the experiment (Fig. 6.2).



**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:

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

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:

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

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  $\pm$  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<sub>50</sub> values were calculated by probit analysis. Statistical analyses was done using software SPSS (version 17.0 for Windows, SPSS Inc.).

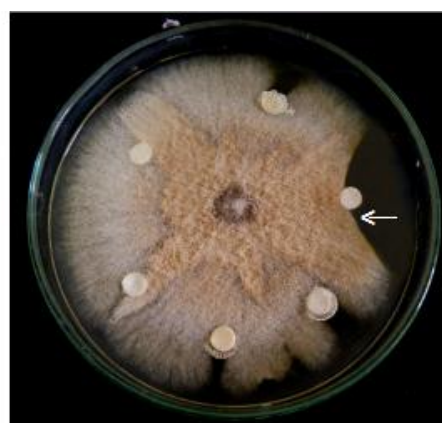
## **6.3 Results**

### **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 crescentic 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*, *Paecilomyces lilacinum* and *Aspergillus versicolor*. Isolate L128 exhibited activity against *Penicillium verrucosum*, *Penicillium commune*, *Geotrichum candidum*, and *Scopulariopsis brevicolis*. Chitinase extract from L92 and M32 exhibited activity against only a single fungal pathogen, *Penicillium verrucosum* and *Scopulariopsis brevicolis* respectively.

**Table 6.1 Antifungal activity of marine actinomycetes**

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

**Penicillium 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

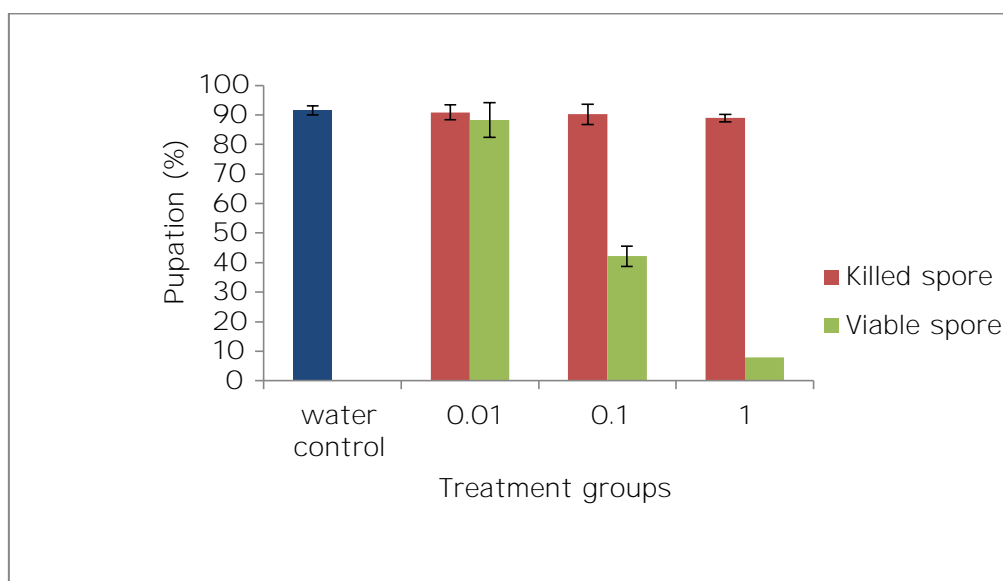
Spore suspensions of marine actinomycetes J18 incorporated into *Drosophila* rearing media exhibited significant reduction in pupation compared to controls in the larval development of *Drosophila melanogaster*.

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 <sup>c</sup> | 8.42 ± 1.54 <sup>a</sup>  | 6.66 ± 0.47 <sup>a</sup> |
| Killed spore (0.01OD)  | 90.89 ± 2.54 <sup>c</sup> | 9.11 ± 2.54 <sup>a</sup>  | 6.3 ± 0.47 <sup>a</sup>  |
| Viable spore(0.01 OD)  | 88.33 ± 6.03 <sup>c</sup> | 11.67 ± 6.03 <sup>a</sup> | 7.6 ± 0.47 <sup>ab</sup> |
| Killed spore (0.1 OD)  | 90.20 ± 3.39 <sup>c</sup> | 9.80 ± 3.39 <sup>a</sup>  | 6.3 ± 0.47 <sup>a</sup>  |
| Viable spore (0.1 OD)  | 42.17 ± 5.89 <sup>b</sup> | 57.83 ± 5.89 <sup>b</sup> | 8.6 ± 0.94 <sup>b</sup>  |
| Killed spore (1 OD)  | 88.94 ± 1.21 <sup>c</sup> | 11.06 ± 1.21 <sup>a</sup> | 7 ± 0.81 <sup>a</sup>    |
| Viable spore (1 OD)  | 7.87 ± 3.41 <sup>a</sup>  | 92.13 ± 3.41 <sup>c</sup> | 8.6 ± 0.47 <sup>b</sup>  |
| <b>IC<sub>50</sub>=0.07±0.02 (1.9 x 10<sup>6</sup>) spores /ml</b> |                           |                           |                          |

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.

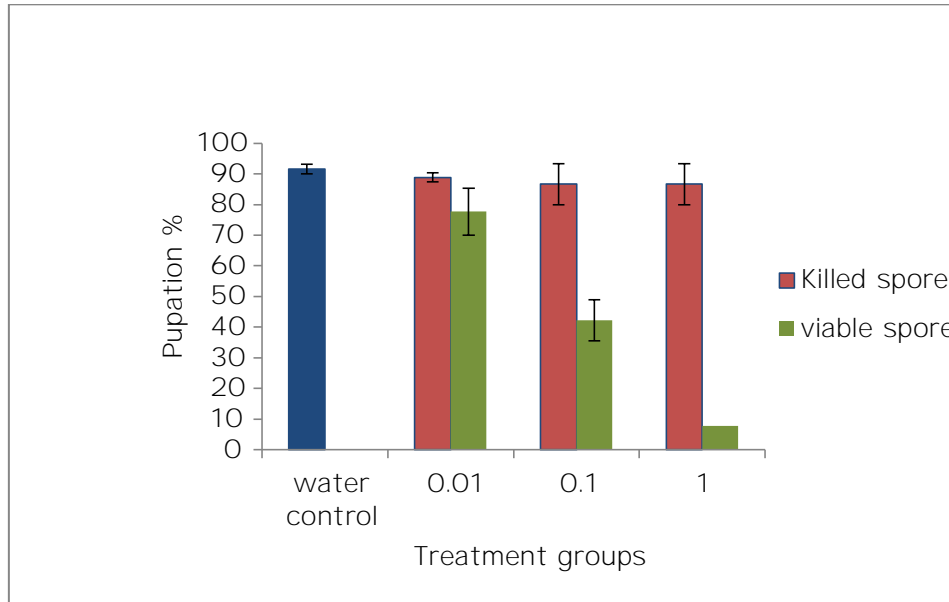
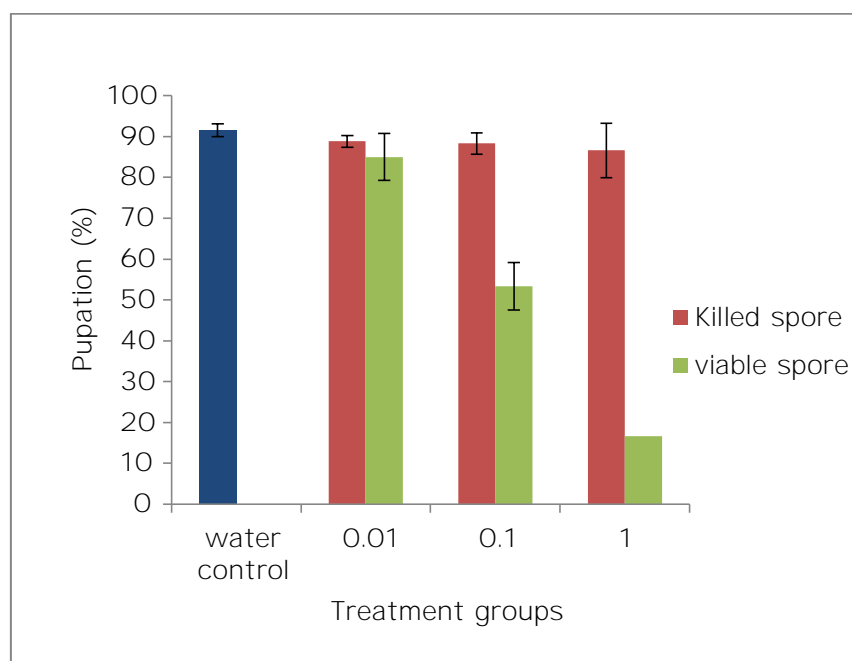


Fig. 6.5 Effect of different spore concentrations of M32 on pupation of *Drosophila melanogaster*

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 <sup>d</sup>  | 8.42 ± 1.54 <sup>a</sup>   | 6.66 ± 0.47 <sup>a</sup> |
| Killed spore (0.01OD)   | 88.87 ± 1.43 <sup>d</sup>  | 11.13 ± 1.43 <sup>a</sup>  | 6.33 ± 0.47 <sup>a</sup> |
| Viable spore(0.01OD)  | 77.67 ± 4.04 <sup>c</sup>  | 22.33 ± 4.04 <sup>b</sup>  | 7.33 ± 0.47 <sup>a</sup> |
| Killed spore (0.1 OD)   | 86.63 ± 6.65 <sup>cd</sup> | 13.37 ± 6.65 <sup>ab</sup> | 6.66 ± 0.47 <sup>a</sup> |
| Viable spore (0.1 OD)   | 35.29 ± 5.89 <sup>b</sup>  | 64.71 ± 5.89 <sup>c</sup>  | 8.33 ± 0.47 <sup>b</sup> |
| Killed spore (1 OD)   | 86.63 ± 6.65 <sup>cd</sup> | 13.37 ± 6.65 <sup>ab</sup> | 7 ± 0 <sup>a</sup>       |
| Viable spore (1 OD)   | 6.67 ± 6.67 <sup>a</sup>   | 93.33 ± 6.67 <sup>d</sup>  | 8.6 ± 0.47 <sup>b</sup>  |
| <b>IC<sub>50</sub> = 0.045 ± 0.02 OD (1.5 × 10<sup>6</sup> spores/ml)</b> |                            |                            |                          |

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*

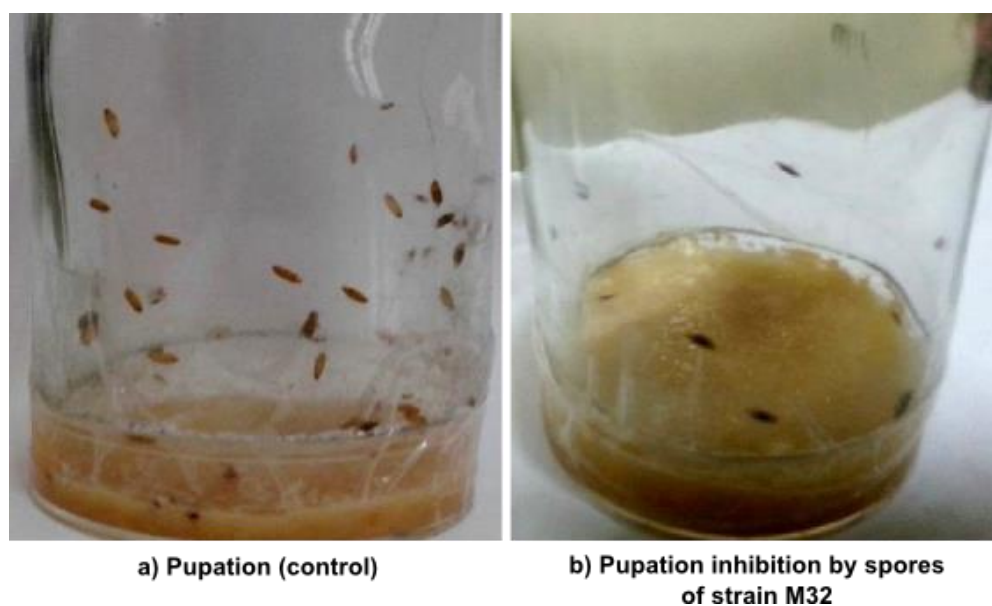
**Table 6.4** Comparison of various spore concentrations of strain L128 on larval development of *D. melanogaster*

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

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  $0.045 \pm 0.02$  approximately  $1.5 \times 10^6$  spores/ml, for strain M32,  $0.07 \pm 0.02$  approximately  $1.9 \times 10^6$  spores /ml for strain J18 and  $0.088 \pm 0.03$  approximately  $2.6 \times 10^6$  spores/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 rectiflexibles (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

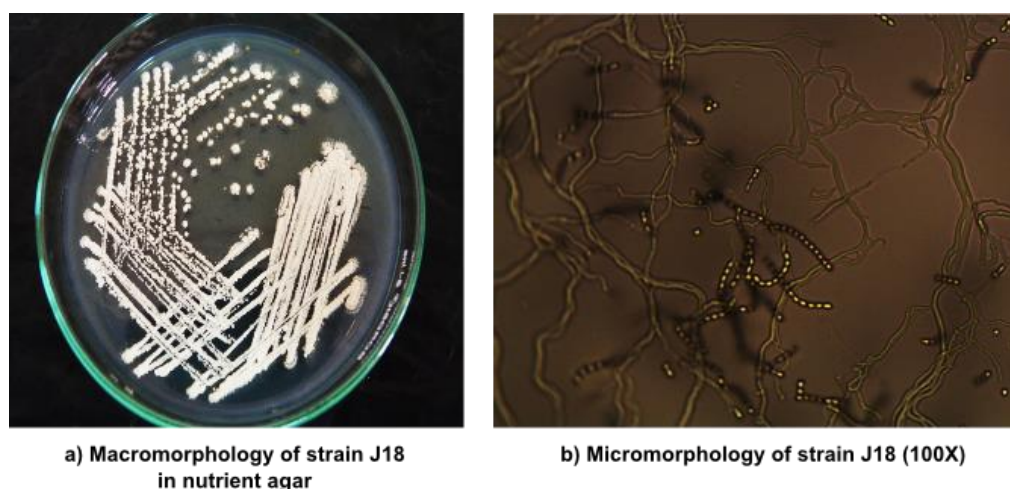


mycelium (Fig. 6.9). Micromorphology of the strain revealed sporophores as rectiflexibles.

### **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<sub>2</sub>S. 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<sub>2</sub>S. 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.**

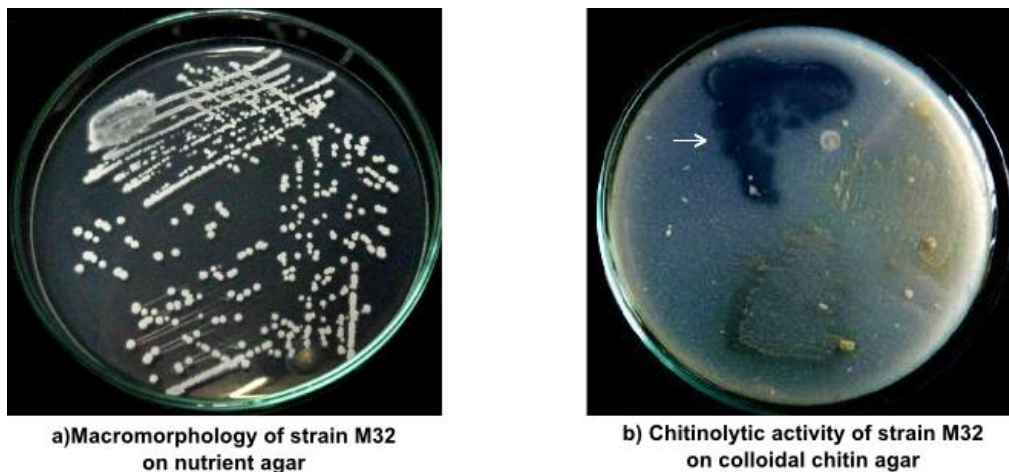
The 1049 bp sequence of culture J18 and the 1023 bp sequence of strain M32 determined were submitted to GenBank database under the accession number **KJ158472** and **KC570321** respectively.

#### **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,  $\beta$ -methyl-D-glucoside, D-piscose, rhamnose, ribose, stachyose, arabinose, cellobiose, fructose, D-galactose,  $\alpha$ -methyl-D-galactoside,  $\beta$ - 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  $\alpha$ -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.



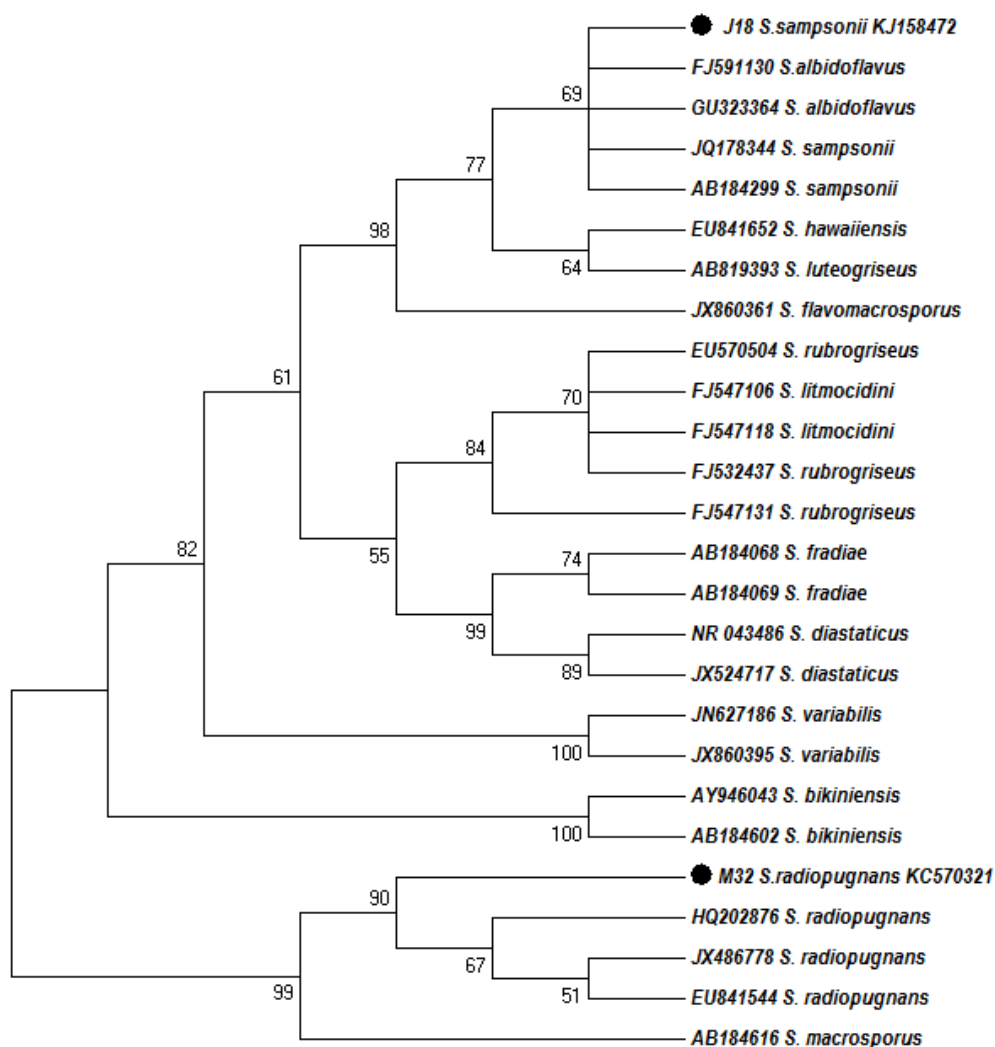
**Fig. 6.9 Morphology and chitinolytic activity of strain M32**

**Table 6.5 Morphological and biochemical characteristics of actinomycete J18 and M32**

| Characteristic                                |  | Result          |         |
|---|--|-----------------|---------|
|   |  | J18             | M32     |
| <b>Morphological</b>                          | 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      |
| <b>Biochemical reactions</b>                  | 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 <sub>2</sub> S production                          | +               | -       |
|   | Urea Hydrolysis                                      | +               | +       |
|   | Citrate utilization                                  | +               | -       |
| <b>Acid Production<br/>from carbohydrates</b> | Lactose  | -               | -       |
|   | Mannitol   | +               | -       |
|   | Glucose  | +               | +       |
|   | Arabinose  | +               | -       |
|   | Rhamnose   | +               | +       |
|   | Galactose  | +               | +       |
|   | Inositol   | -               | -       |
|   | Trehalose  | +               | -       |
|   | Sorbitol   | -               | -       |
|   | Xylose   | +               | -       |
| <b>Resistance to Antibiotics</b>              | Penicillin G (10 i.u)                                | +               | +       |
|   | Tobramycin(10µg)                                     | -               | -       |
|   | Rifampicin (30 µg)                                   | -               | -       |
|   | Cephaloridine( 30µg)                                 | +               | +       |
|   | Streptomycin (25 µg)                                 | -               | -       |
|   | Neomycin (30 µg)                                     | -               | -       |
|   | Gentamicin (10 µg)                                   | -               | -       |
|   | Vancomycin (30 µg)                                   | -               | -       |
| <b>Hydrolytic Enzyme<br/>Activity</b>         | 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 |
| $\alpha$ -Cyclodextrin        | -      | +   | L-Arabinose              | +      | +   | $\alpha$ -D-Lactose               | -      | -   |
| $\beta$ -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              | +      | -   | $\alpha$ -Methyl-D galactoside    | +      | -   |
| N-Acetyl $\beta$ -            | -      | -   | D-Gluconic acid          | +      | -   | B-Methyl-D-galactoside            | +      | -   |
| Amygdalin                     | -      | -   | $\alpha$ -D- Glucose     | +      | +   | 3-Methyl-D glucose                | -      | -   |
| D-Tagatose                    | -      | -   | m-Inositol               | -      | -   | $\alpha$ -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                           | -      | -   |
| $\alpha$ -Hydroxybutyric acid | -      | -   | L-Glutamic acid          | +      | -   | Adenosine-5                       | -      | -   |
| $\beta$ -Hydroxybutyric acid  | -      | -   | Glycyl -L- Glutamic acid | -      | -   | Thymidine5-<br>monophosphate      | -      | -   |
| $\gamma$ -Hydroxybutyric acid | -      | -   | L-Pyrogutamic acid       | -      | -   | Uridine-5-monophosphate           | -      | -   |
| P-Hydroxyphenyl acetic        | -      | -   | L -Serine                | -      | -   | D-Fructose-6-Phosphate            | -      | -   |
| $\alpha$ -Ketoglutaric acid   | -      | -   | Putrescine               | -      | -   | $\alpha$ -D-Glucose-1 phosphate   | -      | -   |
| $\alpha$ -Ketovaleric acid    | -      | -   | 2,3-Butanediol           | -      | -   | D-glucose-6- phosphate            | -      | -   |
| $\beta$ -methyl-D glucoside   | +      | -   | Glycerol                 | +      | +   | D-L- $\alpha$ -glycerol Phosphate | -      | -   |
| $\alpha$ -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, *Pseudomonas 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 2<sup>nd</sup> and 3<sup>rd</sup> 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  $3 \times 10^6$  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 mosquito *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. luteogriseus*. 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:

- 3 Morphological, biochemical and physiological characterization of marine actinomycetes (230 Nos.) revealed *Streptomyces* (76%) as the dominant genera followed by *Nocardiopsis* sp. (24%).
- 3 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.

- <sup>3</sup> Pentose sugar rhamnose induced heavy growth and sporulation in marine actinomycetes.
- <sup>3</sup> *Streptomyces* and *Nocardioopsis* were almost equivocal in their biochemical and decomposition profile.
- <sup>3</sup> Self-immobilization property (biogranulation) was exhibited by marine actinomycetes (82%) in shake flask cultures in nutrient broth.
- <sup>3</sup> 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 *Nocardioopsis*, the *Streptomyces* isolates displayed better ability to degrade compounds such as chitin, pectin and lignin.
- <sup>3</sup> Melanin production ability was exhibited by only genus *Streptomyces* (4%).
- <sup>3</sup> 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.

- 3 A total of eight polymorphic patterns were obtained for *Sau3A1*, ten patterns for *Taq1* and only six patterns for *Hinf1* digestions of 16S amplicons of the actinomycete isolates.
- 3 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 *Nocardiopsis*.
- 3 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.
- 3 *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.
- 3 Actinomycete isolate *Streptomyces rubrolavendulae* M56 from Bay of Bengal exhibited broad spectrum antibacterial activity.
- 3 *Streptomyces rubrolavendulae* (M56) could significantly inhibit *Vibrio* spp. by *in vitro* co-culture exclusion experiments.
- 3 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.

- <sup>3</sup> 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.
- <sup>3</sup> 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<sup>-1</sup> (CH<sub>2</sub>-CH<sub>3</sub> bending) characteristic of melanin pigment.
- <sup>3</sup> 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.
- <sup>3</sup> The most abundant element detected in M8 melanin is iron followed by calcium, sodium, potassium and magnesium.
- <sup>3</sup> 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).

- 3 Melanin exhibited significant antioxidant activity as exemplified by lipid peroxidation inhibition ( $IC_{50}=73.70 \pm 12.6 \mu\text{g ml}^{-1}$ ), ABTS ( $IC_{50} = 25.09 \pm 5.08 \mu\text{g ml}^{-1}$ ) and DPPH ( $IC_{50} = 35.49 \pm 6.19 \mu\text{g ml}^{-1}$ ) radical scavenging assays.  $IC_{50}$  of the melanin (M8) on ABTS radicals was only 12 times lower than the standard trolox ( $2.2 \pm 0.2 \mu\text{g ml}^{-1}$ ); the activity can be improved on purification.
- 3 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  $156.25 \mu\text{g ml}^{-1}$ .
- 3 Antioxidant activity of melanin (M8) in human cancer cells (HEp-2) was confirmed by super oxide dismutase and super oxide anion assays.
- 3 Crude Melanin (M8) at  $200 \mu\text{g ml}^{-1}$  could scavenge 44 % reactive oxygen species of HEp-2 cells generated on UV exposure, which was comparable to positive control synthetic melanin at  $50 \mu\text{g ml}^{-1}$ .
- 3 Crude melanin extracted from *S. bikiniensis* (M8) has proved to be a promising antioxidant and photoprotectant.
- 3 Chitinolytic actinomycete J18 from sediments of Arabian Sea identified as *Streptomyces sampsonii* exhibited remarkable antifungal property.

- <sup>3</sup> Actinomycete M32 isolated from Bay of Bengal identified as *Streptomyces radiopugnans* exhibited potent insecticidal activity with an IC<sub>50</sub> of 1.5 x10<sup>6</sup> spores/ml.
- <sup>3</sup> 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 *Nocardiopsis*. 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 Streptomyces 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.

## Bibliography

---

- Acinas, S.G., Klepac Ceraj, V., Hunt, D.E., Pharino, C., Ceraj, I., Distel, D.L., Polz, M.F., 2004. Fine scale phylogenetic architecture of a complex bacterial community. *Nature* 430, 551–554.
- Adegboye, M.F., Babalola, O., 2012. Taxonomy and ecology of antibiotic producing actinomycetes. *African J. Agric. Res.* 7, 2255–2261.
- Adegboye, M.F., Babalola, O.O., 2013. Phylogenetic characterization of culturable antibiotic producing *Streptomyces* from rhizospheric soils. *Mol. Biol.* 2, 1–7.
- Agate, A.D., Bilimoria, M.H., Bhat, J.V., 1962. Pectin transeliminase activity in *Streptomyces viridochromogenes*. *Curr. Sci.* 31, 462–463.
- Agodi, A., Stefani, S., Corsaro, C., Campanile, F., Gribaldo, S., Sichel, G., 1996. Study of a melanic pigment of *Proteus mirabilis*. *Res. Microbiol.* 147, 167–174.
- Aime, S., Fasano, M., Terreno, E., Groombridge, C.J., 1991. NMR Studies of melanins: Characterization of a soluble melanin free acid from Sepia ink. *Pigment Cell Res.* 4, 216–221.
- Ai-Min, M., Xin, L., Cheng-Jie, M., 2009. Isolation and Identification of high yield melanin producing actinomycetes G-HD-4. *Food Sci.* 30, 185–188.
- Alam, M., Merlo, M., Takano, E., Breitling, R., 2010. Genome-based phylogenetic analysis of *Streptomyces* and its relatives. *Mol. Phylogenet. Evol.* 54, 763–772.
- Alexander, M., 1977. Introduction to soil Microbiology, 2nd edn. John Wiley and sons, Newyork.
- Allen, D., Caviedes, R., Cardenas, A.M., Shimahara, T., Segura Aguilar, J., Cavides, P.A., 2005. Cell lines as in vitro models for drug screening and toxicity studies. *Drug Dev. Ind. Pharm.* 31, 757–768.
- Al-Tai, A.M., Ji-Sheng, R., 1994. *Nocardiopsis halophila* sp. nov., a new halophilic actinomycete isolated from soil. *Int. J. Syst. Bacteriol.* 44, 474–478.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.
- Al-Zarban, S.S., Abbas, I., Al-Musallam, A. A, Steiner, U., Stackebrandt, E., Kroppenstedt, R.M., 2002. *Nocardiopsis halotolerans* sp. nov., isolated from salt marsh soil in Kuwait. *Int. J. Syst. Evol. Microbiol.* 52, 525–9.
- Amabile, C.F., Gardenas, G.M., Ludger, M., 1995. Antibiotic resistance. *Am. Sci.* 83, 320–329.
- Amal, A.M., Abeer, K.A., Samia, H.M., Nadia, A.E.H., Ahmed, K.A., 2011. Selection of Pigment ( Melanin ) production in *Streptomyces* and their application in printing and dyeing of Wool Fabrics. *Res. J. Chem. Sci.* 1, 22–28.
- Anderson, A.S., Wellington, E.M.H., 2001. The taxonomy of *Streptomyces* and related genera. *Int. J. Evol. Microbiol.* 51, 797-814

- Anitha, A., Rabeeth, M., 2010. Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of *Streptomyces griseus*. African J. Plant Sci. 4, 61–66.
- Anzai, K., Nakashima, T., Kuwahara, N., 2008. Actinomycete bacteria isolated from the sediments at coastal and offshore area of Nagasaki Prefecture, Japan: Diversity and biological activity. J. Biosci. Bioeng. 106, 215–217.
- Apak, R., Gorinstein, S., Bohm, V., Schaich, K.M., Ozyurek, M., Guçlu, K., 2013. Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure Appl. Chem. 85, 957–998.
- Apte, M., Girme, G., Bankar, A., Ravikumar, A., Zinjarde, S., 2013. 3, 4-dihydroxy-L-phenyl alanine derived melanin from *Yarrowia lipolytica* mediates the synthesis of silver and gold nanostructures. J. Nanobiotechnol. 11, 1-9
- Ara, I., Daram, D., Baljinova, T., Yamamura, H., Bakir, M.A., Suto, M., Ando, K., 2013. Isolation , classification, phylogenetic analysis and scanning electron microscopy of halophilic, halotolerant and alkaliphilic actinomycetes isolated from hypersaline soil. African J. Microbiol. Res. 7, 298–308.
- Arai, T., Mikami, Y., 1972. Chromogenicity of *Streptomyces*. Appl. Microbiol. 23, 402–406.
- Arasu, M.V., Duraipandiyar, V., Agastian, P., Ignacimuthu, S., 2008. Antimicrobial activity of *Streptomyces* spp. ERI-26 recovered from Western Ghats of Tamil Nadu. J. Med. Mycol. 18, 147–153.
- Arifuzzaman, M., Khatun, M.R., Rahman, H., 2010. Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity. African J. Biotechnol. 9, 4615–4619.
- Asha, N.K., Devi, Jeyarani, M., Balakrishnan, K., 2006. Isolation and identification of marine actinomycetes and their potential in antimicrobial activity. Pakistan J. Biol. Sci. 9, 470–472.
- Atta, H., 2007. Production of vitamin B12 by *Streptomyces fulvissimus*. Egypt. J. Biomed. Sci. 23, 1–19.
- Atta, H.M., 2012. Biochemical studies on antibiotic production from *Streptomyces* sp: Taxonomy, fermentation, isolation and biological properties. J. Saudi Chem. Soc.
- Atta, H.M., El-Sehrawi, M.H., Awany, N.M., El-Mesady, N.I., 2011. Microbiological Studies on Cultural, Physiological characteristics and antimicrobial activities of *Streptomyces Cyaneus* AZ-13Zc. Researcher 3, 80–90.
- Attimarad, S.L., Ediga, G.N., Karigar, A.A., Karadi, R., Chandrashekhar, S., Chandrashekhar, N., 2012. Screening , isolation and purification of antibacterial agents from marine actinomycetes. Int. Curr. Pharm. J. 1, 394–402.
- Austin, B., Billaud, A., 1990. Inhibition of the fish pathogen, *Serratia liquefaciens*, by an antibiotic producing isolate of *Planococcus* recovered from seawater. J. Fish Dis. 13, 553–556.
- Austin, B., Stuckey, L.E., Robertson, P.A.W., Effendi, I., Griffith, D.R.W., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing disease caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalli*. J. Fish Dis. 18, 93–96.

- 
- Babalola, O., Kirby, B., Le Roes-Hill, M., Cook, A., Cary, S., Burton, S., DA, C., 2009. Phylogenetic analysis of actinobacterial populations associated with Antarctic dry valley mineral soils. *Environ. Microbiol.* 11, 566–576.
- Babitskaya, V.G., Scherba, V. V., Filiminova, T. V., Grigorchuk, E.A., 2000. Melanin pigments from the fungi *Paecilomyces variotii* and *Aspergillus carbonarius*. *Appl. Biochem. Microbiol.* 36, 128–133.
- Babu, J., Pramod, W.R., George, T., 2008. Cold active microbial lipases: Some hot issues and recent developments. *Biotechnol. Adv.* 26, 457–470.
- Balachandran, C., Duraipandiyar, V., Ignacimuthu, S., 2012. Purification and characterization of protease enzyme from actinomycetes and its cytotoxic effect on cancer cell line (A549). *Asian Pac. J. Trop. Biomed.* 2, S392–400.
- Ballav, S., Dastager, S. G., Kerkar, S., 2012. Biotechnological significance of Actinobacterial research in India . *Recent. Res. Sci. Techol.* 4, 31–39.
- Baltz, R., 2008. Renaissance in antibacterial discovery from actinomycetes. *Curr. Opin. Pharmacol.* 8, 557–563.
- Baltz, R.H., 2005. Antibiotic discovery from actinomycetes: will a renaissance follow the decline and fall? *SIM News* 55, 186–196.
- Baltz, R.H., 2007. Antimicrobials from Actinomycetes: Back to the future. *Microbe* 2, 125–131.
- Banerjee, S., Devaraja, T.N., Shariff, M., Yusoff, F.M., 2007. Comparison of four antibiotics with indigenous marine *Bacillus* spp . in controlling pathogenic bacteria from shrimp and *Artemia*. *J. Fish Dis.* 30, 383–389.
- Barboza-Corona, J., Nieto-Mazzocco, E., Velazquez-Robledo, R., Salcedo-Hernandez, R., Bautista, M., Jimenez, B., 2003. Cloning, sequencing and expression of the chitinase gene *chiA74* from *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 69, 1023–1029.
- Barcina, I., Iriberry, J., Egea, L., 1987. Enumeration, isolation and some physiological properties of actinomycetes from sea water and sediment. *Syst. Appl. Microbiol.* 10, 85–91.
- Baskaran, R., Vijayakumar, Mohan, P., 2011. Enrichment method for the isolation of bioactive actinomycetes from mangrove sediments of Andaman Islands India. *Malays. J. Microbiol.* 7, 26–32.
- Bell, A., Wheeler, M., 1986. Biosynthesis and functions of fungal melanins. *Annu. Rev. Phytopathol.* 24, 411–451.
- Berd, D., 1973. Laboratory Identification of Clinically Important Aerobic Actinomycetes. *Appl. Microbiol.* 25, 665–681.
- Berdy, J., 2005. Bioactive microbial metabolites. *J. Antibiot.* 58, 1–26.
- Bernan, V., Filpula, D., Herber, W., Bibb, M., Katz, E., 1985. The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product. *Gene* 37, 101–110.
-

- Beyazova, M., Lechevalier, M.P., 1993. Taxonomic utility of restriction endonuclease fingerprinting of large DNA fragments from *Streptomyces* strains. *Int. J. Syst. Bacteriol.* 43, 674–682.
- Beyer, M., Diekmann, H., 1985. The chitinase system of *Streptomyces* sp. ATCC 11238 and its significance for fungal cell wall degradation. *Appl. Microbiol. Biotechnol.* 23, 140–146.
- Bhave, S. V., Shanbhag, P. V., Sonawane, S.K., Parab, R.R., Mahajan, G.B., 2013. Isolation and characterization of halotolerant *Streptomyces radiopugnans* from Antarctica soil. *Letts. Appl. Microbiol.* 56, 348–355.
- Bibb, M., 2005. Regulation of secondary metabolism in Streptomycetes. *Curr. Opin. Microbiol.* 8, 208–215.
- Bilgihan, A., Bilgihan, M.K., Akata, R.F., Aricioglu, A., Hasanreisolu, B., 1995. Antioxidative role of ocular melanin pigment in the model of lens induced uveitis. *Free Radic. Biol. Med.* 19, 883–885.
- Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 181, 1199–1200.
- Boers, S., van der Reijden, W., Jansen, R., 2012. High-throughput multilocus sequence typing: Bringing molecular typing to the next level. *PLoS One* 7, pone.0039630.
- Borodina, I., Krabben, P., Nielsen, J., 2005. Genome-scale analysis of *Streptomyces coelicolor* A3(2) metabolism. *Genome Res.* 15, 820–829.
- Bosshard, P.P., Abels, S., Zbinden, R., Bottger, E.C., Altwegg, M., 2003. Ribosomal DNA sequencing for identification of aerobic Gram-positive rods in the clinical laboratory (an 18-Month Evaluation). *J. Clin. Microbiol.* 41, 4134–4140.
- Bream, A.S., Ghazal, S.A., El-Aziz, Z.K.A., Ibrahim, S.Y., 2001. Insecticidal activity of selected actinomycetes strains against the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Biol. Wet.* 66, 503–544.
- Bredholdt, H., Galatenko, O., Engelhardt, K., Fjaervik, E., Terekhova, L.P., Zotchev, S.B., 2007. Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: Isolation, diversity and biological activity. *Environ. Microbiol.* 9, 2756–2764.
- Brenner, D.J., Staley, J.T., Krieg, N., 2001. Classification of prokaryotic organisms and the concept of bacterial speciation, in: Garrity, G.M. (Ed.), *Bergey's Manual of Systematic Bacteriology*. Vol 1. Springer-Verlag, pp. 27–31.
- Briggs, J.N., 1960. Reduction of adult house fly emergence by the effective *Bacillus* sp. on the development of immature forms. *J. insect Pathol.* 2, 418–432.
- Bruhlmann, F., Kim, K.S., Zimmermann, W., Fiechter, A., 1994. Pectinolytic enzymes from actinomycetes for the degumming of ramie bast fibres. *Appl. Environ. Microbiol.* 60, 2107–2112.
- Brunnegard, J., Grandel, S., Stahl, H., Tengberg, A., Hall, J., 2004. Nitrogen cycling in deepsea sediments of the porcupine abyssal plain NeAtlantic. *Prog. Oceanogr.* 63, 159–181.



- Brzezinska, M.S., Jankiewicz, U., Walczak, M., 2013. Biodegradation of chitinous substances and chitinase production by the soil actinomycete *Streptomyces rimosus*. Int. Biodeterior. Biodegradation 84, 104–110.
- Bull, A., Stach, J., Ward, A., Goodfellow, M., 2005. Marine actinobacteria: perspectives, challenges, future directions. Antonie Van Leeuwenhoek 87, 65–79.
- Bull, A.T., Alan, C.W., Michael, G., 2000. Search and discovery strategies for biotechnology: the paradigm shift. Microbiol. Mol. Biol. Rev. 64, 573–606.
- Bull, A.T., Stach, J.E.M., 2007. Marine actinobacteria: new opportunities for natural product search and discovery. Trends Microbiol. 15, 491–499.
- Burja, A.M., Hill, R.T., 2001. Microbial symbionts of the Australian Great Barrier Reef sponge *Candidaspongia flabellate*. Hydrobiologia 461, 41–47.
- Butler, M.J., Day, A.W., 1998. Fungal melanins: a review. Can. J. Microbiol. 44, 1115–1136.
- Case, R.J., Boucher, Y., Dahlof, I., Holmstrom, C., Doolittle, W.F., Kjelleberg, S., 2007. Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. Appl. Environ. Microbiol. 73, 278–288.
- Castillo, U., Myera, S., Brown, L., Strobel, G., Hess, W.M., Hanks, J., Reay, D., 2005. Scanning electron microscopy of some endophytic Streptomyces in snakevine-*Kennedia nigricans*. Scanning 27, 305–311.
- Chacko, S., Sharma, V., Avid, E., 2012. A comparative study on selected marine actinomycetes from Pulicat, Muttukadu, and Ennore estuaries. Asian Pac. J. Trop. Biomed. 2, S1827–S1834.
- Chakraborty, S., Khopade, A., Biao, R., Jian, W., Liu, X., Mahadik, K., 2011. Characterization and stability studies on surfactant, detergent and oxidant stable  $\alpha$ -amylase from marine haloalkaliphilic *Saccharopolyspora* sp. A9. J. Mol. Catal. B Enzym. 68, 52–58.
- Chakraborty, S., Khopade, A., Kokare, C., Mahadika, K., Chopade, B., 2009. Isolation and characterization of novel  $\alpha$ -amylase from marine *Streptomyces* sp. D1. J. Mol. Catal. B Enzym. 58, 17–23.
- Chakraborty, S., Raut, G., Khopade, A., Mahadik, K., Kokare, C., 2012. Study on calcium ion independent  $\alpha$ -amylase from haloalkaliphilic marine *Streptomyces* strain A3. Indian J. Biotechnol. 11, 427–437.
- Chen, L., Leu, W., Wang, K., Wu-Lee, Y., 1992. Copper transfer and activation of the *Streptomyces* apoxyrosinase are mediated through a complex formation between apoxyrosinase and its trans-activator MelC1. J. Biol. Chem. 267, 20100–20107.
- Chen, Y., Xie, M.Y., Nie, S.P., Li, C., Wang, Y.X., 2008. Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. Food Chem. 107, 231–241.
- Chi, Z., Zhang, T., Liu, G., Li, J., Wang, X., 2009. Production, characterization and gene cloning of the extracellular enzymes from the marine derived yeasts and their applications. Biotechnol. Adv. 27, 236–255.

- Chiaki, I., Syunpei, M., Takeshi, K., Naoko, H.S., Takuji, N., 2010. Isolation and characterization of marine and terrestrial actinomycetes using a medium supplemented with NaCl. *Actinomycetologica* 24, 12–17.
- Chun, J., Bae, K., Moon, E.Y., Jung, S.O., Lee, H.K., Kim, S.J., 2000. *Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete isolated from a saltern. *Int. J. Syst. Evol. Microbiol.* 50, 1909–1913.
- Chye, M., Zhao, K., He, Z., Ramalingam, S., Fung, K., 2005. An agglutinating chitinase with two chitin-binding domains confers fungal protection in transgenic potato. *Planta* 220, 717–730.
- Chythanya, R., Karunasagar, I., Karunasagar, I., 2002. Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. *Aquaculture* 208, 1–10.
- Clardy, J., Fischbach, M.A., Walsh, C.T., 2006. New antibiotics from bacterial natural products. *Nat. Biotechnol.* 24, 1541–1550.
- Clarke, S.D., Ritchie, D.A., Williams, S.T., 1993. Ribosomal DNA restriction fragment analysis of some closely related *Streptomyces* species. *Syst. Appl. Microbiol.* 16, 256–260.
- Clarridge, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev.* 17, 840–862
- Claus, H., Decker, H., 2006. Bacterial tyrosinases. *Syst. Appl. Microbiol.* 29, 3–14.
- Clayton, R.A., Sutton, G., Hinkle, P.S., Bult, C., Fields, C., 1995. Intraspecific variation in small-subunit rRNA sequences in GenBank: Why single sequences may not adequately represent prokaryotic taxa. *Int. J. Syst. Bacteriol.* 45, 595–599.
- Cloud, J.L., Conville, P.S., Croft, A., Harmsen, D., Witebsky, F.G., Carroll, K.C., 2004. Evaluation of partial 16S ribosomal DNA sequencing for identification of *Nocardia* species by using the Micro Seq 500 system with an expanded database. *J. Clin. Microbiol.* 42, 578–584.
- Colwell, R.R., 1970. Polyphasic taxonomy of bacteria. In: Iizuka, H., and Hazegawa, T. (Eds.), *Culture collections of microorganisms*, University Park Press, Baltimore. pp. 421–436
- Cook, A., Meyers, P., 2003. Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *Int. J. Syst. Evol. Microbiol.* 53, 1907–1915.
- Copping, L.G., 2004. *The Manual of biocontrol agents*. British Crop Prot. Council.
- Costa, T.G., Younger, R., Poe, C., Farmer, P.J., Szpoganicz, B., 2012. Studies on Synthetic and Natural Melanin and Its Affinity for Fe(III) Ion. *Bioinorg. Chem. Appl.* Article ID 712840.
- Cummins, C.S., Harris, H., 1956. A comparison of cell wall composition in *Nocardia*, *Actinomyces*, *Mycobacterium* and *Propionibacterium*. *J. Gen. Microbiol.* 15: IX.
- Dahiya, N., Tewari, R., Hoondal, G., 2006. Biotechnological aspects of chitinolytic enzymes: a review. *Appl. Microbiol. Biotechnol.* 71, 773–782.

- Dalisay, D.S., Torres, J.L., Penaverde, F.V., Rosales, S.V., 1997. *In vitro* studies on the activity of commercial probiotics against *Vibrio harveyi* and *Vibrio anguillarum*. J. Nat. Sci. 2, 91–101.
- Dang, H., Zhu, H., Wang, J., Li, T., 2009. Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough. World J. Microbiol. Biotechnol. 25, 71–79.
- Das, S., Lyla, P., Khan, S., 2006. Marine microbial diversity and ecology: Importance and future perspectives. Curr. Sci. 90, 1325–1335.
- Das, S., Lyla, P.S., Ajmal, K.S., 2008. Distribution and generic composition of culturable marine actinomycetes from the sediments of Indian continental slope of Bay of Bengal. Chinese J. Oceanol. Limnol. 26, 166–177.
- Das, S., Ward, L.R., Burke, C., 2010. Screening of marine *Streptomyces* spp. for potential use as probiotics in aquaculture. Aquaculture 305, 32–41.
- Dasgupta, N., De, B., 2004. Antioxidant activity of Piper beetle leaf extract *in vitro*. Food Chem. 88, 219–224.
- Dastager, S., Wen-Jun, L.A., Shu-Kun, T., Xin-Peng, T., Xiao-Yang, Z., Li-Hua, X., Cheng-zin, J., 2006. Separation, identification and analysis of pigment (melanin) production in *Streptomyces*. African J. Biotechnol. 5, 1131–1134.
- Decker, H., Terwilliger, N., 2000. Cops and robbers: putative evolution of copper oxygen binding proteins. J. Exp. Biol. 203, 1777–1782.
- Dehnad, A., Reza, Yeganeh, L.P., Bakhshi, R., Mokhtarzadeh, A., Soofiani, S., 2010. Investigation of antibacterial activity of Streptomyces isolates from soil samples, West of Iran. African J. Microbiol. Res. 4, 1542–1549.
- Del Marmol, V., Beermann, F., 1996. Tyrosinase and related proteins in mammalian pigmentation. FEBS Lett. 381, 165–168.
- Demain, A., Fang, A., 1995. Emerging concepts of secondary metabolism in actinomycetes. Actinomycetologica 9, 98–117.
- Demain, A., Sanchez, S., 2009. Microbial drug discovery: 80 years of progress. J. Antibiot. 62, 5–16.
- Demain, A.L., Zhang, L., 2005. Natural products and drug discovery. In: Zhang, L., Demain, A. (Eds.), Natural products: Drug discovery and therapeutics medicines. Totowa, NJ, Humana Press, pp. 3–32.
- Deng, W., Xi, D., Mao, H., Wanapat, M., 2008. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: a review. Mol. Biol. Rep. 35, 265–274.
- Devan, S.V., 1999. Environmental impact assessment. Studies on microbial population of Veli Lake. Indian J. Exp. Biol. 10, 46–58.
- Devi, N., Jeyarani, M., Balakrishnan, K., 2006. Isolation and identification of marine actinomycetes and their potential in microbial activity. Pakistan J. Biol. Sci. 9, 470–472.

- Dhanasekaran, D., Sakthi, V., Thajuddin, N., Panneerselvam, A., 2010. Preliminary evaluation of Anopheles mosquito larvicidal efficacy of mangrove actinobacteria. *Int. J. Appl. Biol. Pharm. Technol.* 1, 374 – 381.
- Dhanasekaran, D., Selvamani, S., Panneerselvam, A., Thajuddin, N., 2009. Isolation and characterization of actinomycetes in Vellar Estuary, Annagkoil, Tamil Nadu. *African J. Biotechnol.* 8, 4159–4162.
- Dharmaraj, S., 2010. Marine *Streptomyces* as a novel source of bioactive substances. *World J. Microbiol. Biotechnol.* 26, 2123–2139.
- Dhevendaran, K., Annie, M.K., 1999. Antibiotic and L-asparaginase activity of *Streptomyces* sp isolated from fish, shell fish and sediment of Veli estuarine Lake along the Kerala coast. *Indian J. Mar. Sci.* 28, 335–337.
- Dixit, V., Pant, A., 2000. Comparative characterization of two serine endopeptidases from *Nocardioopsis* sp. NCIM 5124. *Biochim. Biophys. Acta* 1523, 261–268.
- Dobner, P., Feldmann, K., Rifai, M., Loscher, T., Rinder, H., 1996. Rapid identification of mycobacterial species by PCR amplification of hypervariable 16S rRNA gene promoter region. *J. Clin. Microbiol.* 34, 866–869.
- Drancourt, M., Bollet, Carlioz, A., Martelin, R., Gayral, J.P., Raoult, D., 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clin. Microbiol.* 38, 3623–3630.
- Dulekgurgen, E., Ovez, S., Artan, N., Orhon, D., 2003. Enhanced biological phosphate removal by granular sludge in a sequencing reactor. *Biotechnol. Lett.* 25, 687–693.
- Duo-Chuan, L., 2006. Review of fungal chitinases. *Mycopathologia* 161, 345–360.
- Eisenman, H.C., Casadevall, A., Mc Clelland, E.E., 2007. New insights on the pathogenesis of invasive *Cryptococcus neoformans* infection. *Curr. Infect. Dis. Rep.* 9, 457–464.
- El-Khawagh, M.A., Hamadah, K.S., El-Sheikh, T.M., 2011. The insecticidal activity of actinomycete metabolites, against the mosquito *Culex pipiens*. *Egypt. Acad. J. Biol. Sci.* 4, 103–113.
- Ellaiah, P., Adinarayan, K., Naveen Babu, A., Thaer, B., Srinivasulu, T., Prabhakar, T., 2002. Bioactive actinomycetes from marine sediments of Bay of Bengal near Machilipatnam. *Geobios* 29, 97–100.
- Ellaiah, P., Ramana, T., Bapi Raju, K.V.V.S.N., Sujatha, P., Uma Sankar, A., 2004. Investigation on marine actinomycetes from Bay of Bengal near Kakinada coast of Andhra Pradesh. *Asian J. Microbiol. Biotechnol. Environ. Sci.* 6, 53–56.
- Ellaiah, P., Reddy, A., 1987. Isolation of actinomycetes from marine sediments of Visakhapatnam East Coast of India. *Indian J. Mar. Sci.* 16, 134–135.
- El-Tarabily, K., Soliman, M.H., Nassar, A.H., Al-Hassani, H.A., Sivasithamparam, K., McKenna, F., 2000. Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. *Plant Pathol.* 49, 573–583.
- El-Tarabily, K.A., Sivasithamparam, K., 2006. Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biol. Biochem.* 38, 1505–1520.

- Embley, T., Stackebrandt, E., 1994. The molecular phylogeny and systematics of the actinomycetes. *Annu. Rev. Microbiol.* 48, 257–289.
- Engelhardt, K., Degnes, K.F., Kemmler, M., Bredholt, H., Fjaervik, E., Klinkenberg, G., Sletta, H., Ellingsen, T.E., Zotchev, S., 2010. Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species. *Appl. Environ. Microbiol.* 76, 4969–4976.
- Erikson, D., 1940. Pathogenic anaerobic micro organisms of the actinomyces group. *Spec. Rept. Ser. Med. Res. Co, London.* 240, 1- 63
- Euzeby, J.P., 2008. Genus *Streptomyces*. List of Prokaryotic names with Standing in Nomenclature <http://www.bacterio.cict.fr/s/streptomycesa.html>
- Evtushenko, L.I., Taran, V. V., Akimov, V.N., Kroppenstedt, R.M., Tiedje, J.M., Stackebrandt, E., 2000. *Nocardiopsis tropica* sp. nov., *Nocardiopsis trehalosi* sp. nov., nom. rev. and *Nocardiopsis dassonvillei* subsp. *albirubida* subsp. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* 50, 73–81.
- Farris, M., Duffy, C., Findlay, R., Olson, J., 2011. *Streptomyces scopuliridis* sp. nov., a bacteriocin-producing soil streptomycete. *Int. J. Syst. Evol. Microbiol.* 61, 2112–2116.
- Fehling, R.H., Buchanan, G.O., Mincer, T.J., Kauffman, C.A., Jensen, P.R., Fenical, W., 2003. Salinosporamide A: A highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*. *Angew. Chemie Int. Ed. English* 42, 355–357.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution.* 39, 783–791.
- Fenical, W., Jensen, P.R., 1993. Marine microorganisms a new biomedical resource, In: Attaway, D., Zaborsky, O. (Eds.), *Pharmaceutical and Bioactive Natural Products*. Plenum Press, New York, pp. 419–457.
- Fenical, W., Jensen, P.R., 2006. Developing a new resource for drug discovery: Marine actinomycete bacteria. *Nat. Chem. Biol.* 2, 666–673.
- Fiedler, H.P., Bruntner, C., Bull, A.T., Ward, A.C., Goodfellow, M., Potterat, O., Puder, C., Mihm, G., 2005. Marine actinomycetes as a source of novel secondary metabolites. *Antonie van Leeuwenhoek* 87, 37–42.
- Franco-Correa, M., Quintana, A., Duque, C., Suarez, C., Rodríguez, M., Barea, J., 2010. Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. *Appl. Soil Ecol.* 45, 209–217.
- Fuller, R., 1989. A review: Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365–378.
- Fulton, T.R., Losada, M.C., Fluder, E.M., Chou, G.T., 1995. Ribosomal-RNA operon restriction derived taxa for streptomycetes (RIDITS). *FEMS Microbiol. Lett.* 125, 149–158.
- Gadd, G., 1982. Effects of media composition and light on colony differentiation and melanin synthesis in *Microdochium bolleyi*. *Trans. Br. Mycol. Soc.* 78, 115–122.
- Gadelhak, G., El-Tarabily, K., Al-Kaabi, F., 2005. Insect control using chitinolytic soil actinomycetes as biocontrol agents. *Int. J. Agric. Biol.* 7, 627–633.

- Gao, X., Lu, Y., Xing, Y., Ma, Y., Lu, J., Bao, W., Wang, Y., Xi, T., 2012. A novel anticancer and antifungus phenazine derivative from a marine actinomycete BM-17. *Microbiol. Res.* 167, 616–622.
- Garriques, D., Arevalo, G., 1995. An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in commercial production of *Penaeus Vannamei* postlarvae in Ecuador, In: Browdy, C.L., Hopkins, J. (Eds.), *Swimming through troubled water. Proceedings of the special session on prawn farming, Aquaculture 95.* World Aquaculture Society, Baton Rouge, USA, pp. 53–59.
- Gatesoupe, F., 1994. Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic *Vibrio*. *Aquat. Living Resour.* 7, 277–282.
- Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. *Aquaculture* 180, 147–165.
- Geng J., Yuan P., Shao C., Yu S. B., Zhou B., Zhou P., and Chen X. D., 2010. Bacterial melanin interacts with double-stranded DNA with high affinity and may inhibit cell metabolism in vivo, *Arch. Microbiol.*, 192, 321–329
- Genilloud, O., Gonzalez, I., Salazar, O., Martín, J., Tormo, J.R., Vicente, F., 2011. Current approaches to exploit actinomycetes as a source of novel natural products. *J. Ind. Microbiol. Biotechnol.* 38, 375–389.
- Ghanem, N., Sabry, S., El-Sherif, Z., Abu El-Ela, G., 2000. Isolation and enumeration of marine actinomycetes from sea water and sediments in Alexandria. *J. Gen. Appl. Microbiol.* 46, 105–111.
- Ghasemi, S., Ahmadian, G., Babaeian, J.N., Rahimian, H., Ghandili, S., Dehestani, A., 2010. Antifungal chitinases from *Bacillus pumilus* SG2: preliminary report. *World J. Microbiol. Biotechnol.* 26, 1437–1443.
- Ghazal, S., Bream, A., Abdel-Aziz, Z., Ibrahim, S., 2001. Preliminary studies on insecticidal activities of actinomycete strains propagated on solid and broth media using *Musca domestica* (Diptera: Muscidae). *Biol. Wet.* 66, 559–570.
- Gherbawy, Y., Elhariry, H., Altalhi, A., El Deeb, B., Khiralla, G., 2012. Molecular screening of *Streptomyces* isolates for antifungal activity and family 19 chitinase enzymes. *J. Microbiol.* 50, 459–468.
- Gibello, A., Ferrer, E., Sanz, J., Martin, M., 1995. Polymer production by *Klebsiella pneumoniae* 4-hydroxy phenyl acetic acid hydroxylase genes cloned in *Escherichia coli*. *Appl. Environ. Microbiol.* 61, 4167–4171.
- Glen, E.P., Brooks, P.R., Kurtboke, I., 2008. The occurrence of bioactive Micromonosporaceae in aquatic habitats of the sunshine coast in Australia. *Mar. Drugs* 6, 243–261.
- Goarant, M.F., Berthe, Mermound, I., Perolet, P., 1999. Arbitrarily primed PCR to type *Vibrio* spp. pathogenic for shrimp. *Appl. Environ. Microbiol.* 65, 1145–1151.
- Gohel, V., Singh, A., Vimal, M., Ashwini, P., Chhatpar, H.S., 2006. Bioprospecting and antifungal potential of chitinolytic microorganisms. *African J. Biotechnol.* 5, 54–72.
- Gontang, E. A, Fenical, W., Jensen, P.R., 2007. Phylogenetic diversity of Gram-positive bacteria cultured from marine sediments. *Appl. Environ. Microbiol.* 73, 3272–3282.
- Goodfellow, M., Ferguson, E., Sanglier, J., 1992. Numerical classification and identification of *Streptomyces* species—a review. *Gene* 115, 225–233.

- 
- Goodfellow, M., Fiedler, H.P., 2010. A guide to successful bioprospecting, informed by actinobacterial systematics. *Antonie Van Leeuwenhoek* 98, 119–142.
- Goodfellow, M., Haynes, J.A., 1984. Actinomycetes in marine sediments. In: Biological, biochemical, and biomedical aspects of actinomycetes. Ortiz-Ortiz, L., Bojalil, L.F., and Yakoleff, V. (Eds.), Academic Press, Orlando, pp. 453–472.
- Goodfellow, M., Lacey, J., Todd, C., 1987. Numerical classification of thermophilic streptomycetes. *J. Gen. Microbiol.* 133, 3135–3149.
- Goodfellow, M., O'Donnell, A.G., 1989. Search and discovery of industrially significant actinomycetes: Proceeding of the 44th Symposium on Society for General Microbiology. Cambridge Univ. Press. Cambridge, pp.343–383.
- Goodfellow, M., Simpson, K.E., 1987. Ecology of Streptomycetes. *Front. Appl. Microbiol.* 2, 97–125.
- Goodfellow, M., Williams, S.T., 1983. Ecology of actinomycetes. *Ann. Rev. Microbiol.* 37, 189–216.
- Goshev, I., Gousterova, A., Vasileva-Tonkova, E., 2005. Characterization of the enzyme complexed produced by two newly isolated thermophilic actinomycete strains during growth on collagen rich materials. *Process Biochem.* 40, 1627–1631.
- Goulden, E.F., Hall, M.R., Bourne, D.G., Pereg, L.L., Hoj, L., 2012. Pathogenicity and infection cycle of *Vibrio owensii* in larviculture of the ornate spiny lobster (*Panulirus ornatus*). *Appl. Environ. Microbiol.* 78, 2841–2849.
- Gram, L., Melchiorson, J., Bruhn, J., 2010. Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms. *Mar. Biotechnol.* 12, 439–451.
- Gram, L., Melchiorson, J., Spanggaard, B., Huber, I., Nielsen, T.F., 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* strain AH2—a possible probiotic treatment of fish. *Appl. Environ. Microbiol.* 65, 969–973.
- Gregg, C.T., 1991. Bioluminescence in clinical microbiology. In W.H. Nelson (ed.), *Physical Methods for Microorganisms Detection* CRC Press, Boca Raton, Florida. pp. 1-28.
- Grein, A., Meyers, S.P., 1958. Growth characteristics and antibiotic production of actinomycetes isolated from littoral sediments and materials suspended in sea water. *J. Bacteriol.* 76, 457–463.
- Gulve, R., Deshmukh, A., Sonajirao, K., Alias, K., Arts, K., 2012. Antimicrobial activity of the marine actinomycetes. *Int. Multi-disciplinary Res. J.* 2, 16–22.
- Gunderson, J.H., Sogin, M.L., Wollett, G., Hollingdale, M., Delacruz, V.F., Waters, A.P., Mccutchan, T.F., 1987. Structurally distinct, stage specific ribosomes occur in *Plasmodium*. *Science* . 238, 933–937.
- Guo, Y., Zheng, W., Rong, X., Huang, Y., 2008. A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: Use of multilocus sequence analysis for streptomycete systematics. *Int. J. Syst. Evol. Microbiol.* 58, 149–159.
- Gupta, R., Saxena, R.K., Chatuverdi, P., Viridi, J.S., 1995. Chitinase production by *Streptomyces viridificans*: Its potential in fungal cell wall lysis. *J. Appl. Bacteriol.* 78, 378–383.
-

- Gurtler, V., Wilson, V.A., Mayall, B.C., 1991. Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J. Gen. Microbiol.* 137, 2673–2679.
- Hain, T., Ward-Rainey, N., Kroppenstedt, R.M., Stackebrandt, E., Rainey, F. a., 1997. Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S-23S ribosomal DNA intergenic spacers. *Int. J. Syst. Bacteriol.* 47, 202–206.
- Hall, T., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Halliwell, B., Gutteridge, J., 1989. Free radicals in biology and medicine. Oxford University Press.
- Hamed, J., Mohammadipanah, F., Ventosa, A., 2013. Systematic and biotechnological aspects of halophilic and halotolerant actinomycetes. *Extremophiles.* 17, 1-13.
- Han, Y., Yang, B., Zhang, F., Miao, X., Z, L., 2009. Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with South China Sea Sponge *Craniella Australiensis*. *Mar. Biotechnol.* 11, 132–140.
- Harvey, I., Cormier, Y., Beaulieu, C., Akimov, V.N., Me riaux, A., Duchaine, C., 2001. Random amplified ribosomal DNA restriction analysis for rapid identification of thermophilic actinomycete like bacteria involved in hypersensitivity pneumonitis. *Syst. Appl. Microbiol.* 24, 277–284.
- Harwani, D., 2013. Biodiversity of rare thermophilic actinomycetes in the Great Indian Thar Desert: An Overview. *Indo Am. J. Pharm. Res.* 3, 9349–9356.
- Havenaar, R., Veld, H. int, J.H.J., 1992. Probiotics: a general view, In: Wood, B.J. (Ed.), *The Lactic Acid Bacteria*. Vol. 1. Elsevier, London, pp. 151–169.
- He, H., Ding, W.D., Bernan, V.S., Richardson, A.D., Ireland, C.M., Greenstein, M., Ellestad, G., Carter, G.T., 2001. Lomaivitins A and B, potent antitumor antibiotics from *Micromonospora lomaivitensis*. *J. Am. Chem. Soc.* 123, 5362–5363.
- Hentschel, U., Hopke, J., Horn, M., Friedrich, A.B., Wagner, M., Hacker, J., Moore, B.S., 2002. Molecular evidence for a uniform microbial community in sponges from different oceans *Appl Environ Microbiol* 68, 4431–4440.
- Hernandez, I.L.C., Macedo, M.L., Berlinck, R.G.S., Ferreira, A.G., Godinho, M.J.L., 2004. Dipeptide metabolites from the marine derived bacterium *Streptomyces acrymicini*. *J. Braz. Chem. Soc.* 15, 441–444.
- Heuer, H., Krsek, M., Baker, P., Smalla, K., Wellington, E.M.H., 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl. Environ. Microbiol.* 63, 3233–3241.
- Hewedy, M.A., Ashour, S.M., 2009. Production of a melanin like pigment by *Kluyveromyces marxianus* and *Streptomyces Chibaensis*. *Aust. J. Basic Appl. Sci.* 3, 920–927.
- Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K., De Vos, P., 1996. Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J. Microbiol. Methods* 26, 247–259.



- Higginbotham, S., Murphy, C., 2010. Identification and characterisation of a *Streptomyces* sp. isolate exhibiting activity against methicillin-resistant *Staphylococcus aureus*. *Microbiol. Res.* 165, 82–86.
- Hill, H.Z., 1992. The function of melanin or six blind people examine an elephant. *BioEssays* 14, 49–56.
- Hill, R.T., 2004. Microbes from marine sponges: a trove of biodiversity of natural products discovery, In: Bull, A. (Ed.), *Microbial Diversity and Bioprospecting*. ASM Press, pp. 177–190.
- Hirsch, P., Mauchline, T., Clark, I., 2010. Culture-independent molecular techniques for soil microbial ecology. *Soil Biol. Biochem.* 42, 878–887.
- Hirsh, A.M., McKhann, H.I., Reddy, A., Liao, J., Fang, Y., Marshall, C.R., 1995. Assessing horizontal transfer of *nif* HDK genes in eubacteria: Nucleotide sequence of *nifK* from *Frankia* strain HFPCcI3. *Mol. Biol. Evol.* 12, 16–27.
- Hoang, K.C., TH, Lai, Lin, C.S., Chen, Y.T., Liau, C.Y., 2011. The chitinolytic activities of *Streptomyces* sp. TH-11. *Int. J. Mol. Sci.* 12, 56–65.
- Holding, A., Collee, J., 1971. Routine biochemical tests. *Methods Microbiol.* 6A, 1–31.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Stanley, J. T., Williams, S. T., 1994. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Williams & Wilkins, Co., Baltimore.
- Hong, K., Gao, A.H., Xie, Q.-Y., Gao, H., Zhuang, L., Lin, H.-P., Yu, H.-P., Li, J., Yao, X.-S., Goodfellow, M., Ruan, J.-S., 2009. Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar. Drugs* 7, 24–44.
- Hong, L., Simon, J.D., 2006. Insight into the binding of divalent cations to *Sepia* eumelanin from IR absorption spectroscopy. *Photochem. Photobiol.* 82, 1265–1269.
- Hopwood, D., Buttner, M., Bibb, M., Kieser, T., Charter, K., 2000. Antibiotic production by *Streptomyces*. *Pract. Streptomyces Genet.* 1, 1–42.
- Hoster, F., Schmitz, J.E., Daniel, R., 2005. Enrichment of chitinolytic microorganisms: Isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. *Appl. Microbiol. Biotechnology* 66, 434–442.
- Hozzein, W., Goodfellow, M., 2011. *Actinopolyspora egyptensis* sp. nov., a new halophilic actinomycete. *African J. Microbiol. Res.* 100–105.
- Hozzein, W., Li, W., Ali, M., Hammouda, O., Mousa, A., Xu, L., Jiang, C., 2004. *Nocardiopsis alkaliphila* sp. nov, a novel alkaliphilic actinomycete isolated from desert soil in Egypt. *Int. J. Syst. Evol. Microbiol.* 54, 247–252.
- Hsieh, P., Lien, T., 2012. Study of the physico-chemical properties and antioxidant activity of extracted melanins. *J. Agric. Sci.* 4, 217–229.
- Huber, M., Hintermann, G., Lerch, K., 1985. Primary structure of tyrosinase from *Streptomyces glaucescens*. *Biochem.* 24, 6038–6044.
- Huddleston, A.S., 1995. The distribution and diversity of streptomycin-producing streptomycetes in Brazilian soil. Ph.D. thesis University of Warwick.

- Huddleston, A.S., Cresswell, N., Neves, M.C.P., Beringer, J.E., Baumberg, S., Thomas, D.I., Wellington, E.M.H., 1997. Molecular detection of streptomycin producing streptomycetes in Brazilian soils. *Appl. Environ. Microbiol.* 63, 1288–1297.
- Hughes, M.S., Skuce, R.A., Beck, L.A., Neil, S., 1993. Identification of mycobacteria from animals by restriction enzyme analysis and direct DNA cycle sequencing of polymerase chain reaction amplified 16S rRNA gene sequences. *J. Clin. Microbiol.* 31, 3216–3222.
- Hung, Y., Sava, V., Golkin, B., Hong, M.Y., Huang, G., 2002. Protective activity of melanin-like pigment derived from tea on *Drosophila melanogaster* against the toxic effects of benzidine. *Food Res. Int.* 35, 619–626.
- Hussain, A.A., Mostafa, S.A., Ghazal, S.A., Ibrahim, S.Y., 2002. Studies on antifungal antibiotic and bioinsecticidal activities of some actinomycete isolates. *African J. Mycol. Biotechnol.* 10, 63–80.
- Hutchinson, C.R., 2003. Polyketide and non-ribosomal peptide synthase: falling together by coming apart. *Proc. Natl. Acad. Sci. U. S. A.* 100, 3010–3012.
- Igarashi, Y., 2004. Screening of novel bioactive compounds from plant-associated actinomycetes. *Actinomycetologica* 18, 63–66.
- Inagaki, F., Suzuki, M., Takai, K., Oida, H., Sakamoto, T., K, A., Nealon, K., Horikoshi, K., 2003. Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk. *Appl. Environ. Microbiol.* 69, 7224–7235.
- Inbar, J., Chet, I., 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. *Soil Biol. Biochem.* 23, 973–978.
- Jacob, N., Niladevi, K.N., Anisha, G.S., Prema, P., 2008. Hydrolysis of pectin: an enzymatic approach and its application in banana fiber processing. *Microbiol. Res.* 163, 538–544.
- Jacobson, E.S., 2000. Pathogenic roles for fungal melanins. *Clin. Microbiol. Rev.* 13, 708–717.
- Jain, R., Agrawal, S.C., Jain, P.C., 2009. Proteolytic actinomycetes from Indian habitats. *J. Cult. Collect.* 6, 28–37.
- Jankiewicz, U., Brzezinska, M.S., Saks, E., 2012. Identification and characterization of a chitinase of *Stenotrophomonas maltophilia*, a bacterium that is antagonistic towards fungal phytopathogens. *J. Biosci. Bioeng.* 113, 30–35.
- Jasmine, D.J., Agastian, P., 2013. *In vitro* antioxidant activity and *in vivo* alpha glucosidase activity of endophytic actinomycetes isolated from *Catharanthus roseus* (L.) G. Don. *J. Pharm. Res.* 6, 674–678.
- Jayaprakash, N.S., Pai, S.S., Anas, A., Preetha, R., Philip, R., Singh, I.S.B., 2005. A marine bacterium, *Micrococcus* MCCB 104, antagonistic to vibrios in prawn larval rearing systems. *Dis. Aquat. Organ.* 68, 39–45.
- Jayaro, B.M., Dore, J.J.E., Baumbach, G.A., Matthews, K.R., Oliver, S.P., 1991. Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase

- chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. *J. Clin. Microbiol.* 29, 2774–2778.
- Jee, H.J., Kim, H., Kim, A.J., Bae, Y., Bae, S., Yun, J., 2009. UV light induces premature senescence in Akt1-null mouse embryonic fibroblasts by increasing intracellular levels of ROS. *Biochem. Biophys. Res. Commun.* 383, 358–362.
- Jensen, P.R., 2010. Linking species concepts to natural product discovery in the post-genomic era. *J. Ind. Microbiol. Biotechnol.* 37, 219–224.
- Jensen, P.R., Fenical, W., 1991. Distribution of actinomycetes in near-shore tropical marine sediments. *Appl. Environ. Microbiol.* 57, 1102–1108.
- Jensen, P.R., Gontang, E., Mafnas, C., Mincer, T.J., Fenical, W., 2005a. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. *Environ. Microbiol.* 7, 1039–1048.
- Jensen, P.R., Mincer, T., Fenical, W., 2005b. Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus *Salinispora*. *Appl. Environ. Microbiol.* 71, 7019–7028.
- Jensen, P.R., Mincer, T.J., Williams, P.G., Fenical, W., 2005c. Marine actinomycete diversity and natural product discovery. *Antonie Van Leeuwenhoek* 87, 43–48.
- Jensen, P.R., Williams, P., Oh, D., Zeigler, L., Fenical, W., 2007. Species-specific secondary metabolite production in marine actinomycetes of the genus *Salinispora*. *Appl. Environ. Microbiol.* 73, 1146–52.
- Jiang, H.L., Tay, J.H., Tay, S.T.L., 2002. Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol. *Let. Appl. Microbiol.* 35, 439–445.
- Jiang, J., He, X., Cane, D., 2007. Biosynthesis of the earthy odorant geosmin by a bifunctional *Streptomyces coelicolor* enzyme. *Nat. Chem. Biol.* 3, 711–715.
- Jiang, S., Sun, W., Chen, M., Dai, S., Zhang, L., Liu, Y., Lee, K.J., Li, X., 2010. Diversity of culturable actinobacteria isolated from marine sponge *Haliclona* sp. *Antonie Van Leeuwenhoek* 92, 405–416.
- Jing, S., Li, X., Zhang, L., Sun, W., Dai, S., Xie, L., Liu, Y., KJ, L., 2008. Culturable actinobacteria isolated from marine sponge *Iotrochota* sp. *Mar. Biol.* 153, 945–952.
- Joo, G., 2005. Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*. *Biotechnol. Lett.* 27, 1483–1486.
- Jose, P.A., Robinson, S., Jebakumar, D., 2012. Phylogenetic diversity of actinomycetes cultured from coastal multipond solar saltern in Tuticorin, India. *Aquat. Biosyst.* 8, 23.
- Jose, S., Mohandas, A., Philip, R., Singh, I.S., 2010. Primary hemocyte culture of *Penaeus monodon* as in vitro model for white spot syndrome virus titration, viral and immune related gene expression and cytotoxicity assays. *J. Invertebr. Pathol.* 105, 312–321.
- Joshua, D.N., Arturo, C., 2003. The contribution of melanin to microbial pathogenesis. *Cell. Microbiol.* 5, 203–223.

- Julaluk, T., Hataichanoke, N., 2012. Chitinase production and antifungal potential of endophytic *Streptomyces* strain P4. Maejo Int. J. Sci. Technol, 95–104.
- Jung, W.J., An, K.N., Jin, Y.L., Park, R.D., Lim, K.T., Kim, K.Y., Tim, T.H., 2003. Biological control of damping off caused by *Rhizoctonia solani* using chitinase-producing *Paenibacillus illinoisensis* KJA-424. Soil Biol. Biochem. 35, 1261–1264.
- Kahng, H.Y., Chung, B.S., Lee, D.H., Jung, J.S., Park, J.H., Jeon, C.O., 2009. *Cellulophaga tyrosinoxydans* sp. nov., a tyrosinase-producing bacterium isolated from seawater. Int. J. Syst. Evol. Microbiol. 59, 654–657.
- Kakkar, D., Das, B., Vishwanath, D., 1984. A modified spectrophotometric assay of superoxide dismutase. Indian J. Biochem. Biophys. 21, 130–132.
- Kampfer, P., 2006. The family Streptomycetaceae, Part I: Taxonomy, In: Dworkin, M. (Ed.), The Prokaryotes: A Handbook on the Biology of Bacteria. Springer, Berlin, pp. 538–604.
- Kampfer, P., Glaeser, S.P., 2012. Prokaryotic taxonomy in the sequencing era – the polyphasic approach revisited. Environ. Microbiol. 14, 291–317.
- Kannan, R.R., Vincent, S.G.P., 2011. Molecular characterization of antagonistic *Streptomyces* Isolated from a Mangrove Swamp. Asian J. Biotechnol. 3, 237–245.
- Karl, D.M., Holm-Hansen, O., 1978. Methodology and measurement of adenylate energy charge ratios in environmental samples. Mar. Biol. 48, 185–197.
- Karthik, L., Kumar, G., Bhaskara Rao, K. V., 2010. Diversity of marine actinomycetes from Nicobar marine sediments and its antifungal activity. Int. J. Pharm. Pharm. Sci. 2, 199–203.
- Kataoka, M., Ueda, K., Kudo, T., Seki, T., Yoshida, T., 1997. Application of the variable region in 16S rDNA to create an index for rapid species identification in the genus *Streptomyces*. FEMS Microbiol. Lett. 151, 249–255.
- Katritzky, A.R., Akhmedov, N.G., Denisenko, S.N., Denisko, O. V., 2002. <sup>1</sup>H NMR spectroscopic characterization of solutions of Sepia melanin, Sepia melanin free acid and human hair melanin. Pigment Cell Res. 15, 93–97.
- Katz, E., Thompson, C.J., Hopwood, D.A., 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129, 2703–2714.
- Kavitha, A., Vijayalakshmi, M., 2011. Partial purification and antifungal profile of chitinase produced by *Streptomyces tendae* TK-VL 333. Ann. Microbiol. 61, 597–603.
- Kekuda, T., Shobha, K., Onkarappa, R., 2010. Fascinating diversity and potent biological activities of actinomycetes metabolites. J. Pharm. Res. 3, 250–256.
- Khamna, S., Yokota, A., Lumyong, S., 2009. Actinomycetes isolated from medicinal plant rhizosphere soils: Diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. World J. Microbiol. Biotechnol. 25, 649–655.
- Khanna, M., Solanki, R., Lal, R., 2011. Selective isolation of rare actinomycetes producing novel antimicrobial compounds. 2, 357–375.

- Khopade, A., Biao, R., Liu, X., Mahadik, K., Zhang, L., Kokare, C., 2012. Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4. *Desalination* 285, 198–204.
- Kieser, T., Bibb, M., Buttner, M., Chater, K., Hopwood, D., 2000. *Practical Streptomyces Genetics*. John Innes Found, Norwich, 162–170
- Kim, B.S., Oh, M., Kang, H., Park, S, S., Chun, J., 2004. Remarkable bacterial diversity in the tidal flat sediment as revealed by 16S rRNA analysis. *J. Microbiol. Biotechnol.* 14, 205–211.
- Kim, K., Yang, Y., Kim, J., 2003. Purification and characterization of chitinase from *Streptomyces* sp. M-20. *J. Biochem. Mol. Biol.* 36, 185–189.
- Kim, S.B., Falconer, C., Williams, E., Goodfellow, M., 1998. *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydophilic species from soil. *Int. J. Syst. Bacteriol.* 48, 59–68.
- Kirby, W., Bauer, A., Sherris, J., Turk, M., 1966. Antibiotic susceptibility testing by standard single disk method. *Am. J. Clin. Pathol.* 45, 493–496.
- Klappenbach, J., Dunbar, J., Schmidt, T., 2000. rRNA operon copy number reflects ecological strategies of bacteria. *Appl. Environ. Microbiol.* 66, 1328–1333.
- Kobayashi, T., Vieira, W., Potterf, B., Sakai, C., Imokawa, G., Hearing, V., 1995. Modulation of melanogenic protein expression during the switch from eu- to pheomelanogenesis. *J. Cell Sci.* 108, 2301–2309.
- Kohler, G., Ludwig, W., Schleifer, K.H., 1991. Differentiation of *Lactococci* by rRNA gene restriction analysis. *FEMS Microbiol. Lett.* 84, 307–312.
- Kokare, C., Mahadik, K., Kadam, S., Chopade, B., 2004. Isolation of bioactive marine actinomycetes from sediments isolated from Goa and Maharashtra coastlines (west coast of India). *Indian J. Mar. Sci.* 33, 248–256.
- Konig, G.M., Kehraus, S., Seibert, S.F., Abdel-Lateff, A., Müller, D., 2006. Natural products from marine organisms and their associated microbes. *Chem. Biochem* 7, 229–238.
- Korn-Wendisch, F., Schneider, J., 1992. Phage typing – a useful tool in actinomycete systematics. *Gene* 115, 243–247.
- Koroleva, O. V., Kulikova, N.A., Alekseeva, T.N., Stepanova, E. V., Davidchik, V.N., Beliaeva, E.I., 2007. A comparative characterization of fungal melanin and the humin-like substances synthesized by *Cerrena maxima* 0275. *Appl. Biochem. Microbiol.* 43, 69–76.
- Kotob, S., Coon, S., Quintero, E., Weiner, R., 1995. Homogentisic acid is the primary precursor of melanin synthesis in *Vibrio cholerae*, a *Hyphomonas* strain and *Shewanella colwelliana*. *Appl. Environ. Microbiol.* 61, 1620–1621.
- Kramer, K. J., Muthukrishnan, S., Johnson, L. and White, F., 1997. Chitinases for insect control. In: *Advances in Insect Control. The Role of Transgenic Plants*, eds N. Carozzi and M. Koziel, Taylor and Francis Publishers, Washington, D.C. pp. 185–193.
- Krishnakumari, K., Ponnurugan, P., Kannan, N., 2006. Isolation and characterization of *Streptomyces* sp. for secondary metabolite production. *Biotechnology* 5, 478–480.

- Kroken, S., Glass, L., Taylor, J., Yoder, D., Turgeon, B., 2003. Phylogenetic analysis of the type I polyketide synthase genes in pathogenic and saprobic ascomycetes. *Proc. Natl. Acad. Sci.* 100, 15670–15675.
- Kroppenstedt, R.M., 1992. The genus *Nocardiopsis*, In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), *The Prokaryotes*, 2nd Edn. Springer, pp. 1139–1156.
- Kroppenstedt, R.M., Evtushenko, L.I., 2002. The family Nocardiosaceae, In: Dworkin, M., K.H.S., Stackebrandt, E. (Eds.), *The Prokaryotes: An evolving electronic database for the microbial community*, 3rd Edn (release 3.11). Springer, New York.
- Krysciak, J., 1985. Solubility of melanin and humin preparations in polar aromatic solvents. *Folia Biol.* 33, 195–214.
- Kshitija, D.R., 2012. Isolation, characterization of melanin producing organism and extraction of melanin. *Int. J. Sci. Eng. Res.* 3, 1–4.
- Kumar, C., Joo, H.S., Koo, Y.M., Paik, S., Chang, C., 2004. Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus Clausii* Isolate. *World J. Microbiol. Biotechnol.* 20, 351–357.
- Kumar, S., Kannabiran, K., 2010. Diversity and optimization of process parameters for the growth of *Streptomyces* VITSVK9. *J. Nat. Environ. Sci.* 1, 56–65.
- Kumar, Y., Goodfellow, M., 2010. Reclassification of *Streptomyces hygroscopicus* strains as *Streptomyces aldersoniae* sp. nov., *Streptomyces angustmyceticus* sp. nov., comb. nov., *Streptomyces ascomycinicus* sp. nov., *Streptomyces decoyicus* sp. nov., comb. nov., *Streptomyces milbemycinicus* s. Int. *J. Syst. Evol. Microbiol.* 60, 769–775.
- Kumar, C.G., Mongolla, P., Pombala, S., Kamle, A., Joseph, J., 2011. Physicochemical characterization and antioxidant activity of melanin from a novel strain of *Aspergillus bridgeri* ICTF-201. *Lett. Appl. Microbiol.* 53, 350–358.
- Kurtboke, I., 2010. Biodiscovery from microbial resources: Actinomycetes leading the way. *Microbiol. Aust.* 31, 53–57.
- Kuznetsov, V.D., Filippova, S.N., Rybakova, A.M., 1984. Nature of the brown pigment and the composition of the phenol oxidases of *Streptomyces galbus*. *Mikrobiologia* 53, 251–256.
- Labeda, D., Goodfellow, M., Brown, R., Ward, C., Lanoot, B., Vannanneyt, M., Swings, J., Kim, S.-B., Liu, Z., Chun, J., Tamura, T., Oguchi, A., Kikuchi, T., Kikuchi, H., Nishii, T., Tsuji, K., Yamaguchi, Y., Tase, A., Takahashi, M., Sakane, T., Suzuki, K.I., Hatano, K., 2012. Phylogenetic study of the species within the family Streptomycetaceae. *Antonie van Leeuwenhoek* 101, 73–104.
- Labeda, D.P., 1987. Actinomycete taxonomy: generic characterization. *Dev. Ind. Microbiol.* 115–121.
- Labeda, D.P., Testa, R.T., Lechevalier, M.P., Lechevalier, H.A., 1984. *Saccharothrix*: a new genus of the *Actinomycetales* related to *Nocardiopsis*. *Int. J. Syst. Bacteriol.* 34, 426–431.

- 
- Lakshmanaperumalsamy, P., 1978. Studies on actinomycetes with special reference to antagonistic streptomycetes from sediments of Porto Novo coastal zone. Ph.D. thesis. Annamalai University.
- Lakshmi, G.N., 2008. Marine actinomycetes as source of antiviral agents and as probiotics for *Penaeus monodon*, Culture Systems. Ph.D. thesis. Cochin University of Science and Technology. pp 104.
- Lam, K., 2006. Discovery of novel metabolites from marine actinomycetes. Curr. Opin. Microbiol. 9, 245–251.
- Lane, A.L., Moore, B.S., 2011. A sea of biosynthesis: Marine natural products meet the molecular age. Nat. Prod. Rep. 28, 411–428.
- Lane, D. J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., and Goodfellow, M., (Eds.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, United kingdom. pp.115-175.
- Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., Pace, N.R., 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. 82, 6955–6959.
- Langfelder, K., Streibel, M., Jahn, B., Haase, G., Brakhage, A., 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet. Biol. 38, 143–158.
- Laskaris, P., Sekine, T., Wellington, E.M.H., 2012. Diversity analysis of streptomycetes and associated phosphotransferase genes in soil. PLoS One 7, e35756.
- Laurent, F.J., Provost, F., Boiron, P., 1999. Rapid identification of clinically relevant *Nocardia* species to genus level by 16S rRNA gene PCR. J. Clin. Microbiol. 37, 99–102.
- Lawton, P., Whitaker, A., Odell, D., Stowell, J., 1989. Actinomycete morphology in shaken culture. Can. J. Microbiol. 35, 881–889.
- Lazzarini, A., Cavaletti, L., Toppo, G., Marinelli, F., 2000. Rare genera of actinomycetes as potential producers of new antibiotics. Anton van Leeuwenhoek 78, 399–405.
- Leon, J., Liza, L., Soto, I., Cuadra, D., Patino, L., Zerpa, R., 2007. Bioactive actinomycetes of marine sediment from the central coast of Peru. Peruvian J. Biol. 14, 259–270.
- Lewer, P., Chapin, E., Graupner, P., Gilbert, J., Peacock, C., 2003. Tartrolone, C : A novel insecticidal macrodiolide produced by *Streptomyces* sp. CP1130. J. Nat. Prod. 66, 143–145.
- Li, H.W., Zhi, X.Y., Yao, J.C., Zhou, Y., Tang, S.K., Klenk, H.P., Zhao, J., Li, W.J., 2013. Comparative genomic analysis of the genus *Nocardiopsis* provides new insights into its genetic mechanisms of environmental adaptability. PLoS One 8, e61528.
- Li, J., Tan, B., Mai, K., Ai, Q., Zhang, W., Xu, W., Liufu, Z., Ma, H., 2006. Comparative study between probiotic bacterium *Arthrobacter* XE-7 and chloramphenicol on protection of *Penaeus chinensis* post-larvae from pathogenic vibrios. Aquaculture 253, 140–147.

- Li, M., Li, W., Xu, P., Cui, X., Xu, L., Jiang, C., 2004. *Nocardiopsis xinjiangensis* sp. nov., a halophilic actinomycete isolated from saline soil sample in China. *Int. J. Syst. Evol. Microbiol.* 54, 1805–1809.
- Li, W., Tang, S., Wang, D., Zhang, Y., Xu, L., Jiang, C., 2003. Studies of the biological characteristics of some halophilic and halotolerant actinomycetes isolated from saline and alkaline soils. *Actinomycetologica* 17, 6–10.
- Liao, D., Dennis, P.P., 1994. Molecular phylogenies based on ribosomal protein L11, L1, L10, and L12 sequences. *J. Mol. Evol.* 38, 405–419.
- Lightner, D., 1993. Diseases of cultured penaeid shrimp, In: JP, M.V. (Ed.), *CRC Hand Book of Mariculture, Crustacean Aquaculture*, 2nd Edn. Boca Raton, pp. 393–486.
- Lindholm, P., Kortemaa, H., Kokkola, M., Haahtela, K., Salkinojasalonen, M., Valkonen, J., 1997. *Streptomyces* spp. isolated from potato scab lesions under Nordic conditions in Finland. *Plant Dis.* 81, 1317–1322.
- Liu, G.Y., Nizet, V., 2009. Color me bad: microbial pigments as virulence factors. *Trends Microbiol.* 17, 406–13.
- Liu, H., Qin, S., Wang, Y., 2008. Insecticidal action of Quinomycin A from *Streptomyces* sp. KN-0647, isolated from a forest soil. *World J. Microbiol. Biotechnol.* 24, 2243–2248.
- Liu, X., Ashforth, E., Ren, B., Song, F., Dai, H., Liu, M., Wang, J., Xie, Q., Zhang, L., 2010. Bioprospecting microbial natural product libraries from the marine environment for drug discovery. *J. Antibiot.* 63, 415–422.
- Liu, Y., Tay, J.H., 2002. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. *Water Res.* 36, 1653–1665.
- Liu, Y., Tay, J.H., 2004. State-of-the-art of biogranulation technology for wastewater treatment. *Biotechnol. Adv.* 22, 533–563.
- Liu, Y.T., Sui, M.J., Ji, D.D., Wu, I.H., Chou, C.C., Chen, C.C., 1993. Protection from ultraviolet irradiation by melanin of mosquitocidal activity of *Bacillus thuringiensis* var. *Israeliensis*. *J. Invertebr. Pathol.* 62, 131–136.
- Logan, N., Forsyth, G., Lebbe, L., 2002. Polyphasic identification of *Bacillus* and *Brevibacillus* strains from clinical, dairy and industrial specimens and proposal of *Brevibacillus invocatus* sp. nov. *Int. J. Syst. Evol. Microbiol.* 52, 953–966.
- Logan, N.A., 1994. *Bacterial Systematics*. Oxford: Blackwell Scientific Publications.
- Long, R., Azam, F., 2001. Antagonistic interactions among marine pelagic bacteria. *Appl. Environ. Microbiol.* 67, 4975–4983.
- Ludwig, W., Schleifer, K.H., 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol. Rev.* 15, 155–173.
- Lyons, A.J.J., Pridham, T.G., Hesseltine, C.W., 1969. Survey of some actinomycetales for  $\alpha$ -galactosidase activity. *Appl. Environ. Microbiol.* 18, 579–583.
- Magarelli, M., Passamonti, P., Renieri, C., 2010. Purification, characterization and analysis of Sepia melanin from commercial Sepia ink (*Sepia Officinalis*). *Rev. CES Med. Vet. y Zotec.* 5, 18–28.



- Magarvey, N., Keller, J., Bernan, V., Dworkin, M., Sherman, D., 2004. Isolation and characterization of novel marine derived actinomycete taxa rich in bioactive metabolites. *Appl. Environ. Microbiol.* 70, 7520–7529.
- Mahadevan, B., Crawford, D.L., 1997. Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYECIOS. *Enzyme Microb. Technol.* 20, 489–493.
- Maki, J.S., LaCroix, S.J., Hopkins, B.S., Stanley, J.T., 1986. Recovery and diversity of heterotrophic bacteria from chlorinated drinking waters. *Appl. Environ. Microbiol.* 5, 1047–1055.
- Maldonado, L.A., Stach, J.E.M., Pathom-aree, W., Ward, A.C., Bull, A.T., Goodfellow, M., 2005. Diversity of cultivable actinobacteria in geographically widespread marine sediments. *Antonie van Leeuwenhoek* 87, 11–18.
- Manfio, G., Atalan, E., Zakrzewska-Czerwinska, J., Mordarski, M., Rodriguez, C., Collins, M., Goodfellow, M., 2003. Classification of novel streptomycetes as *Streptomyces aureus* sp. nov., *Streptomyces laceyi* sp. nov. and *Streptomyces sanglieri* sp. nov. *Antonie Van Leeuwenhoek* 83, 245–255.
- Manivasagan, P., Gnanam, S., Sivakumar, K., Thangaradjou, T., Vijayalakshmi, S., Balasubramanian, T., 2009. Antimicrobial and cytotoxic activities of an actinobacteria (*Streptomyces* sp. PM- 32) Isolated from offshore sediments of the Bay of Bengal in Tamil Nadu. *Adv. Biol. Res.* 3, 231–236.
- Manivasagan, P., Venkatesan, J., Sivakumar, K., Kim, S., 2013. Marine actinobacterial metabolites : Current status and future perspectives. *Microbiol. Res.* 168, 311–332.
- Manteca, A., Alvarez, R., Salazar, N., Yague, P., Sanchez, J., 2008. Mycelium differentiation and antibiotic production in submerged cultures of *Streptomyces coelicolor*. *Appl. Environ. Microbiol.* 74, 3877–3886.
- Mao, J., Tang, Q., Zhang, Z., Wang, W., Wei, D., Huang, Y., Liu, Z., Shi, Y., Goodfellow, M., 2007. *Streptomyces radiopugnans* sp. nov., a radiation-resistant actinomycete isolated from radiation-polluted soil in China. *Int. J. Syst. Evol. Microbiol.* 57, 2578–2582.
- Maplestone, R.A., Stone, M.J., Williams, D.H., 1992. The evolutionary role of secondary metabolites — a review. *Gene* 115, 151–157.
- Mathew, A., 1995. Studies on antagonistic *Streptomyces* sp associated with fish and shell fish of Veli Lake, Kerala. *Indian J. Exp. Biol.* 12, 32–54.
- Mayer, A.M., Rodríguez, A.D., Berlinck, R.G., Fusetani, N., 2011. Marine pharmacology in 2007–8: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mech. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 153, 191–222.
- McCarthy, B. J. 1991. The use of ATP measurements in biodeterioration studies. In W.H. Nelson (ed.) *Physical Methods for Microorganisms Detection* CRC Press. Boca Raton, Florida. pp. 129–138.
- Mehling, A., Wehmeier, U.F., Piepersberg, W., 1995. Nucleotide sequences of streptomycete 16s ribosomal DNA: towards a specific identification system for streptomycetes using PCR. *Microbiology* 141, 2139–2147.

- Mencher, J.R., Heim, A.H., 1962. Melanin biosynthesis by *Streptomyces lavendulae*. J. Gen. Microbiol. 28, 665–670.
- Menter, J.M., Patta, A.M., Hollins, T.D., Moore, C.L., Willis, I., 1998. Photoprotection of mammalian acid-soluble collagen by cuttlefish *Sepia* melanin *in vitro*. Photochem. Photobiol. 68, 532–537.
- Menter, J.M., Willis, I., 1997. Electron transfer and photoprotective properties of melanins in solution. Pigment. Cell Res. 10, 214–217.
- Meredith, P., Sarna, T., 2006. The physical and chemical properties of eumelanin. Pigment Cell Res. 16, 572–594.
- Meyer, J., 1976. *Nocardiopsis*, a new genus of the order *Actinomycetales*. Int. J. Syst. Bacteriol. 26, 487–493.
- Michael, J.J., Sharon, L.A., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Phrases, perils and pitfalls. J. Clin. Microbiol. 45, 2761–2764.
- Mignard, S., Flandrois, J.P., 2006. 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. J. Microbiol. Methods 67, 574–581.
- Miller, N., Rice-Evans, C., 1997. Factors influencing the antioxidant activity determined by the ABTS radical cation assay. Free Radic. Res. 26, 195–199.
- Mincer, T.J., Jensen, P.R., Kauffman, C.A., Fenical, W., 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. Appl. Environ. Microbiol. 68, 5005–5011.
- Ming, Y., Geng-yi, G., Lu, Y., Sheng, S., Hui-yan, W., Liu, Y., 2014. Purification, structure and anti-radiation activity of melanin from *Lachnum* YM404. Int. J. Biol. Macromol. 63, 170–176.
- Mishima, K., Nakamura, M., 1991. No Title Self-immobilization of aerobic activated sludge - A pilot study of the aerobic upflow sludge blanket process in municipal sewage treatment. Water Sci. Technol. 23, 981–990.
- Mo, S.J., Kim, J.H., Cho, K., 2009. Enzymatic properties of an extracellular phospholipase C purified from a marine streptomyccete. Biosci. Biotechnol. Biochem. 73, 2136–2137.
- Mohanraj, G., Sekar, T., 2013. Antagonistic activity of marine *Streptomyces* sp LCJ94 against the shrimp pathogens. Ann. Biol. Res. 4, 224–227.
- Moore, E.R.B., Mihaylova, S.A., Vandamme, P., Krichevsky, M.I., Dijkshoorn, L., 2010. Microbial systematics and taxonomy: relevance for a microbial commons. Res. Microbiol. 161, 430–438.
- Moran, M.A., Rutherford, L.T., Hodson, R.E., 1995. Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe. Appl. Environ. Microbiol. 61, 3695–3700.
- Moriarty, D.J.W., 1997. The role of microorganisms in aquaculture ponds. Aquaculture 151, 333–349.
- Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351–358.

- Moyer, C., Tiedje, J., Dobbs, F., Karl, D., 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl. Environ. Microbiol.* 62, 2501–2507.
- Muharram, M., Houssam, A., Reda, B., Salem, B., 2010. Taxonomic Utility of PCR restriction pattern analysis for rapid identification of clinical isolates of aerobic actinomycetes to the genus level. *Aust. J. Basic Appl. Sci.* 4, 151–159.
- Mukherjee, G., Sen, S., 2004. Characterization and identification of chitinase producing *Streptomyces venezuelae* P10. *Indian J. Exp. Biol.* 42, 541-544.
- Mukherjee, G., Sen, S.K., 2006. Purification, characterization, and antifungal activity of chitinase from *Streptomyces venezuelae* P10. *Curr. Microbiol.* 53, 265–269.
- Murray, M., 2011. *Microbial Secondary Metabolites: Comprehensive Biotechnology.* (2nd edn). Academic Press, Burlington.
- Naikpatil, S. V, Rathod, J.L., 2011. Selective isolation and antimicrobial activity of rare actinomycetes from mangrove sediment of Karwar 3, 48–53.
- Naine, J., Srinivasan, M., Devi, S., 2011. Novel anticancer compounds from marine actinomycetes: a review. *J. Pharm. Res.* 4, 1285–1287.
- Nakashima, N., Mitani, Y., Tamura, T., 2005. Actinomycetes as host cells for production of recombinant proteins. *Microb. Cell Fact.* 4, 7.
- Nappi, A., Ottaviani, E., 2000. Cytotoxicity and cytotoxic molecules in invertebrates. *BioEssays* 22, 469–480.
- Narayana, K.J.P., Prabhakar, P., Vijayalakshmi, M., Vekateswarlu, Y., Krishna, P.S.J., 2007. Biological activity of phenylpropionic acid from a terrestrial Streptomyces. *Polish J. Microbiol.* 56, 191–197.
- Newman, D., Cragg, G., 2007. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* 70, 461–477.
- Newman, D.J., Laird, S.A., 1999. The influence of natural products on 1997 pharmaceutical sales figures. In: Kate, K., Laird, S., (Eds.), *The commercial use of biodiversity: Access to genetic resources and benefit sharing.* Commission of the European Communities and Earthscan Publications Ltd., London.
- Nie, S.P., Xie, M.Y., Zhou, P., Cao, S.W., 2007. In vitro antioxidative and anticancer activities of tea glycoprotein in green tea. *Eur. Food Res. Technol.* 224, 437–442.
- Ninawe, A.S., Selvin, J., 2009. Probiotics in shrimp aquaculture: Avenues and challenges. *Crit. Rev. Microbiol.* 35, 43–66.
- Nishimura, T., Meguro, A., Hasegawa, S., Nakagawa, Y., Shimizu, M., Kunoh, H., 2002. An endophytic actinomycete, *Streptomyces* sp. AOK-30, isolated from Mountain Laurel and its antifungal activity. *J. Gen. Plant Pathol.* 68, 390–397.
- Nithya, B., Ponmurugan, P., Fredimoses, M., 2012. 16S rRNA phylogenetic analysis of actinomycetes isolated from Eastern Ghats and marine mangrove associated with antibacterial and anticancerous activities. *African J. Biotechnol.* 11, 12379–12388.

- Nityanand, M., Arvind, K. yadav., Mahesh, S.Y., Dilip, K.A., 2011. Diversity of culturable Streptomycetes from wheat cropping system of fertile regions of Indo-Gangetic Plains, India. *World J. Microbiol. Biotechnol.* 37, 1593–1602.
- Nogami, K., Maeda, M., 1992. Bacteria as biocontrol agent for rearing larvae of crab *Portunus trituberculatus*. *Can. J. Fish. Aquat. Sci.* 49, 2373–2376.
- Nonomura, H., 1974. Key for classification and identification of 458 species of the Streptomycetes included in ISP. *J. Ferment. Technol.* 52, 78–92.
- Nosanchuk, J., Casadevall, A., 1997. Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin and monoclonal antibody binding. *Infect. Immun.* 65, 1836–1841.
- Nosanchuk, J., Casadevall, A., 2006. Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob. Agents Chemother.* 50, 3519–3528.
- Nosanchuk, J.D., Casadevall, A., 2003. The contribution of melanin to microbial pathogenesis. *Cell. Microbiol.* 5, 203–223.
- O'Donnell, A.G., Embley, T.M., Goodfellow, M., 1993. Future of Bacterial Systematics. *Handbook of New Bacterial Systematics*. Academic Press, London.
- Ochi, K., 1995. A taxonomic study of the genus *Streptomyces* by analysis of ribosomal protein AT-L30. *Int. J. Syst. Bacteriol.* 45, 507–514.
- Okami, Y., Hotta, K., 1988. Search and discovery of new antibiotics, In: Goodfellow, M., Williams, S.T., Mordarski, M. (Eds.), *Actinomycetes in biotechnology*. Inc., New York. pp.33-67
- Olano, C., Mendez, C., Salas, J.A., 2009. Antitumor compounds from marine actinomycetes. *Drugs* 7, 210–248.
- Ordentlich, A., Elad, Y., Chet, I., 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* 78, 84–92.
- Osman, G., Mostafa, S., HM, S., 2007. Antagonistic and insecticidal activities of some *Streptomyces* isolate. *Pakistan J. Biotechnol.* 4, 65–71.
- Page, W.J., Shivprasad, S., 1995. Iron binding to *Azotobacter salinestris* melanin, iron mobilization and uptake mediated by siderophores. *Biometals* 8, 59–64.
- Pai, S.S., 2006. Biocontrol of *Vibrio harveyi* in *Penaeus monodon* larval rearing systems employing probiotics and vibriophages. Cochin University of Science and Technology.
- Parkes, R.J., Wellsbury, P., 2004. Deep biospheres. In: Bull A.T.,(Ed.), *Microbial diversity and bioprospecting*. ASM Press.pp. 120-129.
- Parsons, T.R., Maita, Y., Lalli, C.M., 1984. A manual of chemical and biological methods for seawater analysis. Pergamon press.pp.157-161.
- Parthasarathi, S., Sathya, G., Bupesh, R., Samy, D., Mohan, R., Kumar, S., Manikandan, M., Kim, C., Balakrishnan, K., 2012. Isolation and characterization of antimicrobial compound from marine *Streptomyces hygroscopicus* BDUS 49. *World J. Fish Mar. Sci.* 4, 268–277.

- Patil, R., Jayasekharan, G., Shanmugham, S., Shakila, R.J., 2001. 2001 Control of bacterial pathogens associated with fish diseases by antagonistic marine actinomycetes isolated from marine sediments. *Indian J. Mar. Sci.* 30, 264–267.
- Peela, S., Bapiraju, K., Terli, R., 2005. Studies on antagonistic marine actinomycetes from the Bay of Bengal. *World J. Microbiol. Biotechnol.* 21, 583–585.
- Penn, K., Jenkins, C., Nett, M., Udworthy, D.W., Gontang, E.A., McGlinchey, R.P., Foster, B., Lapidus, A., Podell, S., Allen, E.E., Moore, B.S., Jensen, P.R., 2009. Genomic islands link secondary metabolism to functional adaptation in marine actinobacteria. *ISME J.* 3, 1193–1203.
- Peter, R., Jure, Z., Cadez, N., 2007. Validation of yeast identification by in silico RFLP. *J. Rapid Methods Autom. Microbiol.* 15, 267–281.
- Petit, J., Jousel, J., Raynaud, D., Barkov, N., Barnola, J., Basile, I., Benders, M., Chappellaz, J., Davis, M., Delaygue, G., Delmotte, M., 1990. Climate and atmospheric history of the past 420,000 years from the Vostock ice core, Antarctica. *Nature* 399, 429–436.
- Pimentel-Elardo, S.M., Kozytska, S., Bugni, T.S., Ireland, C.M., Moll, H., Hentschel, U., 2010. Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. *Mar. Drugs* 8, 373–380.
- Pleban, S., Chernin, L., Chet, I., 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. *Lett. Appl. Microbiol.* 25, 284–288.
- Plonka, P.M., Grabacka, M., 2006. Melanin synthesis in microorganisms-biotechnological and medical aspects. *Acta Biochim. Pol.* 53, 429–443.
- Poornima, R., Sahu, M., Sivakumar, K., Pushpavalli, V., 2008. Optimization of  $\alpha$ -amylase production by actinomycete strain AE-19 isolated from shrimp pond. *Trends Appl. Sci. Res.* 3, 45–52.
- Powell K. S., Gatehouse A. M. R., Hilder V. A., Gatehouse J. A. 1993. Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinctipes*. *Entomol. Exp. Appl.* 66, 119–126.
- Prapagdee, B., Kuekulvong, C., Mongkolsuk, S., 2008. Antifungal potential of extracellular metabolites produced by *Streptomyces hygroscopicus* against phytopathogenic fungi. *Int. J. Biol. Sci.* 4, 330–337.
- Pridham, T.G., Gottlieb, D., 1948. The utilization of carbon compounds by some actinomycetes as an aid for species determination. *J. Bacteriol.* 56, 107–114.
- Pridham, T.G., Tresner, H.D., 1974. Family VII. Streptomycetaceae, In: Buchanan, R.E., Gibbons, W.E. (Eds.), *Bergey's Manual of Determinative Bacteriology*, 8th Edn. Williams & Wilkins, Baltimore, pp. 747–748.
- Prieto-Davo, A., Fenical, W., Jensen, P., 2008. Comparative actinomycete diversity in marine sediments. *Aquat. Microb. Ecol.* 52, 1–11.
- Proser, B.L.T., Palleroni, N.J., 1978. Nutritional characterization of some selected actinomycetes. *Int. J. Syst. Bacteriol.* 28, 516–522.
- Prota, G., 1992. *Melanins and melanogenesis*. Acad. Press. San Diego, USA.

- Quadri, S.R., Agsar, D., 2012. Detection of melanin producing thermo alkaliphilic *Streptomyces* from limestone quarries of the Deccan traps. World J. Sci. Technol. 2, 08–12.
- Radhakrishnan, M., Balagurunathan, R., 2011. Antioxidant activity of melanin pigment from *Streptomyces* species D5 isolated from Desert. Drug Invent Today. 3, 12–13.
- Rahman, H., Arifuzzaman, M., Khatun, M.R., 2010. Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity. African J. Biotechnol. 9, 4615–4619.
- Rahman, M.A., Islam, M.Z., Islam, M.A.U., 2011. Antibacterial activities of actinomycete isolates collected from soils of rajshahi, Bangladesh. Biotechnol. Res. Int. 2011, Article ID 857925.
- Rainey, F.A., Ward Rainey, N., Kroppenstedt, R.M., Stackebrandt, E., 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiopsaceae fam. Int. J. Syst. Bacteriol. 46, 1088–1092.
- Rajan, P.C., Priya, A.M., Jayapradha, D., Devi, S.S., 2012. Isolation and characterization of marine actinomycetes from West Coast of India for its antioxidant activity and cytotoxicity. Int. J. Pharm. Biol. Arch. 3, 641–645.
- Rajendhran, J., Gunasekaran, P., 2011. Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. Microbiol. Res. 166, 99–110.
- Rajkumar, J., Swarnakumar, N.S., Sivakumar, K., Thangaradjou, T., Kannan, L., 2012. Actinobacterial diversity of mangrove environment of the Bhitharkanika mangroves, east coast of Orissa, India. Int. J. Sci. Res. Publ. 2, 1–6.
- Ramesh, S., Jayaprakashvel, M., Mathivanan, N., 2006. Microbial status in seawater and coastal sediments during pre and post tsunami periods in the Bay of Bengal, India. Mar. Ecol. 27, 198–203.
- Ramesh, S., Mathivanan, N., 2009. Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World J. Microbiol. Biotechnol. 25, 2103–2111.
- Ramesh, S., Rajesh, M., Mathivanan, N., 2009. Characterization of a thermostable alkaline protease produced by marine *Streptomyces fungicidicus* MML1614. Bioprocess Biosyst. Eng. 32, 791–800.
- Rauland, U., Glocker, I., Redenbach, M., Cullum, J., 1995. DNA amplifications and deletions in *Streptomyces lividans* 66 and the loss of one end of the linear chromosome. Mol. Gen. Genet. 246, 37–44.
- Ravel, J., Amoroso, M.J., Clowell, R.R., Hill, R.T., 1998. Mercury resistant actinomycetes from Chesapeake Bay. FEMS Microbiol Lett. 162, 177–184.
- Ravikumar, S., Krishnakumar, S., Inbaneson, S.J., Murugesan, G., 2010. Antagonistic activity of marine actinomycetes from Arabian Sea coast Arch. Appl. Sci. Res. 2, 273–280.
- Reguera, G., Leschine, S.B., 2001. Chitin degradation by cellulolytic anaerobes and facultative aerobes from soils and sediments. FEMS Microbiol. Lett. 204, 367–374.
- Remya, M., Vijayakumar, R., 2008. Isolation and characterization of marine antagonistic actinomycetes from West coast of India. FACTA Univ. Ser. Med. Biol. 15, 13–19.

- 
- Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* 167, 301–313.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (Bacillus S11). *Aquaculture* 191, 271–288.
- Renner, M., Shen, Y., Cheng, X., Jensen, P.R., Frankmoelle, W., Kauffman, C., Fenical, W., Lobkovsky, E., Cladry, J., 1999. Cyclomarins A-C, new anti-inflammatory cyclic peptides produced by a marine bacterium (*Streptomyces* sp.). *J. Am. Chem. Soc.* 121, 11273–11276.
- Rice-Evans C.A., Miller N.J. and Paganga G. Antioxidant properties of phenolic compounds. *Trends Food Sci.* 1997; 2, 152-159
- Ridell, M., Wallerstrom, G., Williams, S.T., 1986. Immuno-diffusion analysis of phenetically defined strains of *Streptomyces*, *Streptoverticillum* and *Nocardioopsis*. *Syst. Appl. Microbiol.* 8, 24–27.
- Riley, P.A., 1997. Melanin. *Int. J. Biochem. Cell Biol.* 29, 1235–1239.
- Robertson, P.A.W., O’Dowd, C., Burrells, C., Williams, P., Austin, B., 2000. Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*Walbaum). *Aquaculture* 185, 235–243.
- Roberts, W..K., Selitrennikoff C.P., 1986. Plant and bacterial chitinases differ in antifungal activity. *J Gen Microbiol* 134, 169–176.
- Roes, L., Meyer, P., 2005. *Streptomyces pharetrae* sp. nov. isolated from soil from the semi-arid Karoo region. *Syst. Appl. Microbiol.* 28, 488–493.
- Rong, X., Huang, Y., 2010. Taxonomic evaluation of the *Streptomyces griseus* clade using multilocus sequence analysis and DNA-DNA hybridization, with proposal to combine 29 species and three subspecies as 11 genomic species. *Int. J. Syst. Evol. Microbiol.* 60, 696–703.
- Rong, X., Huang, Y., 2012. Taxonomic evaluation of the *Streptomyces hygroscopicus* clade using multilocus sequence analysis and DNA-DNA hybridization, validating the MLSA scheme for systematics of the whole genus. *Syst. Appl. Microbiol.* 35, 7–18.
- Rossello-Mora, R., 2011. Towards a taxonomy of Bacteria and Archaea based on interactive and cumulative data repositories. *Environ. Microbiol.* 14, 318–334.
- Rossello-Mora, R., Amann, R., 2001. The species concept for prokaryotes. *FEMS Microbiol. Rev.* 25, 39–67.
- Roth, A., Andrees, S., Kroppenstedt, R.M., Harmsen, D., Mauch, H., 2003. Phylogeny of the genus *Nocardia* based on reassessed 16S rRNA gene sequences reveals under speciation and division of strains classified as *Nocardia asteroides* into three established species and two unnamed taxons. *J. Clin. Microbiol.* 41, 851–856.
- Roth, A., Reischl, U., Streubel, A., Naumann, L. Kroppenstedt, R.M. Habicht, M., Fischer, M., Mauch, H., 2000. Novel diagnostic algorithm for identification of *mycobacteria* using genus- specific amplification of the 16S–23S rRNA gene spacer and restriction endonucleases. *J. Clin. Microbiol.* 38, 1094–1104.
-

- Rożanowska, M., Sarna, T., Land, E.J., Truscott, T.G., 1999. Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals. *Free Radic. Biol. Med.* 26, 518–525.
- Ruzafa, C., Sanchez-Amat, A., Solano, F., 1995. Characterization of the melanogenic system in *Vibrio cholerae*, ATCC 14035. *Pigment Cell Res.* 8, 147–152.
- Sabry, S.A., 2004. *Nocardiopsis aegyptia* sp. nov., isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* 54, 453–456.
- Sacramento, D., Coelho, R., Wigg, M., Linhares, L., Santos, M., Semêdo, L., Silva, A., 2004. Antimicrobial and antiviral activities of an actinomycetes (*Streptomyces* sp.) isolated from a Brazilian tropical forest soil. *World J. Microbiol. Biotechnol.* 20, 225–229.
- Sahu, S., Das, B.K., Mishra, B.K., Pradhan, J., Sarangi, N., 2007. Effects of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *J. Appl. Ichthyol.* 23, 80–86.
- Saintpierre-Bonaccio, D., Amir, H., Pineau, R., Lemriss, S., Goodfellow, M., 2004. *Streptomyces ferralitis* sp. nov., a novel Streptomycete isolated from a New-Caledonian ultramafic soil. *Int. J. Syst. Evol. Microbiol.* 54, 2061–2065.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Microbiol. Evol.* 4, 406–425.
- Sajjan, S., Kulkarni, G., Yaligara, V., Kyoun, L., Karegoudar, T.B.J., 2010. Purification and physicochemical characterization of melanin pigment from *Klebsiella* sp. GSK. *J. Microbiol. Biotechnol.* 20, 1513–1520.
- Sakai, T., Sakamoto, T., Hallaert, J., Vandamme, E.J., 1993. Pectin, pectinase and protopectinase: production, properties and applications. *Adv. Appl. Microbiol.* 39, 213–294.
- Sakakibara, T., Murakami, S., Hattori, N., Nakajima, M., Imai, K., 1997. Enzymatic treatment to eliminate the extra cellular ATP for improving the detectability of bacterial intracellular ATP. *Anal. Biochem.* 250, 157–161.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular cloning. A laboratory Manual* 2nd ed, Cold Spring Harbor, Cold Spring Harbor Laboratory press, New York, USA.
- Sanchez-Amat, A., Ruzafa, C., Solano, F., 1998. Comparative tyrosine degradation in *Vibrio cholerae* strains. The strain ATCC 14035 as a prokaryotic melanogenic model of homogentisate-releasing cell. *Comp. Biochem. Physiol. part B* 119, 557–562.
- Santhi, S., Arul, J., Solomon, J., 2010. Isolation and characterization of antagonistic actinomycetes from mangrove sediments. *Int. J. Curr. Res.* 3, 20–23.
- Sathiyaseelan, K., Stella, D., 2011. Isolation, identification and antimicrobial activity of marine actinomycetes isolated from Parangipettai. *Recent Res. Sci. Technol.* 3, 74–77.
- Sattler, I., Thiericke, R., Zeeck, A., 1998. The manumycin group metabolites. *Nat. Prod. Rep.* 15, 221–240.



- Sava, V., Huang, G.S., Yang, S.M., Hong, M.Y., Yang, P.C., 2001. Isolation and characterization of melanic pigments derived from tea and tea polyphenols. *Food Chem.* 73, 177–184.
- Schutze, E., Kothe, E., 2012. Bio-Geo interactions in metal-contaminated soils, In: Kothe, E., Varma, A. (Eds.), *Soil Biology*. Springer-Verlag, Berlin Heidelberg, pp. 163–182.
- Scribner, E., Tang, T., Bradley, S., 1973. Production of a sporulation pigment by *Streptomyces venezuelae*. *Appl. Microbiol.* 25, 873–879.
- Seiichi, A., Akihiko, Y., Mutsuo, H., 1991. Occurrence of marine bacterial lipase hydrolyzing fish oil. *Agric. Biol. Chem.* 55, 2657–2659.
- Selvam, K., Vishnupriya, B., Bose, V.S.C., 2011. Screening and quantification of marine actinomycetes producing industrial enzymes amylase, cellulase and lipase from South Coast of India. *Int. J. Pharm. Biol. Arch.* 2, 1481–1487.
- Selvin, J., Gandhimathi, R., Shanmugapriya, S., Kiran, G.S., Ravji, T.R., Natarajaseenivasan, K., Hema, T.A., 2009. Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes *Nocardiopsis dassonvelli* MAD08. *Appl. Microbiol. Biotechnol.*
- Sembiring, L., Ward, A., Goodfellow, M., 2000. Selective isolation and characterisation of members of the *Streptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcataria*. *Antonie van Leeuwenhoek* 78, 353–366.
- Shaaban, M.T., Mahmoud, S., El-Sabbagh, M., Asmaa, A., 2013. Studies on an actinomycete producing a melanin pigment inhibiting aflatoxin B1 production by *Aspergillus flavus*. *Life Sci. J.* 10, 1437–1448.
- Shi, W., Shukla, G., Varma, A., 2011. Agricultural and ecological significance of soil enzymes: In: Varma A (Ed.), *Soil carbon sequestration and nutrient cycling soil enzymology*. Springer Berlin Heidelberg, pp. 43-60
- Shirling, E.B., Gottlieb, D., 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16, 313–340.
- Shirling, E.B., Gottlieb, D., 1972. Comparative descriptions of type culture of *Streptomyces* II, Species descriptions from first study. *Int. J. Syst. Bacteriol.* 17, 69.
- Shivprasad, S., Page, W., 1989. Catechol formation and melanization by a Na<sup>+</sup>-dependent *Azotobacter chroococcum*: A protective mechanism for aeroadaption? *Appl. Environ. Microbiol.* 55, 1811–1817.
- Siebel, E., Wang, Y., Egli, T., Hammes, F., 2008. Correlations between total cell concentration, total adenosine triphosphate concentration and heterotrophic plate counts during microbial monitoring of drinking water. *Drink. Water Eng. Sci.* 1, 1-6.
- Simon, J.D., Forest, S.E., Eibest, L.M., 2000. Probing the building blocks of eumelanins using scanning electron microscopy. *Pigment Cell Res.* 13, 179–184.
- Singh, G., Dahiya, N., Hoondal, G., 2005. Optimization of chitinase production by *Serratia marcescens* GG5. *Asian J. Microbiol. Biotechnol. Environ. Sci.* 7, 383-385.

- Singh, K., Wangikar, P., Jadhav, S., 2012. Correlation between pellet morphology and glycopeptide antibiotic balhimycin production by *Amycolatopsis balhimycina* DSM 5908. *J. Ind. Microbiol. Biotechnol.* 39, 27–35.
- Singh, K.P., Mahendra, A.L., 2013. Distribution of live and dead cells in pellets of an actinomycete *Amycolatopsis balhimycina* and its correlation with balhimycin productivity. *J. Ind. Microbiol. Biotechnol.* 40, 235–244.
- Singh, P.P., Shin, Y.C., Park, C.S., Chung, Y.R., 1999. Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. *Phytopathology* 89, 92–99.
- Sivakumar, K., 2001. Actinomycetes of an Indian mangrove environment: An inventory. Ph.D. thesis, Annamalai University, India.
- Sivakumar, K., Sahu, M., Thangaradjou, T., Kannan, L., 2007. Research on marine actinobacteria in India. *Indian J. Microbiol.* 47, 186–196.
- Sklarz, M.Y., Angel, R., Gillor, O., M., S.M.I., 2009. Evaluating amplified rDNA restriction analysis assay for identification of bacterial communities. *AntonieVan Leeuwenhoek* 96, 659–664.
- Snyder, R., Gibbs, P., Palacios, A., Abiy, L., Dickey, R., Lopez, J., Rein, K., 2003. Polyketide synthase genes from marine dinoflagellates. *Mar. Biotechnol.* 5, 1–12.
- Solanki, R., Khanna, M., Rup Lal R, 2008. Bioactive compounds from marine actinomycetes. *Indian J. Microbiol.* 48, 410–431.
- Solano, F., Garcia, E., de Egea, E.P., Sanchez-Amat, A., 1997. Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. *Appl. Environ. Microbiol.* 63, 3499–3506.
- Solans, M., Vobis, G., 2003. Saprophytic actinomycetes associated to the rhizosphere and rhizoplane of *Discaria trinervis*. *Ecol. Aust.* 13, 97–107.
- Soliev, A.B., Hosokawa, K., Enomoto, K., 2011. Bioactive pigments from marine bacteria: Applications and physiological roles. *Evidence-based Complement. Altern. Med.* 670349. doi:10.1155/2011/670349.
- Song, Y.L., Hsieh, Y.T., 1994. Immunostimulation of tiger shrimp *Penaeus monodon* hemocytes for generation of microbicidal substances., analysis of reactive oxygen species. *Dev. Comp. Immunol.* 18, 201–209.
- Spooner, F.R., Hammerschmidt, R., 1989. Characterization of extracellular pectic enzymes produced by *Streptomyces* sp. *Phytopathology.* 79, 1190.
- Srinivas, N., Rao, N.S.S., Vardhan, V., Reddy, P., Pratibha, M.S., Sailaja, B., Kavya, B., Kishore, K.H., Begum, Z., Singh, S.M., Shivaji, S., 2009. Bacterial diversity and bioprospecting for cold active lipases, amylases and proteases, from culturable bacteria of Kongsfjorden and NyAlesund, Svalbard, Arctic. *Curr. Microbiol.* 59, 537–547.
- Srinivasan, M., Laxman, R., Deshpande, M., 1991. Physiology and nutritional aspects of actinomycetes: an overview. *World J. Microbiol. Biotechnol.* 7, 171–184.
- Stach, J.E.M. and Bull, A.T. (2005) Estimating and comparing the diversity of marine actinobacteria. *Antonie Van Leeuwenhoek* 87, 3–9

- 
- Stach, J.E.M., Maldonado, L.A., Masson, D.G., Ward, A.C., Goodfellow, M., Bull, A., 2003. Statistical approaches to estimating bacterial diversity in marine sediments. *Appl. Environ. Microbiol.* 69, 6189–6200.
- Stackebrandt, E., Liesack, W., Witt, D., 1992. Ribosomal RNA and rDNA sequence analyses. *Gene* 115, 255–260.
- Stackebrandt, E., Rainey, F.A., Ward-Rainey, N.L., 1997. Proposal for a new hierarchic classification system, Actinobacteria classis nov. *Int. J. Syst. Bacteriol.* 47, 479–491.
- Stamford, T., Stamford, N., Coelho, L., Araujo, J., 2001. Production and characterization of a thermostable  $\alpha$ -amylase from *Nocardioopsis* sp. endophyte of yam bean. *Bioresour. Technol.* 76, 137–141.
- Stanley, P. E., 1989 A review of bioluminescent ATP techniques in rapid microbiology. *J. Biolumin. Chemilumin.* 4, 375-80.
- Steingrube, V.A., Brown, B.A., Gibson, J.L., 1995. DNA amplification and restriction endonuclease analysis for differentiation of 12 species and taxa of *Nocardia*, including recognition of four new taxa within the *Nocardia asteroides* complex. *J. Clin. Microbiol.* 33, 3096–3101.
- Steingrube, V.A., Wilson, R.W., Brown, B.A., Jost, K.C.J., Blacklock, Z., Gibson, J.L., Wallace, R.J.J., 1997. Rapid identification of the clinically significant species and taxa of aerobic actinomycetes, including *Actinomadura*, *Gordona*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* isolates, by DNA amplification and restriction endonuclease analysis. *J. Clin. Microbiol.* 35, 817–822.
- Strickberger, M.W., 1962. *Experiments in Genetics with Drosophila*. John Wiley and Sons, Inc., New York, U.S.A.
- Subramani, R., Aalbersberg, W., 2012. Marine actinomycetes: an ongoing source of novel bioactive metabolites. *Microbiol. Res.* 167, 571–580.
- Sugita, H., Matsuo, N., Hirose, Y., Iwato, M., Deguchi, Y., 1997. *Vibrio* sp. strain NM10, isolated from the intestine of a Japanese coastal fish, has an inhibitory effect against *Pasteurella piscida*. *Appl. Environ. Microbiol.* 4986–4989.
- Sujatha, P., Kurada, V.B., Terli, R., 2005. Studies on antagonistic marine actinomycetes from the Bay of Bengal. *World J. Microbiol. Biotechnol.* 21, 583–585.
- Sun, W., Dai, S., Jiang, S., Wang, G., Liu, G., Wu, H., 2010. Culture-dependent and culture-independent diversity of Actinobacteria associated with the marine sponge *Hymeniacidon perleve* from the South China Sea. *Anton Van Leeuwenhoek* 98, 65–75.
- Sundarapandian, S., Sundaram, M., Balasubramanian, V., Tholkappian, P., 2002. Mosquitocidal properties of indigenous fungi and actinomycetes against *Culex quinquefasciatus* Say. *J. Biol. Control* 16, 89–91.
- Suthindhiran, K., Kannabiran, K., 2009. Hemolytic activity of *Streptomyces* VITSDK1 spp. isolated from marine sediments in Southern India. *J. Med. Mycol.* 19, 77–86.

- Suthindhiran, K., Kannabiran, K., 2010. Diversity and exploration of bioactive marine actinomycetes in the Puducherry coast of Bay of Bengal, India. *Indian J. Microbiol.* 50, 76–82.
- Sutton, H., Winterbourn, C., 1989. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radic. Biol. Med.* 6, 53–60.
- Taddei, A., Rodríguez, M., Marquez-Vilchez, E., Castelli, C., 2006. Isolation and identification of *Streptomyces* spp. from Venezuelan soils: Morphological and biochemical studies. *Microbiol. Res.* 161, 222–231.
- Taechowisan, T., Peberdy, J.F., Lumyong, S., 2003. Chitinase production by endophytic *Streptomyces aureofaciens* CMUAc130 and its antagonism against phytopathogenic fungi. *Ann. Microbiol.* 53, 447–461.
- Taguchi, S., Kojima, S., Miura, K., Momose, H., 1996. Taxonomic characterisation of closely-related *Streptomyces* spp. based on the amino acid sequence analysis of protease inhibitor proteins. *FEMS Microbiol. Lett.* 135, 169–173.
- Takami, H., Inoue, A., Fuji, F., Horikoshi, K., 1997. Microbial flora in the deepest sea mud of the Mariana Trench. *FEMS Microbiol. Lett.* 152, 279–285.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.
- Tarangini, K., Mishra, S., 2013. Production, characterization and analysis of melanin from isolated marine *Pseudomonas* sp. using Vegetable waste. *Res. J. Eng. Sci.* 2, 40–46.
- Tauqeer, M., Elena, M., Takano, E., Breitling, R., 2010. Molecular phylogenetics and evolution genome-based phylogenetic analysis of *Streptomyces* and its relatives. *Mol. Phylogenet. Evol.* 54, 763–772.
- Terra, W.R., Ferreira, C., 2005. Biochemistry of digestion, in: L.I. Gilbert, K. Iatrou, S.S. Gill (Eds.), *Comprehensive Molecular Insect Science* Elsevier Press, Oxford, UK, pp. 171–224.
- Thangapandian, V., Ponnuragan, S., 2007. Actinomycetes diversity in the rhizosphere soils of different medicinal plants in Kolly Hills, Tamilnadu, India, for secondary metabolite production. *Asian J. Plant Sci.* 6, 66–70.
- Thenmozhi, M., Kannabiran, K., 2012. Antimicrobial and antioxidant properties of marine actinomycetes *Streptomyces* sp VITSTK7. *Oxid. Antioxid. Med. Sci.* 1, 51–57.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Thornburg, C.C., Mark Zabriskie, T., McPhail, K.L., 2010. Deep-sea hydrothermal vents: potential hot spots for natural products discovery. *J. Nat. Prod.* 73, 489–99.
- Thorne, G., Alder, J., 2002. Daptomycin: A novel lipopeptide antibiotic. *Clin. Microbiol. Newslett.* 24, 33–40.
- Tiedje, J.M., Asuming-Brempong, S., Nusslein, K., Marsh, T.L., Flynn, S., 1999. Opening the black box of soil microbial diversity. *Appl. Soil Ecol.* 13, 109–122.

- Tiffin, P., 2004. Comparative evolutionary histories of chitinase genes in the genus *Zea* and family Poaceae. *Genetics* 167, 1331–1440.
- Tindall, B.J., Rossello-Mora, R., Busse, H.J., Ludwig, W., Kampfer, P., 2010. Notes on the characterization of prokaryote strains for taxonomic purposes. *Int. J. Syst. Evol. Microbiol.* 60, 249–266.
- Torrento, M., Torres, J., 1996. *In vitro* inhibition of *Vibrio harveyi* by *Pseudomonas* sp. isolated from aquatic environment UPV. *J. Nat. Sci. Biol. Med.* 1, 130–138.
- Torres, M.A., Barros, M.P., Campos, S.C.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P., 2008. Biochemical biomarkers in algae and marine pollution: a review. *Ecotoxicol. Environ. Saf.* 71, 1–15.
- Tresner, H., Davies, M., Backus, E., 1961. Electron microscopy of *Streptomyces* spore morphology and its role in species differentiation. *J. Bacteriol.* 81, 70–80.
- Tresner, H.D., Backus, E.J., 1963. System of colour wheels for streptomycete taxonomy. *J. Appl. Microbiol.* 11, 335–338.
- Trincon, A., 2011. Marine biocatalysts: enzymatic features and applications. *Mar. Drugs* 9, 478–499.
- Tripathi, A.K., Khanuja, S.P.S., Kumar, S., 2002. Chitin synthesis inhibitors as insect-pest control agents. *J. Med. Aromat. Plant Sci.* 24, 104–22.
- Tropeano, M., Coria, S., Turjanski, A., Cicero, D., Bercovich, A., 2012. Culturable heterotrophic bacteria from Potter Cove, Antarctica, and their hydrolytic enzymes production. *Polar Res.* 31, 18507.
- Tropeano, M., Vázquez, S., Coria, S., Turjanski, A., 2013. Extracellular hydrolytic enzyme production by proteolytic bacteria from the Antarctic. *Polish Polar Res.* 34, 1–15.
- Tsujibo, H., Kubota, T., Yamamoto, M., Miyamoto, K., Inamori, Y., 2003. Characterization of chitinase genes from an alkaliphilic actinomycete. *Nocardioopsis prasina* OPC-131. *Appl. Environ. Microbiol.* 69, 894–900.
- Tsujibo, H., Okamoto, T., Hatano, N., Miyamoto, K., Watanabe, T., Mitsutomi, M., 2000. Family 19 chitinases from *Streptomyces thermoviolaceus* OPC-520: molecular cloning and characterization. *Biosci. Biotechnol. Biochem.* 64, 2445–2453.
- Tsuneda, S., Nagano, T., Hoshino, T., Ejiri, Y., Noda, N., Hirata, A., 2003. Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Res.* 37, 4965–4971.
- Tu, Y., Sun, Y., Tian, Y., Xie, M., Chen, J., 2009. Physicochemical characterisation and antioxidant activity of melanin from the muscles of Taihe Black-bone silky fowl (*Gallus gallus domesticus* Brisson). *Food Chem.* 114, 1345–1350.
- Turick, C., Tisa, L., Caccavo, J.F., 2002. Melanin production and use as a soluble electron shuttle for Fe(III) oxide reduction and as a terminal electron acceptor by *Schewanella algae* BrY. *Appl. Environ. Microbiol.* 68, 2436–2444.
- Usuki, H., Nitoda, T., Okuda, T., Kanzaki, H., 2006. Screening and partial characterization of inhibitors of insect beta N acetyl-glucosaminidase. *J. Pestic. Sci.* 31, 41–6.

- Valan, A.M., Asha, K., Duraipandiyan, V., Ignacimuthu, S., Agastian, P., 2012a. Characterization and phylogenetic analysis of novel polyene type antimicrobial metabolite producing actinomycetes from marine sediments: Bay of Bengal India. *Asian Pac. J. Trop. Biomed.* 2, 803–810.
- Valan, A.M., Ignacimuthu, S., Agastian, P., 2012b. Actinomycetes from Western Ghats of Tamil Nadu with its antimicrobial properties. *Asian Pac. J. Trop. Biomed.* 2, S830–S837.
- Valderrama, B., Oliver, P., Medrano-Soto, A., Vazquez-Duhalt, R., 2003. Evolutionary and structural diversity of fungal laccases. *Antonie van Leeuwenhoek* 84, 289–299.
- Valente, P., Ramos, J., Leoncini, O., 1999. Sequencing as a tool in yeast molecular taxonomy. *Can. J. Microbiol.* 45, 949–958.
- Valli, S., Suvathi, S.S., Aysha, O., Nirmala, P., Vinoth, K.P., Reena, A., 2012. Antimicrobial potential of actinomycetes species isolated from marine environment. *Asian Pac. J. Trop. Biomed.* 2, 469–473.
- Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., Swings, J., 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.* 60, 407–438.
- Vandenbergh, P., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiol. Rev.* 12, 221–238.
- Vaneechoutte, M., Dijkshoorn, L., Tjernberg, I., Elaichouni, A., Vos, P. De, Claeys, G., Verschraegen, G., 1995. Identification of *Acinetobacter* Genomic species by amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* 33, 11–15.
- Vaneechoutte, M., H. De Beenhouwer, C. Claeys, G. Verschraegen, A. De Rouck, N. Paepe, A. Elaichouni, and F. Portaels. 1993. Identification of *Mycobacterium* species with amplified rDNA restriction analysis. *J. Clin. Microbiol.* 31, 2061–2065
- Vanessa, M.C., Christopher, M.M.F., 2004. Analysis of the Endophytic Actinobacterial Population in the Roots of Wheat (*Triticum aestivum*L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA Clones. *Appl. Environ. Microbiol.* 70, 1787–1794.
- Varghese, R., Nishamol, S., Suchithra, R., Aa, M.H., 2012. Biochemical and physiological characteristics of actinomycetes isolated from high altitude shola soils of tropical Montane forest. *Indian J. Innov. Dev.* 1, 142–144.
- Vasanthabharathi, V., Lakshminarayanan, R., Jayalakshmi, S., 2011. Melanin production from marine *Streptomyces*. *African J. Biotechnol.* 10, 11224–11234.
- Venter, J., Remington, K., Heidelberg, J., Halpern, A., Rusch, D., Eisen, J., 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* .304, 66–74.
- Venugopalan, V.P., Nancharaiah, Y. V, Mohan, T.V.K., Narasimhan, S. V., 2005. Biogranulation : Self - immobilised microbial consortia for high performance liquid waste remediation. *BARC News Lett.* 254.
- Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671.

- Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* 1, 1112–1116.
- Vigal, T., Gil, J., Daza, A., Garcia-Gonzalez, M., Martin, J., 1991. Cloning characterization and expression of an alpha amylase gene from *Streptomyces griseus* IMRU 3570. *Mol. Gen. Genet.* 225, 278–88.
- Vimal, V., Rajan, B.M., Kannabiran, K., 2009. Antimicrobial activity of marine actinomycete *Nocardopsis* sp. VITSVK 5 (FJ973467) 1, 57–63.
- Vogelsang, R., Barz, W., 1993. Purification, characterization and differential hormonal regulation of a  $\beta$ -1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.). *Planta* 189, 60–69.
- Waksman, S.A., Woodruff, H.B., 1941. *Actinomyces antibioticus*, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. *J. Bacteriol.* 42, 231–249.
- Walker, K., Lynch, M., 2007. Contribution of an anopheles larvae control to malaria suppression in tropical Africa: Review of achievements and potential. *Med. Vet. Entomol.* 21, 2–21.
- Wan, X., Liu, H.M., Liao, Y., Su, Y., Geng, J., Yang, M.Y., Chen, X.D., Shen, P., 2007. Isolation of a novel strain of *Aeromonas media* producing high levels of DOPA-melanin and assessment of the photoprotective role of the melanin in bioinsecticide applications. *J. Appl. Microbiol.* 103, 2533–2541.
- Wang, A., Marino, A.R., Gasyna, Z., Gasyna, E., Norris, J.J., 2008. Photoprotection by porcine eumelanin against singlet oxygen production. *Photochem. Photobiol.* 84, 679–682.
- Wang, H.S., Pan, Y.M., Tang, X.J., Huang, Z.Q., 2006. Isolation and characterization of melanin from *Osmanthus fragrans* seeds. *Lwt-Food Sci. Technol.* 39, 496–502.
- Wang, Y., Zhang, Z.S., Ruan, J.S., Wang, Y.M., Ali, S.M., 1999. Investigation of actinomycete diversity in the tropical rainforests of Singapore. *J. Ind. Microbiol. Biotechnol.* 23, 178 – 187.
- Ward, A.C., Bora, N., 2006. Diversity and biogeography of marine actinobacteria. *Curr. Opin. Microbiol.* 9, 276–286.
- Watve, M.G., Tickoo, R., Jog, M.M., Bhole, B.D., 2001. How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* 176, 386–390.
- Wayne, L.G., Brenner, D.J., Colwell, R.R., 1987. International Committee on Systematic Bacteriology. Report of the adhoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37, 463–464.
- Webster, J.J., Hampton, G.J., Wilson, J.T., Ghiorse, W.C., Leach, F.R., 1985. Determination of microbial cell numbers in subsurface samples. *Ground Water.* 23, 17-25.
- Webster, N.S., Wilson, K.J., Blackall, L.L., Hill, R.T., 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl. Environ. Microbiol.* 67, 434–444.
- Weisburg, W.G., Barns, S., Pelletier, D.A., Lane, D. J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.

- Wellington, E.M.H., Stackebrandt, E., Sanders, D., Wolstrup, J., Jorgensen, N.O.G., 1992. Taxonomic status of *Kitasatosporia*, and proposed unification with *Streptomyces* on the basis of phenotypic and 16S rRNA analysis and emendation of *Streptomyces* Waksman and Henrici 1943, 339AL. *Int. J. Syst. Bacteriol.* 42, 156–160.
- Weyermann, J., Lochmann, D., Zimmer, A., 2005. A practical note on the use of cytotoxicity assays. *Int. J. Pharmacol.* 288, 369–376.
- Weyland, H., 1981. Distribution of actinomycetes on the sea floor. *Actinomycetes*, *Zbl Bakt* 11 185–193.
- Wheeler, M.H., Bell, A.A., 1988. Melanins and their importance in pathogenic fungi. *Curr. Top. Med. Mycol.* 2, 338–387.
- Whitaker, A., 1992. Actinomycetes in submerged culture. *Appl. Biochem. Biotechnol.* 32, 23–35.
- Willey, J.M., Sherwood, L.M., Woolverton, C., 2010. *Prescott's Microbiology*. 7th edition. McGraw-Hill, New York.
- Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S. V, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18, 6531–6535.
- Williams, S., Vickers, J., 1986. The ecology of antibiotic production. *Microb. Ecol.* 12, 43–52.
- Williams, S.T., Entwistle, S., Kurylowicz, W., 1974. The morphology of streptomycetes growing in media used for commercial production of antibiotics. *Microbios* 11A, 47 – 60.
- Williams, S.T., Goodfellow, M., Alderson, G., 1989. Genus *Streptomyces*, In: Williams, S.T., Sharpe, M.E., Holt, J.G. (Eds.), *Bergey's Manual of Systematic Bacteriology*, Vol. 4. Williams & Wilkins, pp. 2452–2492.
- Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A., Sackin, M.J., 1983a. Numerical classification of *Streptomyces* and related genera. *J. Gen. Microbiol.* 129, 1743–1813.
- Williams, S.T., Goodfellow, M., Wellington, E.M.H., Vickers, J.C., Alderson, G., Sneath, P.H.A., Sackin, M.J., Mortimer, A.M., 1983b. A probability matrix for identification of some streptomycetes. *J. Gen. Microbiol.* 129, 1815–1830.
- Williams, S.T., Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., 1994. Genus *Streptomyces*, in: *Bergey's Manual of Determinative Bacteriology*. 9th Ed. Williams and Wilkins Baltimore. USA, pp. 668–675.
- Wilson, R.W., Steingrube, V.A., Brown, B.A., Wallace, R.J., 1998. Clinical application of PCR-restriction enzyme pattern analysis for rapid identification of aerobic actinomycete isolates. *J. Clin. Microbiol.* 36, 148–152.
- Woese, C.R., 1987. Bacterial evolution. *Microbiol. Rev.* 51, 221–227.
- Woese, C.R., Fox, E., 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci.* 74, 5088–5090.



- Woo, P.C., Leung, P.K., Leung, K.W., 2000. Identification by 16S ribosomal RNA gene sequencing of an *Enterobacteriaceae* species from a bone marrow transplant recipient. *Mol. Pathol.* 53, 211–15.
- Woo, P.C.Y., Ng, K.H.I., Lau, S.K.P., Yip, K.T., Fung, A.M.Y., 2003. Usefulness of the microseq 500 16S ribosomal DNA based identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. *J. Clin. Microbiol.* 41, 1996–2001.
- Wu, X., Walker, M.J., Hornitzky, M., Chin, J., 2006. Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. *J. Microbiol. Methods* 64, 107–119.
- Xiao, J., Luo, Y., Xie, S., Xu, J., 2011. *Serinicoccus profundus* sp. nov., a novel actinomycete isolated from deep-sea sediment and emended description of the genus *Serinicoccus*. *Int. J. Syst. Evol. Microbiol.* 61, 16–199.
- Xiaoyun, J., Chen, D., Shenle, H., Wang, W., Chen, S., Zou, S., 2012. Identification, characterization and functional analysis of a GH-18 chitinase from *Streptomyces roseolus*. *Carbohydr. Polym.* 87, 2409–2415.
- Xiong, L., Li, J., Kong, F., 2004. *Streptomyces* sp. 173, an insecticidal microorganism from marine. *Lett. Appl. Microbiol.* 38, 230–236.
- Xu, Y., He, H., Schulz, S., Liu, X., Fusetani, N., Xiong, H., Xiao, X., Qian, P-Y., 2010. Potent antifouling compounds produced by marine *Streptomyces*. *Bioresour. Technol.* 101, 1331–1336.
- Ye, X., Wang, S., Wu, J., Rao, P., Ng, T., 2005. A chitinase with antifungal activity from the mung bean. *Protein Expr. Purif.* 40, 230–236.
- Yin, P., Wang, Y., Zhang, S., Chu, J., Zhuang, Y., Chen, N., Li, X., Wu, Y., 2008. Effect of mycelial morphology on bioreactor performance and avermectin production of *Streptomyces avermitilis* in submerged cultivations. *J. Chinese Inst. Chem. Eng.* 39, 609–615.
- Yokota, A., 1997. Phylogenetic relationship of actinomycetes. *Atlas of actinomycetes*, Asakura Publishing Co. Ltd., Japan, pp.194 – 197.
- Yong, gang T., Sun, Y., Tian, Y., Xie, M., Chen, J., 2009. Physicochemical characterisation and antioxidant activity of melanin from the muscles of Taihe Black-bone silky fowl (*Gallus gallus domesticus* Brisson). *Food Chem.* 114, 1345–1350.
- You, J., Cao, L., Liu, G., Zhou, S., Tan, H., Lin, Y., 2005. Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments. *World J. Microbiol. Biotechnol.* 21, 679–682.
- You, J., Xue, X., Cao, L., Lu, X., Wang, J., Zhang, L., Zhou, S., 2007. Inhibition of *Vibrio* biofilm formation by a marine actinomycete strain A66. *Appl. Microbiol. Biotechnol.* 76, 1137–1144.
- Young, I.D., Street, G., Brown, J., Ad Willis, P., 1987. The effects of inoculum morphology in production scale fermentations of *Streptomyces aureofaciens*, In: Neijssell, M., Meer, R.R. Van der, Luyben., K.C.A.M. (Eds.), 4th European Congress on Biotechnology, Vol. 3. Elsevier, Amsterdam, pp. 386.

- Young, J.M., 2001. Implications of alternative classifications and horizontal gene transfer for bacterial taxonomy. *Int. J. Syst. Evol. Microbiol.* 51, 945–953.
- Yuan, W., Crawford, D., 1995. Characterization of *Streptomyces lydicus* WYEC 108 as a potential biocontrol agent against fungal root and seed rots. *Appl. Environ. Microbiol.* 61, 3119–3128.
- Zhang, C., Kim, S.K., 2010. Research and application of marine microbial enzymes: Status and prospects. *Mar. Drugs* 8, 1920–1934.
- Zhang, H., Zhang, W., Jin, Y., Jin, M., Yu, X., 2008. A comparative study on the phylogenetic diversity of culturable actinobacteria isolated from five marine sponge species. *Antonie Van Leeuwenhoek* 93, 241–248.
- Zhang, S., Ye, L., Xuexi, T., 2012. Diversity and bioactivity of actinomycetes from marine sediments of the Yellow Sea. *J. Ocean Univ. China* 11, 59–64.
- Zhang, Z., Wang, Y., Ruan, J., 1997. A proposal to revive the genus *Kitasatospora* (Omura, Takahashi, Iwai, and Tanaka 1982). *Int. J. Syst. Bacteriol.* 47, 1048–1054.
- Zhdanova, N.N., Gavriushina, A.L., Vasilevskaia, A.L., 1973. Effect of gamma and UV irradiation on the survival of *Cladosporium* sp. and *Oidiodendron cerealis*. *Mikrobiol. Zh.* 35, 449–452.
- Zheng, Z., Zeng, W., Huang, Y., Yang, Z., Li, J., Cai, H., Su, W., 2000. Detection of antitumor and antimicrobial activities in marine organisms associated actinomycetes isolated from Taiwan Strait, China. *FEMS Microbiol. Lett.* 199, 87–91.
- Zhi, X.Y., Li, W., Stackebrandt, E., 2009. An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int. J. Syst. Evol. Microbiol.* 59, 589–608.
- Zhong, K., Gao, X. L., Xu, Z. J., Gao, H., Fan, S., Yamaguchi, I., Li, L. H., Chen, R. J., 2011. Antioxidant Activity of a Novel *Streptomyces* Strain Eri12 Isolated from the rhizosphere of *Rhizoma curcumae longae*. *Curr. Res. Bacteriol.* 4, 63–72.
- Zhou, M., Chen, X., Zhao, H., Dang, H., Luan, X., Zhang, X., He, H., Zhou, B., Zhang, Y., 2009. Diversity of both the culturable protease producing bacteria and their extracellular proteases in the sediments of the South China Sea. *Microb. Ecol.* 58, 582–590.
- ZoBell, C., 1946. *Marine Microbiology*, In: *Chronica Botanica*. Waltham, MA, USA.
- ZoBell, C.E., Upham, H., 1944. A list of marine bacteria including descriptions of sixty new species. *Bull Scripps Inst. Oceanogr. Univ. Calif.* 5, 239–292.
- Zotchev, S., 2012. Marine actinomycetes as an emerging resource for the drug development pipelines. *J. Biotechnol.* 158, 168–175.
- Zuckerlandl, E., Pauling, L., 1965. Molecules as documents of evolutionary history. *J. Theor. Biol.* 8, 357–366.
- Zughaier, S.M., Ryley, H.C., Jackson, S.K., 1999. A melanin pigment purified from an epidemic strain of *Burkholderia cepacia* attenuates monocyte respiratory burst activity by scavenging superoxide anion. *Infect. Immun.* 67, 908–913.

## Appendices

**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 to 19°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  |

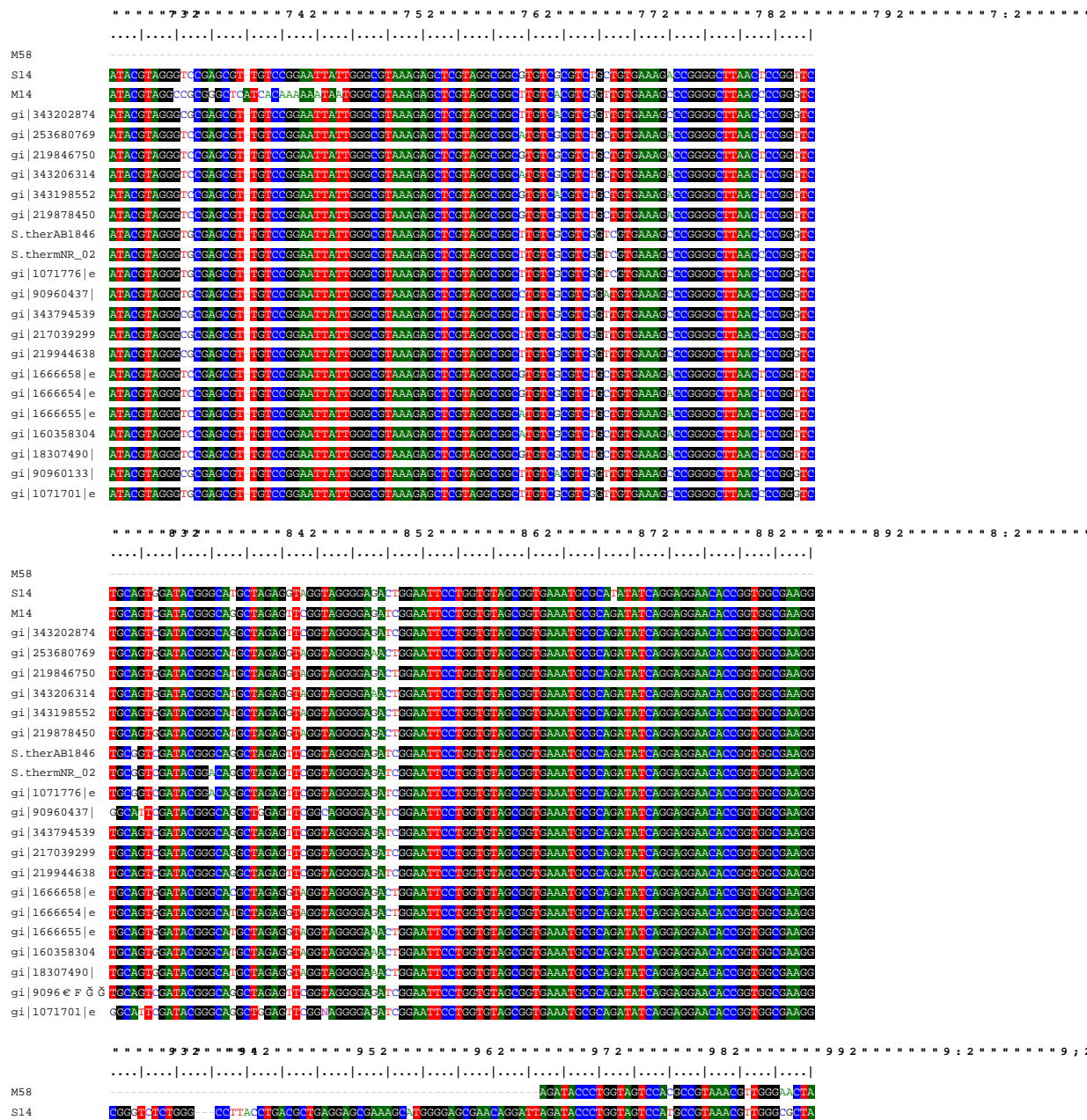
**Appendix 2:** Average *Vibrio* count (Logarithmic value of cfu/ml) during different time intervals in the co-culture experiment

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

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

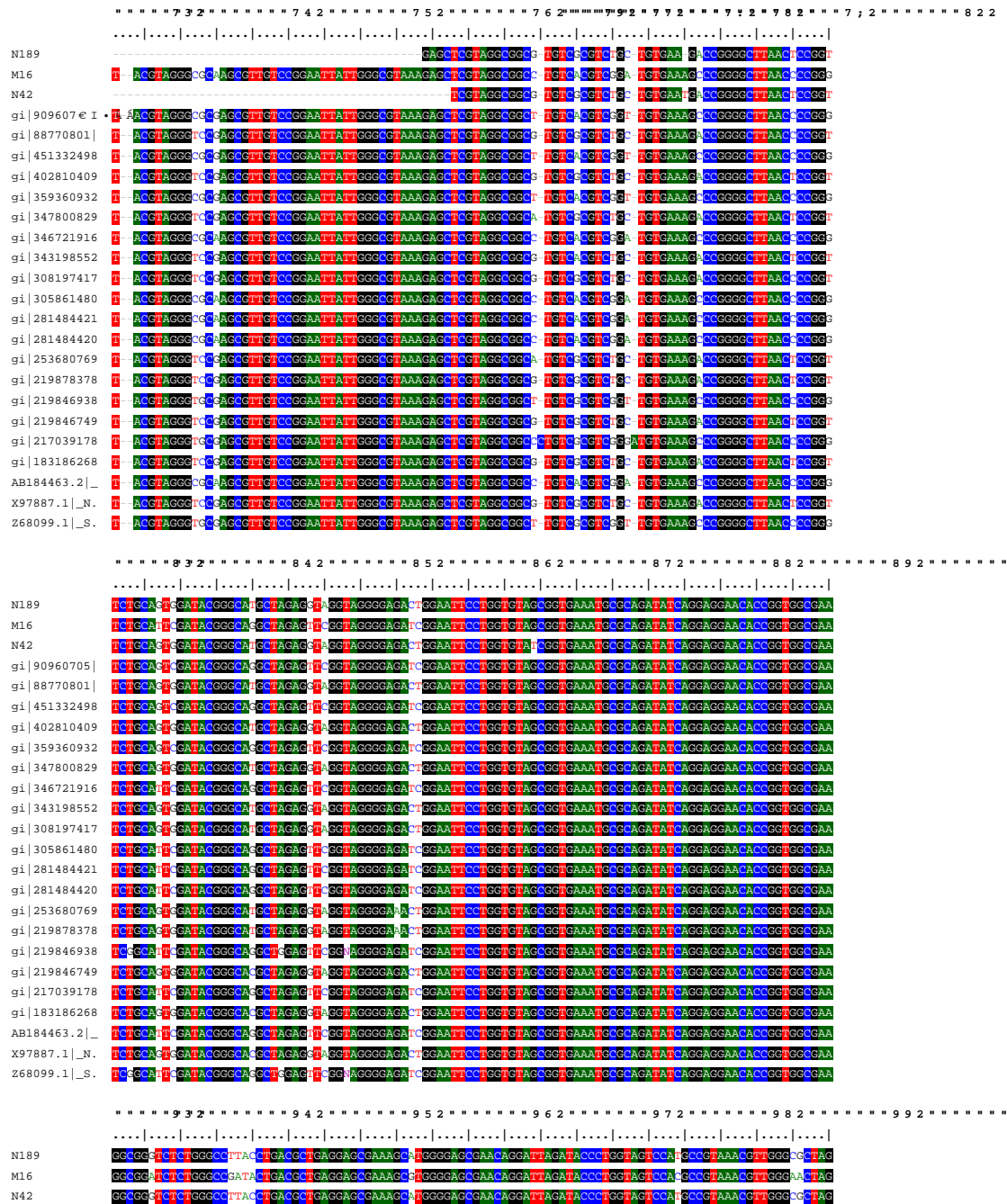
A. Multiple sequence alignment of nucleotide sequences of actinomycetes with green spore mass with closely related *Streptomyces* and *Nocardioopsis* sp. Conserved regions are indicated in colour.







**B. Multiple sequence alignment of nucleotide sequences of pink spore mass actinomycetes with other actinomycetes. Conserved sequences are indicated in colour.**











M3  
gi|90960501|  
gi|343202485|  
gi|344944198|  
gi|1666654|e  
gi|1666655|e  
gi · P U e Ğ I İ  
gi|18307490|  
gi|33302436|  
gi|29501676|  
gi|344944204|  
gi|219878483|  
gi|219878482|  
gi|343200729|  
gi|343200454|  
gi|219878481|  
gi|343202998|  
gi|343 G e e H  
gi|343202871|  
gi|406655113|  
gi|343205036|  
gi|42557951|

\*\*\*\*\*#32\*\*\*\*\* : 42\*\*\*\*\* : 52\*\*\*\*\* : 62\*\*\*\*\* : 72\*\*\*\*\* : 82\*\*\*\*\* : 92\*\*\*\*\* : 2\*\*\*\*\*

D2  
L130\_  
M3  
gi|90960501|  
gi|343202485|  
gi|344944198|  
gi|1666654|e  
gi|1666655|e  
gi|160358304|  
gi|18307490|  
gi|33302436|  
gi|219878482|  
gi|344944204|  
gi|219878483|  
gi|219878482|  
gi|343200729|  
gi|343200454|  
gi|219878481|  
gi|343202998|  
gi|343200457|  
gi|343202871|  
gi|406655113|  
gi|343205036|  
gi|42557951|

\*\*\*\*\*#32\*\*\*\*\* ; 42\*\*\*\*\* ; 52\*\*\*\*\* ; 62\*\*\*\*\* ; 72\*\*\*\*\* ; 82\*\*\*\*\* ; 92\*\*\*\*\* ; 2\*\*\*\*\*

D2  
L130\_  
M3  
gi|90960501|  
gi|343202485|  
gi|344944198|  
gi|1666654|e  
gi|1666655|e  
gi|18307490|  
gi|33302436|  
gi|219878483|  
gi|219878482|  
gi|343200729|

































## 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. *Nocardiosis 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. *Nocardiosis 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

\*\*\*