

**EXPLORING THE BIODIVERSITY OF CULTURABLE
ACTINOMYCETES IN THE SALT PANS OF GOA AND THEIR
ANTI-MICROBIAL POTENTIAL**

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1.	INTORDUCTION	1 - 9
1. 1. Biodiversity of actinomycetes		
1. 2. Characteristics of actinomycetes and their metabolites		
1. 3. Salt pan ecosystems of Goa		
1. 4. Significance of the thesis		
CHAPTER 2.	REVIEW OF LITERATURE	10 - 21
2. 1. Hypersaline environments		
2. 2. Research on hypersaline actinomycetes		
2. 3. Exploration of biodiversity of hypersaline actinomycetes		
2. 4. Bioprospecting for anti-microbial compounds		
2. 5. Anti-microbial profiles of the actinomycetes from marine salt pans		
CHAPTER 3.	MATERIALS AND METHODS	22 - 53
3. 1. Biodiversity		
3. 1. 1. Sampling sites		
3. 1. 1. a. Location and description of the Ribandar saltern		
3. 1. 1. b. Location and description of the Agarvado saltern		
3. 1. 2. Collection of samples		
3. 1. 3. Analysis of physico-chemical parameters		
3. 1. 3. a. Hydrographic parameters		
3. 1. 3. b. Estimation of total organic carbon (TOC)		
3. 1. 3. c. Sedimentology analysis (sand: silt: clay)		
3. 1. 4. Microbiological parameters		

- 3. 1. 4. a. Retrievable counts of heterotrophic bacteria
- 3. 1. 4. b. Estimation of soil microbial biomass
- 3. 1. 5. Standardization of the methodology of actinomycetes isolation
 - 3. 1. 5. a. Antibiotic concentration
 - 3. 1. 5. b. Pre-treatment of the samples
 - 3. 1. 5. c. Selection of the specific media
- 3. 1. 6. Isolation of halophilic and halotolerant actinomycetes
- 3. 1. 7. Purification, maintenance and storage of the isolates
- 3. 1. 8. Classical taxonomy
 - 3. 1. 8. a. Grouping of the halotolerant and halophilic isolates
 - 3. 1. 8. b. Cultural differentiation of *Streptomyces* and rare actinomycetes
- 3. 1. 9. Carbohydrate utilization profiles of halotolerant and halophilic isolates
- 3. 1. 10. Tentative identification of the genera
- 3. 1. 11. Molecular characterization
 - 3. 1. 11. a. 16S rRNA gene sequencing
 - 3. 1. 11. b. Phylogenetic analysis
- 3. 1. 12. Physiological characterization
 - 3. 1. 12. a. Growth optimization
 - 3. 1. 12. b. Utilization of various carbon sources
- 3. 1. 13. Assessment of biodiversity of actinomycetes

3. 2. Bioprospecting

- 3. 2. 1. Screening for the production anti-microbial metabolites
- 3. 2. 2. Sources of the clinical pathogens
- 3. 2. 3. Antibiotic susceptibility test of the bacterial pathogens
- 3. 2. 4. Selection and identification of the potential strains
- 3. 2. 5. Mass scale production and extraction of anti-microbial metabolites

- 3. 2. 5. a. Sequential organic solvent extraction
- 3. 2. 5. b. Resin-methanol extraction
- 3. 2. 6. Characterization of the antibiotic
 - 3. 2. 6. a. Solubility, temperature and pH stability
 - 3. 2. 6. b. Determination of Minimum inhibitory concentration
 - 3. 2. 6. c. TLC and FTIR Spectroscopy
- 3. 2. 7. Structural elucidation of the purified anti-microbial compound
 - 3. 2. 7. a. LC/MS and Q-TOF - ESI MS/MS analysis
 - 3. 2. 7. b. Analysis of LC/MS and Q-TOF ESI-MS/MS data

CHAPTER 4.	RESULTS	53 - 118
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4. 1. Biodiversity

- 4. 1. 1. Physico-chemical parameters
 - 4. 1. 1. a. Hydrographic parameters of the salt pans
 - 4. 1. 1. b. Total organic carbon (TOC) in sediment
 - 4. 1. 1. c. Sedimentology analysis
- 4. 1. 2. Microbiological parameters
 - 4. 1. 2. a. Heterotrophic bacteria in pre and post-monsoon season
 - 4. 1. 2. b. Assessment of soil microbial biomass carbon
- 4. 1. 3. Standardization of actinomycetes isolation procedure
- 4. 1. 4. Total heterotrophic and actinomycete CFU counts
- 4. 1. 5. Percentage of morphologically variant actinomycetes
- 4. 1. 6. Comparison of various media for isolation
 - 4. 1. 7. a. Effect of Salinity on the growth of halotolerant isolates
 - 4. 1. 7. b. Growth of halophilic isolates at various salinities
- 4. 1. 8. Metabolic fingerprinting of hypersaline actinomycetes
- 4. 1. 9. Molecular characterization of the isolates
- 4. 1. 11. Abundance of hypersaline actinomycetes

4. 1. 11. a. Cluster- I: Genus *Actinomadura*
 4. 1. 11. b. Cluster- II: Genus *Streptomonospora*
 4. 1. 11. c. Cluster- III: Genus *Nocardiopsis*
 4. 1. 11. d. Cluster-IV: Genus *Kocuria*
 4. 1. 11. e. Cluster-V: Genus *Streptomyces*
 4. 1. 11. f. Cluster-VI: Genus *Micromonospora*
 4. 1. 11. g. Cluster-VII: Genus *Kitasatospora*
 4. 1. 11. h. Cluster-VIII: Genus *Rhodococcus*
 4. 1. 11. i. Cluster-IX: Genus *Micrococcus*
 4. 1. 11. j. Cluster-X: Genus *Gordonia*
 4. 1. 11. k. Cluster-XI: Genus *Brevibacterium*
 4. 1. 11. l. Cluster-XII: Genus *Arthrobacter*
 4. 1. 11. m. Cluster-XIII: Unclassified isolate SBSKN₅
4. 1. 12. Distribution of various genera in both the salt pans
 4. 1. 13. Diversity indices of Ribandar and Agarvado salt pans

4. 2. Bioprospecting

4. 2. 1. Primary screening of actinomycete isolates
4. 2. 2. Secondary screening and selection of the potential isolates
4. 2. 3. Effect of salinity on the production of anti-microbial compound
4. 2. 4. Characterization of potential strains
 4. 2. 4. a. Microscopy
 4. 2. 4. b. Molecular characterization of the selected isolates
4. 2. 5. Assessment of sequential solvent extraction
4. 2. 6. Production of anti-microbial metabolite by strain SBSK-364
4. 2. 7. Taxonomic characterization of the strain SBSK-364
4. 2. 8. Characterization of the anti-microbial compound
4. 2. 9. TLC and FTIR analysis of ‘SBSKC1’
4. 2. 10. Minimum Inhibitory Concentration (MIC)

- 4. 2. 11. LC/MS and Q-ToF MS/MS analysis
- 4. 2. 12. Structural elucidation of ‘SBSKC1’

CHAPTER 5.

DISCUSSION

119 – 135

- 5. 1. Part-I: Biodiversity
 - 5. 1. 1. Heterotrophic bacteria and actinomycetes
 - 5. 1. 2. Metabolic profiling and molecular characterization
 - 5. 1. 3. Biodiversity of hypersaline actinomycetes
 - 5. 1. 4. Ubiquitous distribution of halophilic and halotolerant actinomycetes
 - 5. 1. 5. Description of extremely halophilic Strain SBSKN₅ (*Actinopolyspora* sp.)
 - 5. 1. 6 Seasonal variation and assessment of biodiversity
- 5. 2. Part-II: Bioprospecting
 - 5. 2. 1. Anti-microbial compounds from hypersaline actinomycetes
 - 5. 2. 2. Salinity affecting the production of anti-microbial compounds
 - 5. 2. 3. Anti-microbial compounds from marine *Micromonospora* species

SUMMARY AND CONCLUSION **136 – 140**

BIBLIOGRAPHY **141 – 161**

APPENDICES **162 – 165**

PUBLICATIONS **166 – 167**

LIST OF FIGURES:

- I. **Fig. 1** Graph showing the percentage of novel species of various genera isolated exclusively from saline and hypersaline environment
- II. **Fig. 2** Locations of Agarvado and Ribandar salt pans in GPS data
- III. **Fig. 3** Map of Goa showing the locations of all the existing salt pans
- IV. **Fig. 4** Sampling site-1, Ribandar salt pan located at Mandovi estuary, Tiswadi taluka, Goa, India
- V. **Fig. 5** A. Salt crystalizer pond of Ribandar salt pan, B. Crystalized salt before harvesting
- VI. **Fig. 6** Sampling site-2: Agarvado salt pan located at Chapora estuary of Pernem taluka, Goa, India
- VII. **Fig. 7** A. Salt crystalizer pond of Agarvado salt pan, B. Crystalized salt before harvesting
- VIII. **Fig. 8** Heterotrophic bacterial counts in the sediments of Ribandar and Agarvado
- IX. **Fig. 9** Variation of SMBC in the sediments of Ribandar and Agarvado salt pan
- X. **Fig. 10** Comparison of total heterotrophic and actinomycete counts in the sediments
- XI. **Fig. 11** Comparison of actinomycete CFU counts in pre and post-monsoon seasons
- XII. **Fig. 12** Actinomycete colonies with diverse morphology on isolation media

- XIII. **Fig. 13** Percentage of actinomycete isolates obtained at 8 different salinity levels
- XIV. **Fig. 14** Comparative assessment of 12 different media used for the isolation of hypersaline actinomycetes
- XV. **Fig. 15** Graph showing growth profile of 5 halotolerant isolates at various salinities
- XVI. **Fig. 16** Graph showing the growth profile of 3 slight halophiles at various salinities
- XVII. **Fig. 17** Graph showing the growth profile of moderate to extreme halophiles at various salinities
- XVIII. **Fig. 18** GP2 Microplate showing carbohydrate utilization by isolate SBSK-364; ‘purple colour’ denotes the positive results
- XIX. **Fig. 19** Genomic DNA profiles of the actinomycete isolates
- XX. **Fig. 20** PCR profile of 16S rRNA gene amplification
- XXI. **Fig. 21** SEM images: Cluster – I, genus *Actinomadura*
- XXII. **Fig. 22** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Actinomadura*
- XXIII. **Fig. 23** SEM images: Cluster – II, genus *Streptomonospora*
- XXIV. **Fig. 24** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Streptomonospora*
- XXV. **Fig. 25** SEM images: Cluster – III, genus *Nocardiopsis*

- XXVI. **Fig. 26** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Nocardiopsis*
- XXVII. **Fig. 27** A. Strain SBSK-221 on SCA agar at 50 psu, B SEM image: Cluster – IV, genus *Kocuria*
- XXVIII. **Fig. 28** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Kocuria*
- XXIX. **Fig. 29** SEM images: Cluster – V, genus *Streptomyces*
- XXX. **Fig. 30** BIOLOG carbohydrate utilization profile of halotolerant strain SBSK-8
- XXXI. **Fig. 31** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Streptomyces*
- XXXII. **Fig. 32** SEM images: Cluster – VI, genus *Micromonospora*
- XXXIII. **Fig. 33** BIOLOG carbohydrate utilization profile of halotolerant strain SBSK-568
- XXXIV. **Fig. 34** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Micromonospora*
- XXXV. **Fig. 35** SEM images: Cluster – VII, genus *Kitasatospora*
- XXXVI. **Fig. 36** BIOLOG carbohydrate utilization profile of halotolerant strain SBSK-430
- XXXVII. **Fig. 37** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Kitasatospora*
- XXXVIII. **Fig. 38** Colony morphology and SEM of strain SBSK-297, Cluster – VIII, genus *Rhodococcus*

- XXXIX. **Fig. 39** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Rhodococcus*
- XL. **Fig. 40** Colony morphology and SEM of strain SBSK-527, Cluster – IX, genus *Micrococcus*
- XLI. **Fig. 42** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Micrococcus*
- XLII. **Fig. 43** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the Cluster – X, genus *Gordonia*
- XLIII. **Fig. 44** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus Cluster – XI, *Brevibacterium*
- XLIV. **Fig. 45** SEM of strain SBSK-401, rod shaped cells growing attached to salt crystals, Cluster – XII, genus *Arthrobacter*
- XLV. **Fig. 46** Phylogenetic tree showing the taxonomic relationship of the isolates with the type strains of the genus *Arthrobacter*
- XLVI. **Fig. 47** A. Colony morphology of isolate SBSKN₅ (Cluster – XIII) on R2A agar with 250 psu salt; B. Light microscopy (100X)
- XLVII. **Fig. 48** Growth of isolate SBSKN₅ at various pH and temperatures
- XLVIII. **Fig. 49** SEM images of strain SBSKN₅, Grown on R2A agar with 250 psu salt
- XLIX. **Fig. 50** Phylogenetic tree showing the position of strain ‘SBSKN5’ along with its closest type strains

- L. **Fig. 51** Pie chart showing percentage of 9 different genera obtained from Ribandar salt pan
- LI. **Fig. 52** Pie chart showing percentage of 12 different genera obtained from Agarvado salt pan
- LII. **Fig. 54** Cell free metabolites of various isolates showing anti-bacterial activity against A. *Escherichia coli*, B. *Proteus mirabilis* and C. *Salmonella typhimurium*
- LIII. **Fig. 55** Anti-fungal activity of the cell free metabolites of various isolates against A. *Aspergillus niger*, B. *Penicillium* sp., C. *Curvularia lunata*
- LIV. **Fig. 56** Graph showing the percentage of isolates showing anti-microbial activity
- LV. **Fig. 57** Graph showing the number of positive isolates from various genera both *Streptomycete* and rare actinomycete genera
- LVI. **Fig. 58** Antibiotic production of 5 selected halotolerant actinomycetes at various salinities against test pathogens
- LVII. **Fig. 59** Antibiotic production of 3 selected halophilic actinomycetes at various salinities against test pathogens
- LVIII. **Fig. 59** SEM images: antibiotic producing strains
- LIX. **Fig. 60** Phylogenetic tree showing taxonomic relationship of 8 potential antibiotic producing isolates belonging to 4 different genera viz. *Kocuria*, *Micromonospora*, *Nocardiopsis* and *Streptomyces*
- LX. **Fig. 61** Activity of concentrated anti-bacterial metabolites extracted from the culture supernatant

- LXI. **Fig. 62** Activity profile of concentrated anti-bacterial metabolites of isolate SBSK-8
- LXII. **Fig. 63** A. Strain SBSK-364 growing on R2A agar supplemented with sea water, B. SEM analysis of the isolate SBSK-364
- LXIII. **Fig. 64** Phylogenetic tree constructed by showing the taxonomic relationship of candidate strain SBSK-364
- LXIV. **Fig. 65** Compound SBSKC1 showing zones of inhibition against A. *Staphylococcus aureus*, B. *Vibrio Cholera*
- LXV. **Fig. 67** Compound SBSKC1 on TLC: A. Detection by iodine vapour; B. Detection by ninhydrine reagent
- LXVI. **Fig. 68** FTIR profile of compound ‘SBSKC1’ showing the peptide groups
- LXVII. **Fig. 69** ESI-TIC profile from the LC-MS/MS of the compound ‘SBSKC1’
- LXVIII. **Fig. 70** A. ESI-MS/MS spectra of ‘Peptide-1’, B. MS/MS at m/z 1130, C. MS/MS at m/z 551 and D. MS/MS at m/z 221
- LXIX. **Fig. 71** Structure of peptide **1** along with ESI-MS data and the conventional order of sequence from *N*-terminal of peptide
- LXX. **Fig. 72** A. ESI-MS of ‘Peptide-2’ and B. MS/MS at m/z 212
- LXXI. **Fig. 73:** Structure of peptide **2** along with ESI-MS data and the conventional order of sequence from *N*-terminal of peptide

LIST OF TABLES:

- I. **Table 1:** List of the various novel genera and species of actinomycetes reported from the hypersaline environments around the world
- II. **Table 2:** List of various genera representing the community structure of hypersaline environments
- III. **Table 3:** Time table for pipette withdrawal to quantify silt and clay in sediment analysis
- IV. **Table 4:** Names of the clinical pathogens, sources of isolation and diseases caused
- V. **Table 5:** Names of the fungal pathogens, sources of isolation and diseases caused
- VI. **Table 6:** Physico-chemical parameters of the selected sediment samples of Ribandar and Agarvado salt pans
- VII. **Table 7:** Isolates of cluster-I (Genus *Actinomadura*): Isolation details, carbon source utilization, salt tolerance and species level identification
- VIII. **Table 8:** Isolates of cluster-II (Genus *Streptomonospora*): Isolation details, carbon source utilization, salt requirement and species level identification
- IX. **Table 9:** Isolates of cluster-III (Genus *Nocardiopsis*): Isolation details, carbon source utilization, salt requirement and species level identification

- X. **Table 10:** Isolates of cluster-IV (Genus *Kocuria*): Isolation details, carbon source utilization, salt tolerance and species level identification
- XI. **Table 11:** Isolates of cluster-V (Genus *Streptomyces*): Isolation details, carbon source utilization, salt tolerance and species level identification
- XII. **Table 12:** Isolates of cluster-VI (Genus *Micromonospora*): Isolation details, carbon source utilization, salt tolerance and species level identification
- XIII. **Table 13:** Isolates of cluster-VII (Genus *Kitasatospora*): Isolation details, carbon source utilization, salt requirement and species level identification
- XIV. **Table 14:** Cluster-XIII (Unidentified species): Strain SBSKNS and the type strains comparative assessment of phenotypic and metabolic characters
- XV. **Table 15:** Shannon and Simpson's diversity index values for species richness both pre and post-monsoon seasons for Ribandar salt pan
- XVI. **Table 16:** SHE analysis values of actinomycetes species diversity for both pre and post-monsoon seasons of Ribandar salt pan
- XVII. **Table 17:** Shannon and Simpson's diversity index values for species richness both pre and post-monsoon seasons for Agarvado salt pan
- XVIII. **Table 18:** SHE analysis values of actinomycetes species diversity for both pre and post-monsoon seasons of Agarvado salt pan
- XIX. **Table 19:** Selected halophilic and halotolerant isolates showing anti-microbial activity against clinical and agricultural pathogens

- XX. **Table 20:** Activity profile of partially purified anti-microbial metabolites by sequential solvent extraction
- XXI. **Table 21:** Activity profile of the cell free metabolite of strain SBSK-364 and Compound ‘SBSKC1’

INTRODUCTION

Biodiversity of Prokaryotes is a topic of immense interest to researchers for the discovery of unexplored microbial communities, untapped bio-geochemical processes, novel and rare microbes with unique physiology and metabolism. Phylum *Actinobacteria* is one of the largest taxonomic clade among 18 major lineages of the domain *Prokaryotae* and represents 5 major subclasses, 6 orders, 14 sub orders, 39 families and 130 genera. The members of this phylum are Gram positive bacteria, with high G+C mol (%) content in their DNA viz. from 51% in few *Corynebacteria* spp. to more than 70% in *Streptomyces* and *Frankia* spp. Order *Actinomycetales* (commonly known as ‘actinomycetes’), contains majority of the families of this phylum, viz. *Micromonosporaceae*, *Frankiaceae*, *Acidothermaceae*, *Sporichthyceae*, *Geodermatophilaceae*, *Microsphaeraceae*, *Pseudonocardiaceae*, *Streptomycetaceae*, *Nocardiaceae*, *Gordoniaceae*, *Mycobacteriaceae*, *Dietziaceae*, *Tsukamurellaceae*, *Corynebacteriace*, *Intrasporangiaceae*, *Dermabacteriaceae*, *Jonesiaceae*, *Brevibacteriaceae*, *Dermatophilaceae*, *Micrococcaceae*, *Promicromonosporaceae*, *Cellulomonadaceae*, *Microbacteriaceae*, *Actinomycetaceae*, *Propionibacteriaceae*, *Nocardioidaceae*, *Streptosporangiaceae*, *Thermomonosporaceae*, *Nocardiopsaceae* and *Glycomycetaceae* [Stackebrandt *et al.*, 1997 and Ventura *et al.*, 2007]. Members of order *Actinomycetales* exist in a wide variety of morphologies viz. coccoid (*Micrococcus*), rod-coccoid (*Arthrobacter*, *Rhodococcus*), fragmenting hyphal forms (*Nocardia*) or highly differentiated and branched substrate mycelia and sporulating aerial mycelia (*Streptomyces*). Major importance of actinomycetes lies in their diverse physiological properties and metabolic pathways which result in the production of

a plethora of compounds as secondary metabolites, sources of various antibiotics, immunosuppressive agents, extracellular enzymes, pigments, enzyme inhibitors, herbicides, anti-parasitics, insecticides, antitumor agents, biofilm inhibitors, antioxidants which have immense applications in the field of biotechnology [Solanki *et al.*, 1997 and Saker *et al.*, 2015]. These microbes are ubiquitous in their distribution and occupy diverse ecological niches; from terrestrial soil to various aquatic habitats (both fresh water and marine), some exists as pathogens (*Bifidobacterium*, *Corynebacterium*, *Mycobacteria*, *Nocardia* and *Propionibacterium*), plant commensals (*Leifsonia*), nitrogen fixing symbionts (*Frankia*). They are also found to colonize in various extreme and unexplored environments viz. deep sea floors, hot springs, arid soil of deserts, soils of Arctic & Antarctica, saline and alkaline lakes, associated with marine invertebrates or as a planktonic community in aquatic biotopes [Stackebrandt *et al.*, 1997, Jensen *et al.*, 2005, Nediakova *et al.*, 2005, Jiang *et al.*, 2010, Augustine *et al.*, 2012, Vicente *et al.*, 2013 and Saker *et al.*, 2015]. Due to this diverse distribution in various habitats, the biodiversity of actinomycetes have always fascinated researchers for the discovery of various novel species under rare genera, novel genera, even novel families with varying community structures of actinomycetes.

1.1. Biodiversity of actinomycetes in various unexplored and extreme habitats:

The community structure of the actinomycetes some unexplored habitats, are found to harbour a unique biodiversity and recently have revealed multiple novel species and genera. Insight into the same, shows their biogeographical distribution and also provides information about members thriving in such environmental extremes. Few noteworthy studies have been mentioned here. In an extensive study by Jensen *et al.* (2005), on the biodiversity of culturable marine

actinomycetes in Pacific sediments, a total of 983 morphological variant actinomycete strains were isolated and 58% of the strains required sea water for growth. The major contribution of this study lead to the discovery of an obligate marine genera '*Salinospora*' was reported under the family *Micromonosporaceae*. The study also contributed two new species under the novel genus *Salinospora* viz. *Salinospora tropica* and *Salinospora arenicola*. Vicente *et al.* (2013), similarly isolated 180 actinomycete strains including 14 new phylotypes which belonged to genera *Micromonospora*, *Verruscosispora*, *Streptomyces*, *Salinospora*, *Solwaraspora*, *Microbacterium* and *Cellulosimicrobium* from 16 different species of Caribbean sponges. In a study on planktonic actinomycete community, analysis by 16S rRNA gene cloning, from a fresh water river and five saline lakes of Tibetan Plateau by Jiang *et al.* (2010) five suborders viz. *Corynebacterineae*, *Frankineae*, *Micrococcineae*, *Propionibacterineae*, *Streptosporangineae* and a group of unclassified actinomycetes have been reported. The study also highlights the distribution of the members of suborder *Micrococcineae* in a wide range of salinities from fresh water up to NaCl saturation.

Actinomycetes were initially thought to be indigenous to soil and fresh water but as time progressed, reports have shown that the members of this order have colonized in various extreme biospheres and have proven to be a dominant population [Bull, 2010]. Global research on the diversity of the extremophilic microbes have revealed several novel species and their structural and chemical adaptations, which allow them thrive in extreme environments [Satyanarayana *et al.*, 2005]. Melkat *et al.* (2011) have reported 52 halophilic actinomycetes from the saline soils of Sahara desert, Algeria, which is constantly subjected to extreme

heat and desiccation. The strains belonged to five different genera viz. *Actinopolyspora*, *Nocardiopsis*, *Saccharomonospora*, *Saccharopolyspora* and *Streptomonospora*. The study also revealed several other halophilic strains which grew in presence of 20% NaCl, & thus probable candidates of novel genera or species [Meklat et al., 2011]. Not only deserts, actinomycetes are found to thrive in the deepest part of the ocean under extreme pressure. Pathom-aree *et al.* (2006) have discovered thirty eight actinomycete strains belonging to six different genera viz. *Dermacoccus*, *Kocuria*, *Micromonospora*, *Streptomyces*, *Tsukamurella* and *Williamsia* from the sediment collected at 10,898 m depth of Challenger Deep of Mariana Trench, Pacific Ocean (deepest point on Earth). The study also revealed the presence of various biosynthetic genes viz. non-ribosomal peptide synthetase and polyketide synthases type-I (PKS-I) among the isolates responsible for the synthesis of various secondary metabolites. Influenced by the significant contributions of the above reports, present study intended to explore and study the community structure as well as biogeographical distribution of actinomycetes in hypersaline environments viz. marine salterns, which are extreme environments and thalassohaline coastal ecosystems with hyper variable saline conditions (32-300 psu).

1.2. Characteristics of actinomycetes and their metabolites:

Not only for the extremely variable community structure and unique morphological architecture, actinomycetes are more investigated for the plethora of the secondary metabolites they secrete which are the products of multiple metabolic pathways and gene clusters which have evolved over a million years. As according to a recent review by Subramani and Aalbersberg (2012), among 22,000 established microbial secondary metabolites, majority have been

discovered from actinomycetes (70%), followed by fungi (20%), various species of *Bacillus* (7%) and other bacteria (1-2%). Genus *Streptomyces* is found to be most valuable member amongst class *Actinomycetales*, contributing to major number (50-55%) of available antibiotics. Baltz (2007) stated that the top 10 cm of the earth surface sediment contains 10^{25} - 10^{26} actinomycetes CFU whereas in the past 50 years, only 10^7 of them have been screened. Hence there is bounteous scope for the discovery of novel chemical entities as anti-microbial compounds from actinomycetes. Although in the past few decades, a significant fraction of microbial research concentrated on exploring the terrestrial actinomycetes as 99% of the known anti-microbial compounds have been derived from them. Marine actinomycetes remained largely neglected with very little incentive to isolate marine strains for the discovery of novel and potent therapeutic agents [Subramani and Aalbersberg, 2013].

Actinomycete from various marine environments have been a major focus of today's research since selected marine actinomycete genera are proven to be a robust source of novel secondary metabolites viz. salinosporamide A, new classes of terpenoids, amino acid-derived metabolites and polyene macrolides [Fenical and Jensen, 2006]. Due to bioprospecting, significant increase in the number of novel metabolites from marine microorganisms reported annually, show that marine habitats are a promising source of biotechnological products with immense commercial importance [Maldonado *et al.*, 2009]. As according to Goodfellow and Fiedler (2010) finding novel antimicrobial metabolites is becoming a tedious task, since in most cases screening 'old friends' (common actinomycetes) has resulted in the costly rediscovery of the same known compounds. Hence, the target should be re-directed towards poorly studied genera or rare actinomycetes

from neglected or un-explored habitats, especially environments with extreme conditions to thrive. Marine salterns, being unique niche is an “ideal environment” to explore the rare actinomycete strains with promising anti-microbial potential. Being a coastal ecosystem marine saltern provide an opportunity to isolate marine strains and perhaps novel actinomycetes thriving in the hypersaline conditions with unique metabolic pathways.

1.3. Salt pan ecosystems of Goa:

Goa being a coastal state and located in the west coast of India surrounded by Arabian Sea on its entire periphery has a well-developed salt manufacturing industry. Marine Salterns of Goa abound the estuarine zones and occupy a low lying area of 18,000 ha i.e. (3715 sq.km.) about 0.5% of the total area of Goa [Kerkar and Loka Bharathi, 2007]. These salterns structurally constitute of a series of linked pans where gradients of salinity occur due to evaporation of sea water: Brine gets concentrated sequentially, demarcating the saltern into primary, secondary, tertiary and finally the crystallizer pond. Salinity in these ponds range from 32-350 psu during the salt manufacturing season. Presently there are 16 active solar salterns in Goa and a few major ones to be named are Ribandar and Batim in Tiswadi, Agarvado in Pernem, Nerul and Arpora in Bardez taluka [Kerkar and Fernandes, 2013]. Over the years, our research group has explored various aspects of the marine salterns of Goa *viz.* Harithsa *et al.* (2002) have reported the mercury and lead tolerance in the sulphate reducing bacteria (SRB) from the salterns, Kerkar and Lokabharathi (2007) has assessed the sulphate reducing activity (SRA) in the hypersaline conditions of SRB surviving in the salt pans of Ribandar saltern, A detailed study was conducted by Kerkar and Lokabharathi (2011) to determine the kinetics of SRA and contribution of SRB to the carbon flux in the salterns of Ribandar, Kamat *et al.*, (2011) have isolated

multiple antibiotic producing bacteria and screened them for specific activities from the salterns of Ribandar and Cavellosim, Pereira *et al.*, (2012) have shown metal tolerance and effect of the metal concentration in the proteomic level of a *Chromohalobacter* sp. and *Bacillus* sp. from the salterns of Ribandar, Kerkar and Fernandes (2013) have assessed the quality of the natural salt for iodine content and micro-nutrients from five different salterns (Agarvado, Arpora, Batim, Nerul and Ribandar), An extensive study on the response of the heterotrophic bacteria to the metal concentrations in the surface sediments of Ribandar saltern, was done by Pereira *et al.*, (2013). However, till date there are no reports on the biodiversity, distribution, community structure of actinomycetes and their bioprospecting for specific anti-microbial compounds from the marine salterns of Goa. Globally minuscule reports are available on the biodiversity of actinomycetes from marine salterns and discovery of promising & novel anti-microbial compounds is an urgent need of the moment.

The present work was thus carried out to explore the biodiversity of culturable hypersaline actinomycetes from the marine salterns of Goa and to evaluate their anti-microbial potential, with the following objectives:

Objectives

1. Isolation of halophilic and halotolerant actinomycetes from the marine salt pan sediment and water samples of Goa.
2. To study the diversity in the actinomycete population of the salt pan ecosystem.
3. Exploring rare actinomycetes and their polyphasic characterization.
4. Identification of the potential bioactive strain and its antimicrobial metabolite.

SIGNIFICANCE OF THE THESIS:

Microbial diversity in the extreme environments has been always a fascinating area of research since it generally results in the discovery of numerous microbes being novel species, novel genera and even novel families. These ‘extreme microbes’ are not only unique in their morphology, physiology and their adaptation strategies, but also in their metabolic pathways which have evolved for millions of years, have been the most prominent resource of novel biomolecules with prime importance in the field of biotechnology. Marine salterns are extreme environments of the coastal ecosystems which harbor both halophilic (salt loving) and halotolerant (can tolerate wide range of salt concentrations but essentially do not require salt for growth) microbial communities. Compared to the other extreme environments, these remain comparatively unexplored since seldom reports are available on the detailed study of the community structure of the halophilic and halotolerant microbes and their bioprospecting.

Actinomycetes are considered to be biotechnologically the most valuable prokaryotes of today, since majority of the antibiotics, anti-fungal compounds, anti-cancer drugs, enzyme inhibitors etc. are isolated from the secondary metabolites they produce. However, the terrestrial and fresh water ecosystems are relatively over explored with regards to bioprospecting of actinomycetes, since most of the times screening “the old friends” have resulted in the discovery of the same known compounds. Targeting the hypersaline (halophilic and halotolerant) actinomycetes from the marine salterns for the discovery of novel anti-microbial compounds was the main interest of the present study. Marine salterns of Goa have been studied for various bio-geochemical processes, distribution of sulphate reducing bacteria, diversity of halophilic archaea,

heterotrophic metal tolerant bacteria and their bioremediation potential but seldom for the biodiversity and bioprospecting of actinomycetes. The present study aimed to develop a baseline data on the diversity and community structure of the hypersaline actinomycetes from the marine salterns of Goa along and bioprospecting for anti-microbial compounds. The prime focus of the study was to explore various culturable rare actinomycete genera producing a broad spectrum anti-microbial compound with possibly a novel chemical structure. The data procured would add into the present information of marine antimicrobial compounds research of any specific actinomycete genus in the hypersaline environments and its multiferrous applications in the field of biotechnology.

2. REVIEW OF LITERATURE:

2.1. Hypersaline environments:

Hypersaline environments are those niches where the salt concentrations are more than that of the sea water (35 psu; 3.5% total dissolved salts). Based on the ionic composition these are environments are of two kinds viz. athalassohaline and thalassohaline. In athalassohaline environments ionic composition of the water varies significantly from the normal sea water eg. Dead sea at Jordan Rift valley where the water contains more divalent cations Mg^{2+} (~1.9 M) and Ca^{2+} (~ 0.4 M) than that of monovalent ones (1.6 M Na^+ and 0.14 M K^+) and pH is slightly acidic (~ 6.0). Thalassohaline environments have similar ionic composition like that of sea water Na^+ and Cl^- being the dominant ions, with pH near neutral to slightly alkaline. These environments are usually located along the coast line and formed due to the evaporation of sea water and concentration of salt [Kerkar, 2004]. Marine salterns are thalassohaline coastal extreme environments, created for manufacturing of salt from sea water by evaporation hence endures a wide variation in salinity (35-300 psu; 3.5-30% of total salt concentration). The varying salinity impacts the microbial community relentlessly, which results in the division of this hypersaline microbial niche in two kinds of organisms, *viz.* halophiles and halotolerants. Halophiles are the organisms which have an obligate requirement of salt for their growth and proliferation whereas halotolerant ones can tolerate wide range of salt concentrations but also can grow in the absence of salt. According to Kushner's classification, (1978) the halophiles in thalassohaline environments are classified into three groups *viz.* weak halophiles (optimum growth at 1.17 – 2.93% of NaCl), moderate halophiles (optimum growth 2.93 – 14.63% of NaCl) and extreme halophiles (optimum growth 14.63 – 30.4% of NaCl). Halotolerant

microbes are adapted to the varying salinity of the hypersaline environments and are found to tolerate 1.17 – 30.45% of NaCl concentration.

2. 2. Research on hypersaline actinomycetes:

In the past few decades' research on biodiversity of actinomycetes, has been restricted to terrestrial environments and fresh water ecosystems due to the easy accessibility. Over exploration of these environments repeatedly result in the rediscovery of the known microbial community for eg. various species of *Streptomyces*. Since this particular genera is predominant in the terrestrial ecosystems and also dwells in the fresh water environments because of the terrestrial run off of the spores during monsoon season. Hence, actinomycetological research needs to be directed towards the unexplored and neglected habitats. Hypersaline environments *viz.* marine salterns possess the advantage of being extreme unexplored habitat for exploration of biodiversity of actinomycetes. As mentioned earlier, the isolates from these environments are either halophilic or halotolerant. Halophilic strains are part of the autochthonous microbial community of these environments where as halotolerants are the ones which have emanated with sea water and over the years got adapted to the varying salinity from low (0.5%/5 psu) to extreme (30%/300 psu). In totality, marine salterns act as 'ecological sink' for various strains of halophilic and halotolerant actinomycetes with diverse and unique physiological features which have not been explored previously. Terrestrial strains from the monsoon run off cannot survive in such hypersaline conditions. Hamedi *et al.*, (2013) has reported that type strains from terrestrial environments of similar species isolated from hypersaline environments, are unable to tolerate NaCl concentration more than 0.7%.

Table. 1 List of the various novel genera and species reported from the hypersaline environments around the world

Sr. No.	Genera and species	Location	Reported by, year and reference
1.	<i>Actinopolyspora alba</i> sp. nov.	Salt field in Xinjiang Province, North-west China	Tang <i>et al.</i> , 2011
2.	<i>Actinopolyspora erythraea</i> sp. nov.	Salt field in Xinjiang Province, North-west China	Tang <i>et al.</i> , 2011
3.	<i>Actinopolyspora lacussalsi</i> sp. nov.	Hypersaline habitat Xinjiang Province, north-west China	Guan <i>et al.</i> , 2013
4.	<i>Actinopolyspora mzabensis</i> sp. nov	Saharan soil of Mzab region, Ghardaïa, Southern Algeria	Meklat <i>et al.</i> , 2013
5.	<i>Actinopolyspora righensis</i> sp. nov	Saharan soil of Djamaâ from El-Oued province, South Algeria	Meklat <i>et al.</i> , 2013
6.	<i>Actinopolyspora xinjiangensis</i> sp. nov.	Hypersaline habitat Xinjiang Province, north-west China	Guan <i>et al.</i> , 2010
7.	<i>Amycolatopsis cihanbeyliensis</i> sp. nov.	Cihanbeyli Salt Mine in the Central Anatolia region of Turkey	Tatar <i>et al.</i> , 2013
8.	<i>Amycolatopsis halophila</i> sp. nov.	Salt lake in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2010
9.	<i>Amycolatopsis salitolerans</i> sp. nov.	Hypersaline habitat of Tarim Basin, Xinjiang Province, north-west China	Guan <i>et al.</i> , 2012
10.	<i>Georgenia halophila</i> sp. nov.	Salt lake in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2010
11.	<i>Glycomyces halotolerans</i> sp. nov.	Hypersaline habitat of Xinjiang Province, north-west China	Guan <i>et al.</i> , 2011
12.	<i>Haloactinobacterium album</i> sp. nov.	Salt lake in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2010

13.	<i>Haloactinopolyspora alba</i> gen. nov., sp. nov.	Salt lake in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2011
14.	<i>Haloactinospora alba</i> gen. nov., sp. nov.	Salt lake in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2008
15.	<i>Haloechinothrix alba</i> gen. nov., sp. nov.	Salt lake in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2010
16.	<i>Haloglycomyces albus</i> gen. nov., sp. nov.	Hypersaline habitat in Xinjiang Province, north-west China	Guan <i>et al.</i> , 2009
17.	<i>Isoptericola halotolerans</i> sp. nov.	Saline soil of Qinghai Province, north-west China	Zhang <i>et al.</i> , 2005
18.	<i>Kocuria aegyptia</i> sp. nov.	Saline alkaline dessert soil of Egypt	Li <i>et al.</i> , 2006
19.	<i>Kocuria halotolerans</i> sp. nov.	Saline soil of Ganjiahu Suosuo Forest National Reserve in Xinjiang Province, north-west China	Tang <i>et al.</i> , 2009
20.	<i>Myceligerans halotolerans</i> sp. nov.	Salt lake in Xinjiang Province, north-western China	Wang <i>et al.</i> , 2011
21.	<i>Nesterenkonia halophila</i> sp. nov.	Saline soil of Xinjiang Province, north-west China	Li <i>et al.</i> , 2008
22.	<i>Nesterenkonia halotolerance</i> sp. nov.	hypersaline soil from west China	Li <i>et al.</i> , 2004
23.	<i>Nesterenkonia lacusekhoensis</i> sp. nov.	Ekho lake of Vestfold Hills, East Antarctica	Collins <i>et al.</i> , 2002
24.	<i>Nesterenkonia suensis</i> sp. nov.	Sua salt pan in Botswana, Southern Africa	Govender <i>et al.</i> , 2013
25.	<i>Nesterenkonia xinjiangensis</i> sp. nov.	Hypersaline soil from west China	Li <i>et al.</i> , 2004
26.	<i>Nocardiopsis baichengensis</i> sp. nov.	Hypersaline soils of China	Li <i>et al.</i> , 2006
27.	<i>Nocardiopsis chromatogenes</i> sp. nov.	Hypersaline soils of China	Li <i>et al.</i> , 2006
28.	<i>Nocardiopsis gilva</i> sp. nov.	Hypersaline soils of China	Li <i>et al.</i> , 2006
29.	<i>Nocardiopsis halophila</i> sp. nov.	Saline soil sample in Iraq	Al-Tai and Ruan, 1994
30.	<i>Nocardiopsis kunsanensis</i> sp. nov.	Saltern of Kunsan, Republic of Korea	Chun <i>et al.</i> , 2000
31.	<i>Nocardiopsis quinghaiensis</i> sp. nov.	Qaidam Basin of	Chen <i>et al.</i> , 2008

		Qinghai Province, north-west China.	
32.	<i>Nocardiopsis rhodophaea</i> sp. nov.	Hypersaline soils of China	Li <i>et al.</i> , 2006
33.	<i>Nocardiopsis rosea</i> sp. nov.	Hypersaline soils of China	Li <i>et al.</i> , 2006
34.	<i>Nocardiopsis salina</i> sp. nov.	Soil sample collected from the hypersaline habitat of Xinjiang Province, China	Li <i>et al.</i> , 2004
35.	<i>Nocardiopsis terrae</i> sp. nov.	Saline soil collected from the Qaidam Basin, north-west China.	Chen <i>et al.</i> , 2010
36.	<i>Nocardiopsis xinjiangensis</i> sp. nov.	Saline soil sample from the Xinjiang Province, People's Republic of China.	Li <i>et al.</i> , 2003
37.	<i>Prauserella alba</i> sp. nov.	Saline soil of Xinjiang Province, West of China	Li <i>et al.</i> , 2003
38.	<i>Prauserella halophila</i> sp. nov.	Saline soil of Xinjiang Province, West of China	Li <i>et al.</i> , 2003
39.	<i>Saccharopolyspora halophila</i> sp. nov.	Saline lake in Xinjiang Province, North-West China	Tang <i>et al.</i> , 2009
40.	<i>Saccharopolyspora lacisalsi</i> sp. nov.	Hypersaline habitat of Tarim Basin in Xinjiang Province, North-West China	Guan <i>et al.</i> , 2011
41.	<i>Saccharopolyspora qijiaojingensis</i> sp. nov.	Salt lake in Xinjiang Province, North-West China	Tang <i>et al.</i> , 2009
42.	<i>Saliactinospora qingdaonensis</i> gen. nov. sp. nov.	Salt pond in Qingdao, China	Chang <i>et al.</i> , 2012
43.	<i>Salinisphaera halophila</i> sp. nov.	Brine of a salt well in Yunnan Province, China	Zhang <i>et al.</i> , 2012
44.	<i>Streptomonospora alba</i> sp. nov.	Xinjiang Province, China	Li <i>et al.</i> , 2003
45.	<i>Streptomonospora amyloytica</i> sp. nov.	Salt lake of North- West China	Cai <i>et al.</i> , 2009
46.	<i>Streptomonospora flavalba</i> sp. nov.	Salt lake of North- West China	Cai <i>et al.</i> , 2009
47.	<i>Streptomonospora halophila</i> sp. nov	Hypersaline soil of Xinjiang Province, China	Cai <i>et al.</i> , 2008

48.	<i>Streptomonospora salina</i> gen. nov., sp. nov.	Salt lake in West of China	Cui <i>et al.</i> , 2001
49.	<i>Streptomyces fukangensis</i> sp. nov.	Dessert soil of Xinjiang Province, North-west China	Zhang <i>et al.</i> , 2013
50.	<i>Streptomyces iconiensis</i> sp. nov.	Salt lake and saltern	Tatar <i>et al.</i> , 2014
51.	<i>Streptomyces smyrnaeus</i> sp. nov.	Salt lake and saltern	Tatar <i>et al.</i> , 2014
52.	<i>Zhihengliuella halotolerans</i> gen. nov., sp. nov.	Saline soil from Qinghai Province, north-west China	Zhang <i>et al.</i> , 2007

Variation in the tolerance to sodium chloride (NaCl) concentration, has been a remarkable category to differentiate between the strains especially for the genus *Streptomyces*. Tresner *et al.*, (1968) has done an extensive study on the NaCl tolerance of 1300 strains of 313 species. Their findings included that 1.8% of the strains could not tolerate 4% of NaCl, whereas 26.9%, 49.7% and 18.8% strains could tolerate up to 4%, 7% and 10% of NaCl concentration, respectively. Spore colouration of the strains was one of the major observation which was correlated with the NaCl tolerance as ‘white’ and ‘yellow’ spore coloured strains showed higher tolerance, whereas ‘red’ ones showed lesser tolerance. The study concluded that since high degree of similarity exists regarding NaCl tolerance between majority of the similar species, so this NaCl tolerance should be used as physiological taxonomic criterion.

In the detailed study of actinobacterial diversity in the saline sediments of Yunnan and Xinjiang province, China Wu *et al.*, (2009) highlighted on the few major points based on their discovery viz. 1. “Geographic distance was not an important factor controlling actinobacterial community structure”, 2. Isolation was done on the media containing 10% NaCl (w/v), so the isolates could be halophilic and halotolerant, 3. Actinobacteria could tolerate wide range of salinity since the explored actinobacterial diversity was inconsistent with the ecological principle “more extreme environments decrease diversity”.

2. 3. Exploration of biodiversity of hypersaline actinomycetes (International and National status):

Johnson *et al.* (1986) first described an extremely halophilic actinomycete, *Actinopolyspora halophila*, discovered by Gochnauer *et al.* (1975) which grew in the presence of 10 to 25% NaCl and also could grow in saturated NaCl.

Sr. No	Sampling location	Actinomycete groups reported	Significance	References
1.	Saline sediments of Yunnan and Xinjiang Provinces, China	Suborders: <i>Corybacterineae</i> , <i>Frankineae</i> , <i>Glycomycineae</i> , <i>Micrococcineae</i> , <i>Micromonosporineae</i> , <i>Pseudonocardineae</i> , <i>Propionibacterineae</i> , <i>Streptomycineae</i> and <i>Streptosporangineae</i> ; <i>Acidimicrobiales</i> and unclassified actinobacteria.	First study of actinobacterial diversity in saline sediments	Wu et. al., 2009
2.	Hypersaline soils of Kolhapur, Maharashtra, India	<i>Actinoplanes</i> , <i>Kitasatospora</i> , <i>Micromonospora</i> , <i>Microbispora</i> , <i>Nocardia</i> , <i>Streptoverticillium</i> and <i>Streptomyces</i>	Screened for anti-microbial compounds	Nakade, 2012
3.	Solar salterns of Tuticorin, Tamil Nadu, India	<i>Micromonospora</i> , <i>Nocardia</i> , <i>Nocardiopsis</i> , <i>Nonomuraea</i> , <i>Saccharopolyspora</i> , <i>Streptomyces</i>	First report on the actinomycete biodiversity of the solar salterns from eastern coastal region of India and genus <i>Nonomuraea</i> was isolated for the first time from the solar saltern	Jose and Jebakumar, 2012

In a very recent and exclusive study on the microbial community analysis of the salt deserts of Kutch, Gujarat, India based on the metagenomics Pandit *et al.* (2015) have shown, actinobacteria constituted about 2.1 – 5.8% of the total microbial community and *Rubrobacter* was found to be the most predominant genera among the other members of the phylum. Till date, a total of 7 genera viz. *Streptomonospora*, *Haloactinospora*, *Haloactinopolyspora*, *Haloechinothrix*, *Haloglycomyces*, *Saliactinospora* and *Zhihnengliuella*, which have been discovered as ‘novel genera’ from hypersaline environment (Table. 1) and they are also obligate halophiles. Fig. 1 shows the percentage of novel species discovered from various marine as well as hypersaline genera.

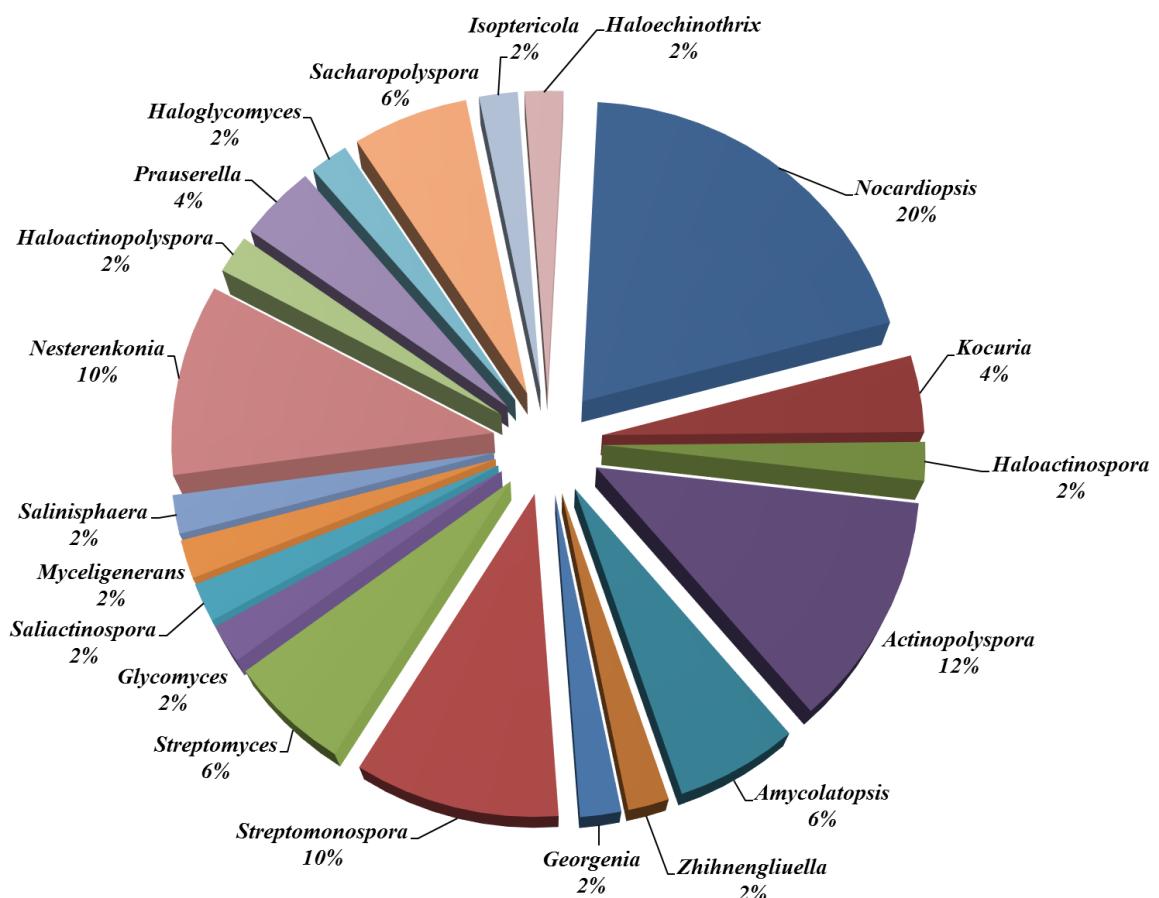


Fig. 1 Graph showing the percentage of novel species of various genera isolated exclusively from saline and hypersaline environment

2. 4. Bioprospecting for anti-microbial compounds of actinomycetes of saline and hypersaline environment:

Biodiversity and bioprospecting programmes go hand in hand for the commercial success of discovering novel biomolecules; one being the resource and the other being the application. As reviewed by Williams (2008) with the hypothesis that “taxonomic diversity to chemical diversity”, exploring microbial diversity to discover novel or unexplored taxa in turn increases the chance of discovering novel chemical entities with immense applications. In past few decades majority of the drug discovery programmes have been focussed on the terrestrial eubacteria including actinomycetes hence this filamentous bacteria accounts for around 45% of the bioactive metabolites discovered from nature. With regards to bioprospecting for secondary metabolites from actinomycetes of marine, saline and hypersaline environments being of immense importance due to high metabolic capacity and possessing unique physiological features of extremophilic nature, only minuscule research interest has been contributed [Hamedi *et al.*, 2013]. Research in past few decades have shown that discovery of novel bioactive compounds from terrestrial actinomycetes have decreased since similar compounds are being re-discovered in multiple reports [Fenical *et al.*, 1999]. Hence, the focus of the bioprospecting should be directed towards actinomycetes from various marine environments which are rarely exploited for the discovery of novel bioactive compounds. As mentioned earlier the actinomycetes are widely distributed. Marine actinomycetes are not exclusively confined in the deep ocean floor but also various coastal ecosystems viz. sandy coastline, mangroves, marine salterns etc. Unlike terrestrial, the marine actinomycetes thrive in extreme harsh environmental conditions such as extreme salinity, pressure, temperature etc. This triggers multiple

metabolic pathways in them resulting in the secretion plethora of secondary metabolites which hold tremendous importance as bioactive compounds in the field of Biotechnology. Though research on marine actinomycetes are still in infant stage and the number of novel bioactive compounds discovered from the marine actinomycetes have surpassed the number from their terrestrial counterparts [Lam, 2006].

Novel anti-microbial compounds are always on crucial demand due to rise of drug resistant pathogens and for the discovery of more efficient broad spectrum antibiotics. In this regards secondary metabolites of marine actinomycetes provide extensively exploitable resource as natural product [Bull and Stach, 2007]. Imada *et al.*, (2010) have reported a series of *Streptomyces* (37%) and *Micromonospora* (26%) isolates from the marine sediment of the neritic zone of the sea surrounding Japan with varying tolerance to NaCl. Tolerance to NaCl was a major characteristic of marine strains compared to their terrestrial counterparts and strains with higher NaCl tolerance were found to produce anti-microbial metabolites in presence of sea water (35 psu; 3.5% salt) against *Bacillus subtilis* and *Candida albicans*. In a report on the marine actinomycetes strains from the coast of Bismark and Solomon Sea of Papua New Guinea, it was found that majority of the strains belonged to new genera from the family *Micromonosporaceae*. The strains also produced metabolites which had bactericidal activity against multi-drug resistant Gram positive pathogens.

2. 5. Anti-microbial profiles of the actinomycetes from marine salt pans:

Halophilic and halotolerant actinomycetes from various marine environment have been to produce potent actinomycetes but anti-microbial compounds from exclusively hypersaline actinomycetes are seldom reported. The first report of a halophilic actinomycete to produce anti-microbial compounds, was shown by Yoshida *et al.*

(1991). They have reported moderately halophilic actinomycetes *Actinopolyspora mortivallis* which was isolated from saline environment to produce a nucleoside antibiotic. In India few attempts have been made to explore the anti-microbial potential of salt pan actinomycetes but the studies mostly restricted to preliminary screening and characterization of the isolate. Dhanasekaran *et al.* (2005) showed production of anti-bacterial metabolites from one *Saccharomonospora* sp. and two *Streptomyces* spp isolated from salt pan sediment. Lakshmiopathy and Kannabiran (2009) reported on three *Streptomyces* spp. from saline soils of Ennore saltpan, Chennai, India showing activity against dermatophytes. Roshan *et al.* (2013) screened the salt pan actinomycetes of Marakanam district, Tamil Nadu, India for anti-microbial activity and reported two *Streptomyces* spp. to produce anti-microbial metabolites against *Staphylococcus aureus*, *Aspergillus niger* and *Aspergillus fumigatus*. In a very recent report Jose and Jebakumar (2015) reported a rare actinomycete belonging to the genus *Actinoalloteichus* from an inland solar saltern located adjacent to Sambhar Lake, Jaipur, India to produce anti-microbial metabolites against methicillin resistant *Staphylococcus aureus* (MRSA), *Pseudomonas* sp., *Proteus vulgaris*, *Escherichia coli*, *Bacillus* sp. and yeast *Candida albicans*. These reports show there is significant potential in the actinomycetes from Indian salt pans and it's an urgent need to explore their secondary metabolites for establishment of novel molecules as anti-microbial drugs.

Hence, the present study holds its importance to explore the community structure of hypersaline actinomycetes in the marine salt pans of Goa and assess their secondary metabolites to establish a ‘novel molecule’ as a potential anti-microbial agent.

All these detailed reports on actinomycetes from India and their potential applications in the field of biotechnology have been compiled and published in a review article.

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Biotechnological significance of Actinobacterial research in India.

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Abstract

Actinobacteria are of special biotechnological interest since they are known to produce chemically diverse compounds with a wide range of biological activity. This distinct clade of Gram-positive bacteria include some of the key antibiotic producers and are also sources of several bioactive compounds, established commercially. The class *Actinobacteria* holds some of the resilient species, capable of growing in extreme, hostile and polluted environments. Their adaptation has been the outcome of several chemical entities which are answers to a number of medicinal and industrial questions of today. In India, actinobacterial research in both marine and terrestrial ecosystems has prospered significantly in past few decades. This valuable class contains large number of genera and demands more attention for exploration. Though substantial work in this field has been carried out, the diversity from the extreme environments in the Indian Peninsula remain unexplored. Marine actinobacterial research has been restricted to the coastal ecosystem while the deep sea oceanic floors remain untapped. Substantial bioprospecting of actinobacteria for bioactive molecules, has not been explored in extremophilic environments in India and the molecular mechanisms for the production of various bioactive compounds are yet to be reported. The present review enlists the prolific metabolites from culturable actinobacteria and attempts have been made to focus on the potentially feasible aspects of actinobacterial research in this field.

Keywords: Actinobacteria, antibiotic, bioprospecting, diversity, enzymes, extreme.

3. MATERIALS AND METHODS:

3. 1. BIODIVERSITY:

3. 1. 1. Sampling sites:

Two sampling sites were selected based on the previous reports on bacterial diversity, geographical location and influence of the anthropogenic effect. Two different locations: salt pans of Ribandar and Agarvado were selected and geographical position of both the sites were recorded by a hand held Global Positioning System (GPS) [eTrexVista™ personal navigator®, Garmin International, Inc., USA].

3. 1. 1. a. Description of Ribandar salt pan:

Ribandar salt pan is situated Tiswadi Taluka along the Mandovi estuary near the city of Panjim, Goa. This salt pan area is surrounded by mangrove swamps and the most prevalent majorly found mangroves species are *Sonneratia alba*, *Aegiceras corniculatum*, *Avicennia officinalis*, *Avicennia marina*, *Acrostichum aureum*, *Acanthus ilicifolius*, *Bruguiera gymnorhiza*, *Excoecaria agallocha*, *Kandelia candel*, *Thespesia populnea*, *Rhizophora mucronata*, *Xylocarpus* spp and *Acacia intsia*. Due to the transport of ferro-manganese ore by the barges and ships, the Mandovi estuary is under the influence of metal influx. The salt pan area is being situated near the city, the estuarine water entering the salt pan also receives the metals, effluents and pollutants entering the estuary. The sampling points in Ribandar salt pan were (N 15° 30'07.9" and E 073° 51'18.2"), (N 15° 29'58.1" and E 073° 50' 49.2"), (N 15° 30' 8.1" and E 073° 51' 19.6") and (N 15° 28'45.6" and E 073° 48' 41.6").

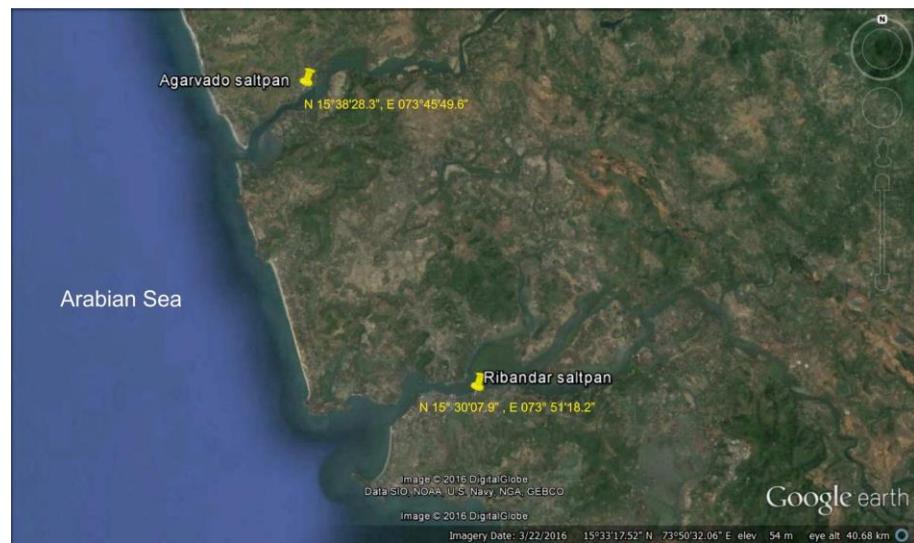


Fig. 2 Locations of Agarvado and Ribandar salt pan in GPS data

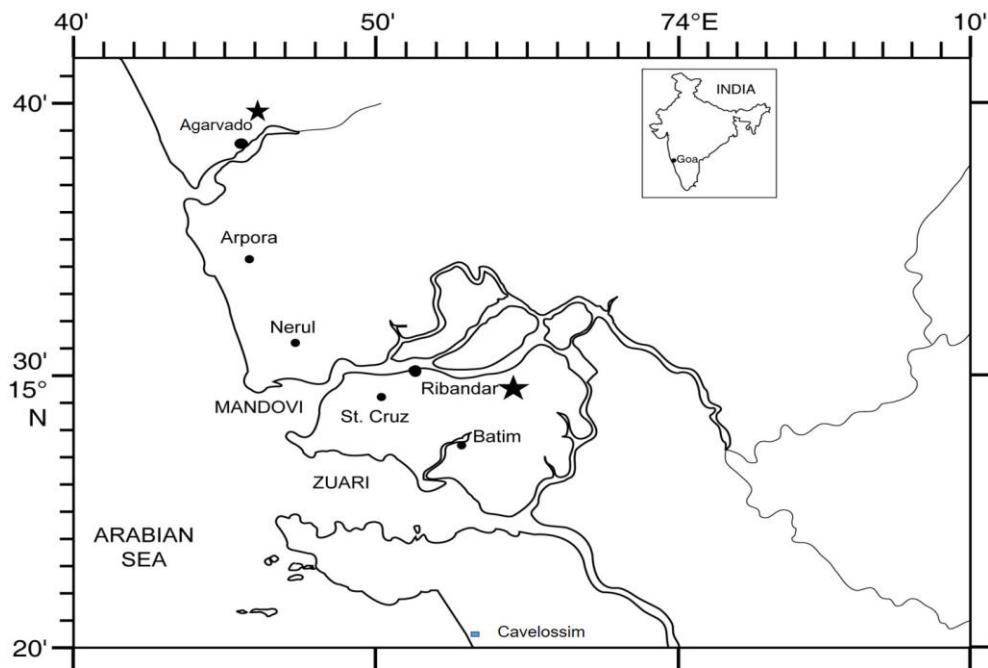


Fig. 3 Map of Goa showing the locations of all the existing salt pans, Ribandar and Agarvado are highlighted with (*)



Fig. 4 Sampling site – 1 Ribandar salt pan located at Mandovi estuary, Tiswadi taluka, Goa, India



Fig. 5 A. Salt crystalizer pond of Ribandar salt pan, B. Crystalized salt before harvesting

3. 1. 1. b. Description of Agarvado salt pan:

Agarvado is a pristine salt pan situated in the Chapora estuary of Pernem Taluka, Goa. Mangrove plantation are scanty in Agarvado and the salt pan is surrounded with coconut trees. This area is located away from the city and not much influenced by the anthropogenic activity. Neighbouring the salt pan are discrete lands of paddy fields and villages of the farmers. The GPS locations of the sampling points in Agarvado salt pan were: 1. N-15°38'28.3", E-073°45'49.6", 2. N-15°38'28.6", E-073°45'52.5", 3. N-15°38'25.9", E-073°45'53.0", 4. N-15°38'25.2", E-073°45'50.3".

3. 1. 2. Sample collection:

Sediment samples were collected with the help of the sterile cores (0 – 10 cm) using a 1.5 inch diameter, 15 cm long graduated PVC pipe. The cores were sealed at both ends with sterile core caps to prevent direct contact with air. Water samples were collected in sterile BOD bottles, which were sealed immediately after collection. Both sediment and water samples were transported to the laboratory in an icebox for further physico-chemical and microbiological analyses. Samples were collected during peak salt manufacturing seasons both pre-monsoon (April – May) and post-monsoon (November – December) for three years 2011, 2012 and 2013. Data obtained from the observations made, was subsequently grouped to tally the three year period.



Fig. 6 Sampling site – 2: Agarvado salt pan located at Chapora estuary of Pernem taluka, Goa, India



Fig. 7 A. Salt crystalizer pond of Agarvado salt pan, B. Crystalized salt before harvesting

3. 1. 3. Analysis of various physico-chemical parameters:**3. 1. 3. a. Hydrographic parameters:**

Temperature, salinity, pH and dissolved oxygen of the overlying salt pan water were measured at the sampling site in the salt crystallizer ponds. For temperature measurements, a field thermometer (76 mm immersion, ZEAL, England) and for pH a digital pH meter (pH Tester 30, Eutech Instruments, Thermo Scientific, USA) was used. The pH meter was calibrated with the standard buffers as per manufacturer's instructions, before sampling. For salinity, a hand held refractometer (S/Mill-E, ATAGO Co. Ltd., Japan) was used after calibration with milliQ water. Dissolved Oxygen was measured using hand held multi-parameter device (CyberScan PC 650, Eutech Instruments, Thermo Scientific).

3. 1. 3. b. Estimation of total organic carbon (TOC):

Total organic carbon the sediment samples were estimated by the titrimetric method as suggested by Allen *et al.* (1976). The method involves complete oxidation of the organic matter in the sediment sample by chromic acid. Sediment samples were dried at 70°C in an oven for overnight and then the dried sediment were ground to a fine powder using a mortar and pestle. 0.5 gm of this powdered sediment sample were taken in a 250 mL conical flask, 25 mL of the acid dichromate was added and the flasks were incubated at 60°C in a water bath for 1 hour. After incubation 100 mL of distilled water was added to the flasks and this solution was titrated against ferrous ammonium sulphate (Mohr's salt) with

diphenylamine indicator (approx. 0.5 mL or 15 drops). The end point was denoted by change in the colour (dark blue to green). Glucose (0.01 gm) was used as standard and blanks were processed without the sediment samples. Values were subsequently calculated and TOC was expressed as percentage.

3. 1. 3. c. Sedimentology analysis (sand: silt: clay):

The amount of the sand, silt and clay in the sediment samples of both the salt pan sediment samples was determined using wet sieving method for sand and pipette method for silt and clay contents [Day, 1965; Carver, 1971]. For analysis, 15 gm of sediment sample (in triplicates from both the salt pans) was dried in an oven at 60°C and was then transferred to a 1000 mL beaker. Distilled water was added to the beaker up to the rim and then the sediment suspension was stirred with a glass rod to break if any clump remained. Then it was allowed to settle for 24 hours. The next day the water was removed using a decanting pipe and fresh distilled water was added. This step was repeated till all the salt content was removed from the sediment samples. Once all the salt was removed, maximum water was decanted and 10 mL of 10% sodium hexametaphosphate was added to suspension (role of sodium hexametaphosphate to dissociate clay particles). Next day 5 mL of 30% H₂O₂ was added to oxidize the organic matter. Contents were mixed by swirling the beaker and was allowed to stand for 30 mins. Contents of the beaker was then poured through a 63 µm (230 mesh) sieve and the filtrate was collected into a 1000 mL measuring cylinder. Content of the beaker was washed off with distilled water and passed through the sieve, till the solution became clear. Then

water volume of the cylinder was made up to the mark (1000 mL). This filtrate in the cylinder was used for pipette analysis. The sand which remained on the sieve was transferred to a pre-weight beaker, oven dried (60°C) to remove the moisture and then again the weight of the beaker was taken followed by calculation of the amount of the sand. For pipette analysis, before withdrawing the filtrate from the measuring cylinder, the contents were homogenized by stirring for about 2 mins using a stirrer. This was then allowed to settle for definite time as per room temperature referring to Table. 1. The stirring and pipetting time were noted. 25 mL of the solution was pipetted out at 8 Ø by inserting pipette up to 10 cm depth from the cylinder. Pipetted solution was then transferred in to pre-weighed 100 mL beaker and dried at 60°C overnight. After drying weight of beakers containing clay was recorded followed by calculation of the percentage of the sand, silt and clay of the sediment samples.

Table. 3 Time table for pipette withdrawal to quantify silt and clay in sediment analysis

Sieve size (Ø)	Depth to which the pipette has to be inserted	Time after which water is to be pipetted out at various temperatures				
		Hours: Minutes: Seconds				
		28°C	29°C	30°C	31°C	32°C
4	20	0:00:48	0:00:46	0:00:46	0:00:44	0:00:44
5	10	0:01:36	0:01:34	0:01:32	0:01:29	0:01:28
6	10	0:06:25	0:06:15	0:06:06	0:06:57	0:05:52

7	10	0:25:40	0:25:02	0:24:25	0:24:49	0:23:57
8	10	1:42:45	1:40:13	1:37:42	1:37:15	1:33:51
9	10	6:30:00	6:40:40	6:32:50	6:32:10	6:11:30
10	10	27:06:00	26:30:00	-	-	-

3. 1. 4. Microbiological parameters:

3. 1. 4. a. Retrievable counts of heterotrophic bacteria:

Quantification of the culturable heterotrophic bacteria was carried out from both sediment and water samples. Retrievable bacterial counts were estimated by standard serial dilution and the plate count method. Sediment (10 g) from total homogenized core samples were added to the 90 ml of the sterile salt pan water (32 psu) in 250 mL conical flask (sealed) and were allowed to shake at 120 rpm for 30 mins to obtain a uniform suspension. Sediment suspension (10 mL) were further added to 90 mL of sterile salt pan water for serial dilution. For the water samples, the collected sample was considered as stock and from there further dilutions were made in sterile salt pan water. From each dilution, 100 µL was spread plated on to Zobell marine agar (Appendix – I) and Nutrient agar (Appendix – I) media plates prepared with 8 different levels of salinity viz. 32, 50, 75, 100, 150, 200, 250 and 300 psu. Spread plating was done with 10^{-2} , 10^{-3} , 10^{-4} dilutions of the sediment samples and stock, 10^{-1} , 10^{-2} dilutions of the water

samples in triplicates. To create the salinity gradients natural sea salt (collected during sampling from each site) was used to mimic the ecobiome and hence used in isolation media. Plates were incubated at $28^{\circ}\text{C} \pm 2$ up to 3 – 4 weeks depending on the increased appearance of the colonies.

3. 1. 4. b. Estimation of soil microbial biomass:

Sediment sample (10g) was taken in two 50 mL glass beakers. One beaker was incubated at 4°C for 24 hours at room temperature ($28^{\circ}\text{C} \pm 2$). Chloroform 20 mL was added to another beaker and it was incubated in the desiccator for 24 hours. After incubation, sediments from both the beakers were transferred to two separate 150 mL conical flasks and 25 mL of 0.5 M K_2SO_4 was added to both. The flasks were incubated on shaker incubator at 150 rpm for 30 mins and at room temperature ($28^{\circ}\text{C} \pm 2$). After incubation these sediment suspension was filtered using Whatman No. 1 filter paper to separate the sediment particles from the filtrate. 10 mL of the filtrate from each flask were taken to two separate 150 mL conical flasks and 5 mL of 0.2 N $\text{K}_2\text{Cr}_2\text{O}_7$ were added followed by 10 mL of H_2SO_4 and 5 mL of ortho-phosphoric acid. Then the flasks were kept on a hot plate at 100°C for 30 mins. After that it was allowed to cool to 30 - 35°C and 250 mL of MilliQ water was added followed by 2 – 3 drops of diphenylamine indicator. After 2 mins once a deep purple colour developed, it was titrated against 0.05 N Ferrous ammonium sulphate. Titration point was recorded once the solution turned colourless.

3. 1. 5. Standardization of the methodology of actinomycetes isolation:

3. 1. 5. a. Antibiotic concentration:

For isolation of actinomycetes, Nalidixic acid was used as an anti-bacterial compound to inhibit all other bacteria except Actinomycetes and Nystatin as anti-fungal compound to inhibit the growth of fungi respectively. Nalidixic acid with 1 M NaOH stock was prepared in sterile MilliQ water and to dissolve was added. Nystatin was dissolved directly in sterile MilliQ water and both these stock solutions were stored at -20°C. Nutrient agar plates were prepared with various concentrations of both the antibiotics (10 – 100 µg/mL) and the saltpan sediment sample dilutions (as described above) were spread plated. The plates were incubated at room temperature (28°C ± 2) upto 2 weeks and the emerging colonies were counted. The lowest concentrations of both the antibiotics where highest number of actinomycete counts were obtained, was considered as standard/optimized antibiotic concentration for isolation of hypersaline actinomycetes.

3. 1. 5. b. Pre-treatment of the samples:

Both sediment and water samples were pre-treated to decrease the abundant bacterial load. The samples were heat treated at 60°C for 10 mins to decrease the load of fast growing Gram negative bacteria as described by Solano *et al.* (2009). After temperature treatment, 1% calcium carbonate (CaCO_3) as suggested by Alferova and Terekhova (1988) & Otoguro *et al.*, (2001) was added to both sediment and water samples, mixed well with sterile glass rod, mortar and pestle followed by incubation at room temperature (28°C ± 2) for overnight.

3. 1. 5. c. Selection of the specific media:

The media used for the isolation of the hypersaline actinomycetes were selected based on the literatures available on isolation of marine as well as hypersaline actinomycetes [McNeil *et al.* (1992), Lu *et al.* (2003), Kämpfer *et al.* (2008), Ara *et al.* (2008), He *et al.* (2012), Tian *et al.* (2012), Hu *et al.* (2012) and Lohum *et al.* (2016)]. The media which were preferably used for the isolation of marine actinomycetes as well as halophilic and halotolerant actinomycetes from other environments viz. Starch-casein agar, Yeast extract-malt extract agar, Glycerol-asparagine agar, Actinomycete isolation agar, Oatmeal agar, Tryptone yeast extract agar, Inorganic salt starch agar, R2A agar, Kuster's agar, Tyrosine agar, Peptone yeast extract iron agar and Zobell marine agar. These 12 different media were selected for the retrieval of maximum number of isolates which would include the exclusively marine halophilic as well as adapted halotolerant actinomycetes isolates.

3. 1. 6. Isolation of halophilic and halotolerant actinomycetes:

The isolation was carried out on 12 different isolation media mentioned above at 8 different levels of salinity viz. 32, 50, 75, 100, 150, 200, 250 and 300 psu. Saline water was prepared with the crude natural salt collected during the sampling. The isolation media was supplemented with Nalidixic acid 40 µg/mL and Nystatin 25 µg/mL. For sediment samples, 10 g of the sediment from the mixed core samples were added to the 90 mL of the sterile salt pan water (32 psu) in 250 mL conical flask (sealed) and were allowed to shake at 120 rpm for 30 mins to obtain a uniform suspension. 10 mL of this sediment suspension were further added to 90 mL of sterile salt pan water for serial dilution. For the water samples, the collected sample was

considered as stock and from there further dilutions were made in sterile salt pan water. From each dilution, 100 µL was spread plated on to the respective agar plates in triplicates. The plates were incubated at 28°C ± 2 up to 6 – 8 weeks depending on the appearance of the colonies.

3. 1. 7. Purification, maintenance and storage of the isolates:

Each of the emerging actinomycete colonies were monitored, their morphology was noted and based on the variation in the morphology, representative colonies were picked. Each of the colonies were streaked (quadrant) in their respective media plates with respect to their isolation salinity, and incubated at 28°C ± 2 for 2 – 4 weeks depending on their growth. After sufficient growth their detailed colony morphology, presence or absence of the spores, colour of the aerial and substrate mycelium was noted. Isolates were further sub cultured from the isolated colonies and individual ‘culture code’ was assigned to each of the isolates. The isolates were maintained in their respective isolation media and salinity. For storage and preservation, isolates were kept in plates and slants at 4°C and as glycerol stocks at -80°C. Glycerol stocks were prepared by growing the cultures in the respective broth media and then mixing the cells/mycelial filaments with glycerol (20% w/v), followed by aliquoting them in sterile Cryo Vials (Tarsons), immediate freezing and storage at -80°C.

3. 1. 8. Classical taxonomy:

3. 1. 8. a. Grouping of the halotolerant and halophilic isolates:

Eight selected isolates showing maximum production of anti-bacterial metabolite(s) were further studied for their salt requirement for growth and proliferation at 0 psu (no salt), 10, 20, 35, 50, 75 and 100 psu salt supplement in the broth medium. The isolates were spread plated onto their respective agar media and then allowed to grow for 2 weeks at $28^{\circ}\text{C} \pm 2$. Spores were collected from agar plates by adding either 1 ml of sterile saline water of the respective salinity in the experimental broth medium (in case of non-sporulating cultures, vegetative cells were used). The spore and cell suspensions were collected in sterile eppendorf tubes and the spore or cell density was adjusted ~ 0.3 at OD_{590} with sterile saline water and 100 μl of this suspension was inoculated into 50 ml of the respective isolation media broths in their respective salt concentrations. The flasks were kept in shaker incubator for 2 weeks ($28^{\circ}\text{C} \pm 2$). The cells and mycelia were pelleted down, lyophilized and the dry weight was calculated.

3. 1. 8. b. Cultural differentiation of *Streptomyces* and rare actinomycetes:

Actinomycetes isolates were studied for growth and sporulation on the 7 specific growth media viz. Tryptone yeast extract agar (ISP-1), Yeast extract-malt extract agar (ISP-2), Oatmeal agar (ISP-3), Inorganic salt starch agar (ISP-4), Glycerol Asparagine agar (ISP-5) Peptone yeast extract iron agar (ISP-6), and Tyrosine agar (ISP-7) as suggested by International *Streptomyces* Project to group the isolates as *Streptomyces* and rare actinomycetes. The colour of the aerial and substrate mycelia along with sporulation pattern, presence or absence of extracellular pigments of each isolates were noted and compared with reports of Shirling and Gottlieb (1966).

3. 1. 8. c. Microscopic characterization:

For microscopic characterization both light and scanning electron microscopy (SEM) was carried out. The isolates were grown on to the coverslips embedded on a slanting position (45° angle) on to the agar surface, as described by Williams and Davies (1967) to recover the mycelia fragments, spore chains and free spores with minimum disturbance to the morphological architecture. Gram staining technique was used to visualize. Detailed microscopy was carried out using light microscopy (at 100X) and scanning electron microscopy (SEM-Hitachi TM 3000 Table Top Electron Microscope). Based on the observations of architecture of mycelial filaments, sporulation pattern, shapes of the spores and spore chain structures for selected isolates were grouped under various genera.

3. 1. 9. Carbohydrate utilization profiles of halotolerant and halophilic isolates:

Halophilic isolates have a major requirement of salt (35 to 250 psu) for their growth, since the utilization of the carbon source was checked by supplementing the respective carbon sources (1% w/v) into their basal growth media. All the sugars viz. L-Arabinose, Galactose, Glucose, Mannitol, Mannose, Melibiose, Rhamnose and Xylose (1%, w/v) were added to the pre-sterilized basal growth media followed by filter sterilization with 0.2 µm filter (Millipore, USA). Similar cell/spore suspension were prepared as described earlier in sterile sea water and 150 µl cell/spore suspension into 50 mL respective broth media. The flasks were incubated at room temperature (28°C ± 2) at 120 rpm for 96 hrs. Cells/mycelial filaments were harvested by centrifuging at 10,000 rpm for 10 mins in pre-weighed centrifuge tubes,

followed by decanting the supernatant and then dry cell biomass were calculated after drying the cell/mycelial pellet at 37°C overnight.

Carbohydrate utilization profiles of the halotolerant isolates were assessed by BIOLOGTM GP2 MicroPlates (Hayward, CA 94545, USA). The strains were grown on Bacterial Universal Growth (BUG) agar and on R2A agar media at room temperature (28°C ± 2). In case of non-sporulating cultures, cells were harvested from agar plate after 24 - 48 hrs and sporulating cultures were observed for aerial and substrate mycelium development followed by harvesting the spores by scraping from agar surface. Spores and cells were suspended in inoculation fluid (GN/GP) and the cell density was adjusted at 600 nm to 0.3. The BIOLOGTM GP2 microplate was immediately inoculated with 150 µl cell/spore suspension per well with the help of a multi-channel pipette and incubated at room temperature (28°C ± 2) in sealed plastic bags for 96 hrs. All wells resembling the blank were scored as negative and all wells with noticeable purple colour were scored as positive. Few of the halotolerant isolates (non-spore formers) were also checked by the carbohydrate utilization kit (HiMedia, India).

3. 1. 10. Grouping of the isolates based on the tentative identification of the genera:

The data obtained from all the above characterization viz. colony morphology, requirement of salt for growth, growth rate & temperature, growth on selective media, colour of aerial & substrate mycelia, sporulation pattern, structural architecture on SEM analysis and carbohydrate utilization pattern was compiled and tabulated. These data were compared with the reports available from the type strains of the various genera of

actinomycetes from the journal International Journal of Systematic and Evolutionary Microbiology (IJSEM) and a presumptive identification was made with respect to their genera. Few distinct ones were under each genera were selected for further molecular characterization.

3. 1. 11. Molecular characterization:

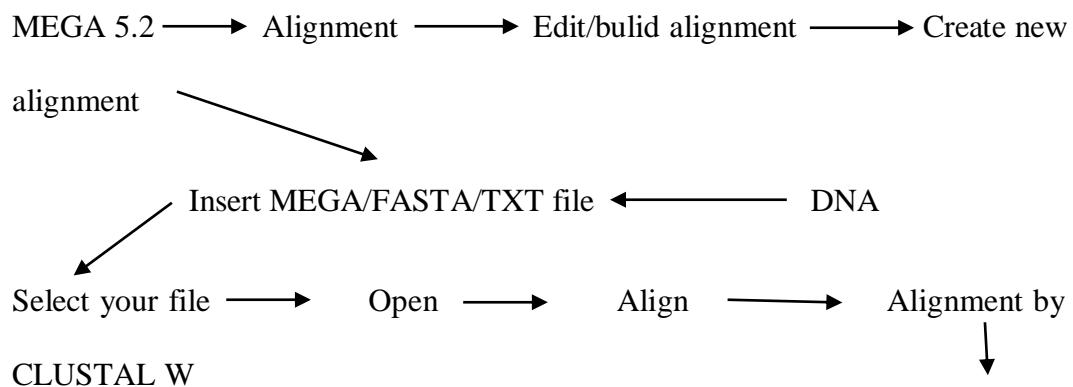
3. 1. 11. a. 16S rRNA gene sequencing:

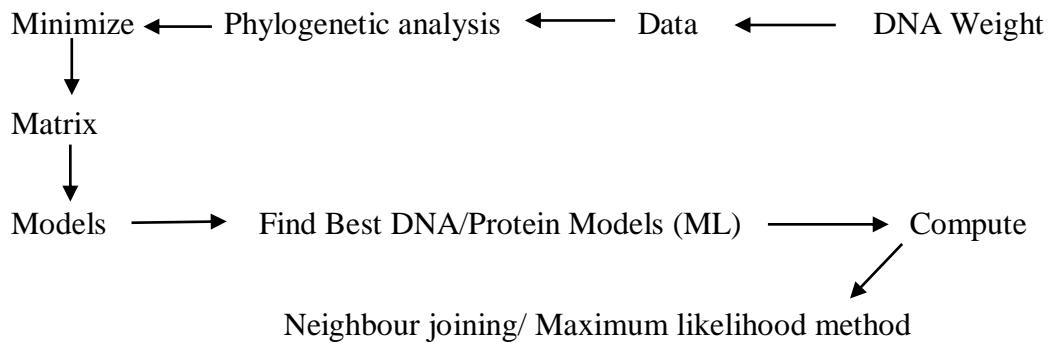
Selected representative isolates from each genera were identified by 16S rRNA gene sequencing. Genomic DNA was isolated by Axygen Bacterial genomic DNA isolation kit/Chromous Biotech fungal genomic DNA isolation kit/manual protocols. Genomic DNA was visualized in 0.8% TBE agarose gel. Using these DNA as templates, 16S rRNA gene of the isolates were amplified by PCR using universal bacterial primers 27F: AGAGTTGATCCTGGCTCCAG and 1492R: TACGGTTACCTTGTACGACTT. The PCR products were run on 1% TBE agarose gel along with 1 kb DNA ladder (NEW ENGLAND BioLabs. Inc). The expected 1.5 kb length band was excised with the help of a sterile scalpel and gel elution was done using GE Health Care, illustraTM GFXTM PCR DNA and Gel band purification kit. The eluted products were used as a template for sequencing PCR using Applied Biosystem Kit with the bacterial universal primers and also internal primer sets (357f: 5'-CTCCTACGGGAGGCAGCAG-3', 704f: 5'-TAGCGGTGAAATGCGTAGA-3', 685r: 5'-TCTACGCATTCACCGCTAC-3', and 1110r: 5'-GGGTTGCGCTCGTTG-

3'). The amplified PCR products were sequenced using Applied Biosystem DNA analyser and the sequences were screened using BioEdit software and aligned with Clustal W [Larkin *et al.*, 2007].

3. 1. 11. b. Phylogenetic analysis:

The obtained 16S rRNA gene sequences of the actinomycete isolates were analysed by BLAST analysis and by EzTaxon server. The aligned sequence was submitted to GenBank after analysis. The phylogenetic tree was constructed with the 16S rRNA gene sequences type strains of the same genera and other related isolates based on the BLAST and EzTaxon results, in MEGA 5.2 using Neighbour joining method and Tamura-Nei model with bootstrap values of 1000 replicates [Saitou and Nei (1984) and Kumar *et al.* (2008)]. All the selected isolates for biodiversity and bioprospecting study were identified using the same technique mentioned above.





3. 1. 12. Physiological characterization of extremely halophilic rare actinomycete isolates:

3. 1. 12. a. Growth optimization in various temperatures, pH and synthetic media:

Growth optimization was done of selected extremely halophilic isolates with delayed growth period (up to 4 – 6 weeks). Temperature (10 – 50°C, at regular intervals) and pH (2 – 10) were employed to optimize the growth in the broth media. The media was inoculated with 100 µL of spore suspension prepared in 250 psu salt pan water containing 10⁶ spores/mL. For growth in synthetic media, the basal medium used was of inorganic salt starch broth with varying carbon and nitrogen sources (either at a time). Cells/mycelial filaments were harvested by centrifuging at 10,000 rpm for 10 mins, followed by decanting the supernatant and then the dry cell biomass was calculated after drying the cell/mycelial pellets.

3. 1. 12. b. Utilization of various carbon sources:

Halophilic isolates had a major requirement of salt (35 to 250 psu) for the growth so the utilization of the carbon source was checked by supplementing the respective carbon sources (1% w/v) into their growth media. Similar cell/spore suspension were prepared as described earlier in sterile sea water and 150 µL cell/spore suspension into 50 mL broth media (prepared with all the components except the carbon source) and supplemented with respective carbon source. The flasks were incubated at 28°C ± 2 and 120 rpm for 96 hrs. Dry cell biomass was calculated after drying the cell/mycelial pellets.

To check growth in completely synthetic media, the salt solution of Inorganic salt starch agar was used as basal media supplemented with various concentrations of synthetic NaCl, KCl and MgCl₂ along with carbon and nitrogen sources. Inoculation was done the same way as mentioned above, followed by incubation and dry cell biomass calculation.

3. 1. 13. Assessment of biodiversity of actinomycetes in the salt pan ecosystem:

All the above mentioned techniques contributed to the phenotypic, metabolic and molecular characterization of the isolates. The obtained data were processed, to determine the diversity of the various genera of actinomycetes in the marine salt pans. Among the total number of isolates collected, the percentage of *Streptomyces* and rare actinomycete genera were calculated to obtain the community structure profile in both sediment and water of both Ribandar and Agarvado salt pans. To assess the biodiversity in both the salt pans in pre-

monsoon and post-monsoon seasons the ‘Shannon index (H)’, Simpson’s diversity index and SHE (S -species richness; H information – the Shannon-Wiener diversity index and E - evenness as measured using the Shannon-Wiener evenness index or Pielou J) were employed [Shannon and Weaver (1949), Pielou (1969) and Magurran (1988)].

3. 2. BIOPROSPECTING:

3. 2. 1. Screening for the production anti-microbial metabolites:

Actinomycete isolates were grown in the respective broth media (50 mL) at respective salinities. After sufficient growth, the mycelial filaments/cells were separated by centrifuging the culture broth at 10,000 rpm for 10 mins at 4°C. The activity of cell free metabolites was assessed against 15 pathogenic bacteria and 7 phyto & human pathogenic fungi (Bacteria: *Escherichia coli*, *Aeromonas hydrophila*, *Acinetobacter baumanii*, *Citrobacter freundii*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Morganella morganii*, *Proteus vulgaris*, *Salmonella typhi*, *Salomonella typhimurium*, *Shigella dysenteriae*, *Vibrio cholera*, *Shigella boydii*, *Staphylococcus aureus* and *Staphylococcus citreus*) and the fungi being *Aspergillus niger*, *Aspergillus fumigatus*, *Curvularia lunata*, *Penicillium* sp., *Rhizopus stolonifer*, *Phoma* sp. and *Vermicularopsiella* sp. respectively) by disc

diffusion assay. The assay were performed by loading cell free metabolite (10 µL) on to sterile Whatman No. 1 filter paper discs (5 mm) followed by drying and placing on to seeded bacterial and fungal plates. The bacterial and fungal assay plates were incubated at 37°C and room temperature (28°C ± 2) respectively. Assays were repeated in triplicates with respective media control and salinity to confirm the consistent production of anti-microbial metabolites. The zone of inhibition were measured using Hi-Antibiotic ZoneScale (HiMedia) and the data was tabulated as mean ± standard deviation (SD).

3. 2. 2. Sources of the clinical pathogens:

The clinical pathogens were obtained from Department of Microbiology, Goa Medical College (GMC) & Hospital, Goa. The bacterial isolates were grown and maintained on Nutrient agar plates and slants. The fungal pathogens were obtained from Goa University Fungal Culture Collection Centre (GUFCC) and they were grown and maintained on potato dextrose agar plates and slants. The pathogens were stored at 4°C and were repeated sub cultured at interval of 7 days for bacteria and 15 days for fungi.

Table 5. Names of the clinical pathogens, sources of isolation and diseases caused

Sr. No.	Name of the pathogen	Isolated from	Disease caused
1.	<i>Acinetobacter baumanii</i>	Blood, Urine	Nosocomial bacteremia
2.	<i>Aeromonas hydrophila</i>	Stool	Dysenteric gastroenteritis, cellulitis

3.	<i>Citrobacter freundii</i>	Stool	Diarrhea, food poisoning (due to enterotoxins)
4.	<i>Escherichia coli</i>	Stool, Urine	Gastroenteritis, food poisoning, urinary tract infections, neonatal meningitis, bowel necrosis, peritonitis, mastitis and septicemia
5.	<i>Escherichia coli</i> ATCC strain	ATCC 25922	Type strain
6.	<i>Klebsiella pneumoniae</i>	Blood	Pneumonia, Bacteremia and meningitis
7.	<i>Morganella morganii</i>	Urine	Urinary tract infection
8.	Methicillin resistant <i>Staphylococcus aureus</i> (MRSA)	Pus	Furunculosis, pyemia, abscesses of the wounds and food poisoning.
9.	<i>Pseudomonas aeruginosa</i>	Lung mucus	Nosocomial infections, sepsis, folliculitis and opportunistic infections on burn wounds & cystic fibrosis
10.	<i>Pseudomonas aeruginosa</i> ATCC strain	ATCC 27855	Type strain
11.	<i>Staphylococcus aureus</i>	Pus	Skin infections, wound abscesses and respiratory infections
12.	<i>Staphylococcus aureus</i> ATCC strain	ATCC 25923	Type strain
13.	<i>Proteus vulgaris</i>	Urine	Urinary tract infection
14.	<i>Salmonella typhi</i>	Blood	Typhoid
15.	<i>Salmonella typhimurium</i>	Blood	Food poisoning
16.	<i>Shigella flexineri</i>	Stool	Bacillary dysentery
17.	<i>Staphylococcus citreus</i>	Pus	Abscesses of the wounds, furunculosis
18.	<i>Vibrio cholerae</i>	Stool	Cholera

Table 6. Names of the fungal pathogens, sources of isolation and diseases caused

Sr. No.	Name of the pathogen	Isolated from	Disease caused
1.	<i>Aspergillus niger</i> strain GUFCC 4998	Soil	Common post-harvest pathogen; “Black mould” disease of onion, apricots, grapes and “Aspergillosis” in humans
2.	<i>Rhizopus stolonifer</i> strain GUFCC 3425	Cattle dung	Soft rot in sweet potatoes and opportunistic zygomycosis infections in humans
3.	<i>Curvularia lunata</i> strain GUFCC 1293	Leaf litter of <i>Careya arborea</i>	Seedling blight and seed germination failure in sugarcane, rice, millet and maize; leaf spot on angiosperms [94, 95], severe allergy and Phaeohyphomycosis in immunocompromised patients [96]
4.	<i>Penicillium</i> sp. strain GUFCC 396	Leaf litter of <i>Caryota urens</i>	Post-harvest pathogen for citrus fruits; systemic infections in immunocompromised patients
5.	<i>Phoma</i> sp. strain GUFCC 216	Leaf litter of <i>Dendrocalamus strictus</i>	Leaf chlorosis, defoliation, twig die backs, stem blight in Olive plants [Papachatzis and Vagelias (2008)], Dry rot in potatoes and tubers [Persley et al. (2010)]
6.	<i>Acremonium</i> sp. strain GUFCC 1013	Leaf litter of <i>Dendrocalamus strictus</i>	Systemic infection and wilting of Sorghum plants [Bandyopadhyay et al. (1987)], Mycetoma in humans, infection and

			colonization in the eye, lungs and gastro-intestinal tract [Fincher <i>et al.</i> (1991)]
7.	<i>Pestalotiopsis</i> sp. strain GUFCC 1251	Leaf litter of <i>Elaeis guineensis</i>	Scab disease on Guava [Keith <i>et al.</i> (2006)], severe infection as “Keratitis” [Monden <i>et al.</i> (2013)]
8.	<i>Guignardia bidwellii</i> strain GUFCC 241	Fresh leaf of <i>Saraca ascoa</i>	Leaf spots, blotch and rot disease of orchids, “Black rot” of Grapes, black spots on citrus fruits,

3. 2. 3. Antibiotic susceptibility test of the bacterial pathogens:

The bacterial pathogens were isolated from clinical wounds were subjected to antibiotic susceptibility test against 30 different antibiotics using standard discs (HiMedia). The bacterial isolates were grown in Muller Hinton broth at 37°C up to OD₆₀₀ of 0.3 and 100 µl of this cells suspension were seeded on to Muller Hinton agar plates with sterile cotton swabs. Antibiotic susceptibility were checked by disc diffusion assay as described above. The names and the concentration of the standard antibiotics are as follows: Ampicillin (10 µg/disc) [A10], Amoxyclav (Amoxycillin/Clavuranic acid) (30: 20/10 µg/disc) [Ac30], Amikacin (30 µg/disc) [Ak30], Ampicillin/Sulbactum (10/10 µg/disc) [As 10/10], Chloramphenicol (30 µg/disc) [C30], Ceftazidime (30 µg/disc) [Ca30], Clindamycin (2 µg/disc) [Cd2], Cephotaxime (30 µg/disc) [Ce30], Ciprofloxacin (5 µg/disc) [Cf5], Cephalothin (30 µg/disc) [Ch30], Co-Trimoxazole (Sulpha/Trimethoprim) (25: 23.75/1.25 µg/disc) [Co25], Cephalexin (30 µg/disc) [Cp30], Cefuroxime (30 µg/disc) [Cu30], Doxycycline Hydrochloride (30 µg/disc) [Dx30].

µg/disc) [Do30], Erythromycin (15 µg/disc) [E15], Gentamicin (10 µg/disc) [G10], Kanamycin (30 µg/disc) [K30], Lincomycin (10 µg/disc) [L10], Levofloxacin (5 µg/disc) [Le5], Methicillin (5 µg/disc) [M5], Neomycin (30 µg/disc) [N30], Nalidixic acid (30 µg/disc) [Na30], Nitrofurantoin (100 µg/disc) [Nf100], Ofloxacin (5 µg/disc) [Of5], Oxacillin (1 µg/disc) [Ox1], Penicillin-G (10 units) [P10], Streptomycin (10 µg/disc) [S10], Tetracycline (30 µg/disc) [T30], Tobramycin (10 µg/disc) [Tb10] and Vancomycin (30 µg/disc) [Va30]. After placing the discs the plates were incubated at 37°C for 24 hours. The inhibitions zones were measured using Hi-Antibiotic ZoneScale (HiMedia) and susceptibility & resistance was determined by comparing with HiMedia catalogue as per manufacturer's protocol.

3. 2. 4. Selection and identification of the potential strains:

The anti-microbial metabolite production profiles of the isolates were tabulated and compared, to select the potential isolates with a broad spectrum of antagonistic activity. Selection was done based on the size of zones of inhibition of the cell free metabolites (in triplicates) against the test pathogens. The growth rate, temperature, salt requirement for growth and classical taxonomy characterization were also considered as a criteria to select the isolates as “de-replicated” and previously unreported strains. Six rare actinomycete strains (presumptive identification by classical taxonomy) and few of the *Streptomyces* isolates were also selected.

3. 2. 5. Mass scale production and extraction of anti-microbial metabolites:**3. 2. 5. a. Sequential organic solvent extraction:**

Anti-bacterial metabolite(s) production by each isolate was scaled up for extraction. Each culture was inoculated into 25 mL culture broth at 35 psu (being the optimum salt concentration for antibiotic production) and grown for 7 days in a shaker incubator at 140 rpm and 28°C ± 2°C temperature. This media was scaled up to 500 mL using the above 25 mL as an inoculum and incubated for 2 weeks. Each time to scale up to 2 – 3 Litres, 500 mL culture was grown in flasks and then pulled in for extraction. The culture broth was then centrifuged at 13,000 rpm for 5 min at 4°C. The culture supernatant was decanted and collected. Sequential extraction was carried out with six different organic solvents ranging from non-polar to polar solvents, (petroleum ether> hexane> diethyl ether> chloroform> ethyl acetate> butanol) in a volume ratio of 1:3 (culture supernatant: organic solvent). The organic layer was evaporated to dryness using a rotary vacuum evaporator (EQUITRON® Roteva) at 45°C and the resultant residue was then dissolved in 1 ml of the respective organic solvent and then checked for its bioactivity against pathogens.

3. 2. 5. b. Resin-methanol extraction:

Cell free culture supernatant (as described above) was collected for metabolite extraction. 22.5 gm of resin (Diaion® HP-20, Sigma Aldrich) were added to 500 mL of culture supernatant and allowed to shake at 120 rpm for 1 hour. The resin granules were filtered out and poured into a syringe column. Elution of the

metabolites were done using 250 mL of HPLC grade methanol, by adding 50 mL of methanol at intervals to the column. This procedure was followed with a slight modification to the manufacturer's protocol. The elutant was collected up to 1 L volume to obtain sufficient quantity of the anti-microbial compound. The metabolites were concentrated using rotary vacuum evaporator (EQUITRON® Roteva) at 45°C in a pre-weighed round bottom flask till complete drying to a brown/yellowish residue. The amount of the residue was calculated and this was then dissolved in 2 – 5 mL of HPLC grade methanol and aliquoted in 2 mL Eppendorf vials. This residue was checked for bioactivity against the target pathogens (as described above).

3. 2. 6. Characterization of the antibiotic:

3. 2. 6. a. Solubility, temperature and pH stability:

The solubility of the anti-microbial compound was checked in various polar and non-polar solvents viz. butanol, chloroform, diethyl ether, ethyl acetate, hexane, petroleum ether and water by adding 1 mg of the compound in the Eppendorf tubes containing 1 mL of the solvent. The tubes were subjected to brief vortexing and was then observed for the clear solution, which in turn confirmed the solubility. In case of compound forming a suspension, the tube was centrifuged at

5,000 rpm for 1 min and formation of precipitation pellet suggested the insolubility of the compound in the solvent tested.

Temperature stability was checked by exposing the compound (in aqueous solution) various temperatures (10 – 100°C) at interval of 10°C. The aqueous solution of the compound was prepared by dissolving 1 mg of the compound in 1 mL of the MilliQ water in Eppendorf tubes. The tubes were sealed with parafilm and incubated in a temperature water bath for one hour. After that activity of the treated samples were checked against the target pathogens and compared with the activity of untreated compound (1 mg/mL). The assay was repeated in triplicates to obtain consistent results.

The compound was exposed to various buffer solutions with varying pH (2 – 10, with interval of 1) to check for the pH stability. Buffer solutions (1 mL) were taken into Eppendorf tubes and then 1 mg of the compound was added to each tube. After one hour of incubation, the compound in buffer solutions was assessed by disc diffusion assay with the test pathogen along with the buffer solutions of respective pH as controls. The assay was repeated in triplicates to obtain consistent results.

3. 2. 6. b. Determination of Minimum inhibitory concentrations (MICs):

Minimum inhibitory concentrations of the anti-microbial compound was determined against all the target pathogens by micro-titre plate assay with minor modification to the protocols suggested by Clinical and Laboratory Standard Institute (M7-A5 and M27-A3). The compound was dissolved in the respective

solvent and dilutions from 10 µg to 100 µg/mL were prepared. Further lower dilutions were made based on the results obtained. The bacterial pathogens were grown in nutrient broth media and overnight grown culture broth were centrifuged at 5000 rpm for 5 mins. Pelleted bacterial cells were diluted up to 10^6 cells/mL concentration in PBS. For fungal pathogens, the spores were scrapped from grown slants by using 1% peptone water solution. This spore suspension was diluted up to 10^6 spores/mL in 1% peptone water. Dilutions of the anti-microbial compound were added to the wells of the micro-titre plate, followed by 100 µL of cell/spore suspension. For bacterial growth 100 µL of sterile nutrient broth and for fungus 100 µL of sterile potato dextrose broth was added. Complete inhibition of growth at the lowest concentration was considered as MIC.

3. 2. 6. c. Thin layer chromatography (TLC) and Fourier transformed Infra-Red (FTIR) Spectroscopy:

Purified anti-microbial compound was further analyzed by TLC in various solvent systems viz. Methanol: water (1:1), Cholorform: methanol: water (65:25:4) and Butanol: acetic acid: water (6:1:2). The plates were developed in an iodine chamber, Ninhydrin reagent and Rhodamine B. Individual spots migrated were marked under UV, scraped off dissolved in H₂O and checked for bioactivity. The compound was subjected to FTIR (SHIMADZU-FTIR instrument) analysis by the

compound was placed between two KBr pellets and IR spectra were recorded with 50 scans within the range of $400 - 4000 \text{ cm}^{-1}$.

3. 2. 7. Structural elucidation of the purified anti-microbial compound:

3. 2. 7. a. Liquid Chromatography – Mass spectrometry (LC/MS) and Q-TOF - ESI MS/MS analysis:

The purified anti-microbial compound was digested in solution as described by Krishnaswamy and Damare (Unpublished work). In short, 6 M Urea was added to the sample with 200 mM DTT. After one hour incubation, 200 mM IAA was added and further incubated for 45 min at room temperature for acetamidation. To this mixture, acidified trypsin was added and incubated overnight at 37°C . The digested samples were further acidified to stop the digestion and the linearized trypsin digested peptides were injected in the Agilent nano Chip-LC coupled with Ultra High Definition Mass Spectrometer (6538) system. . The solvent system used for separation was water and acetonitrile (90%) with formic acid for protonation. The separation was carried out on C-18 matrix using ProtID-Chip-150 II which had a nano-ESI tip as ionization source. A linear gradient of 90% acetonitrile was used to separate the peptides and these were ionized for further MS analysis. The MS-MS scans were carried out in positive mode in the range of 100 to 1200 m/z. The spectra generated were further analysed using Spectrum Mill MS Proteomics Workbench (Agilent Systems, USA) ver. B.04.01.141.

3. 2. 7. b. Analysis of LC/MS and Q-TOF ESI-MS/MS data:

The m/z values of the respective ms/ms peaks were further used to determine the structural components of the compound. The fragment ion peaks were labeled according to the nomenclature proposed by Roepstorff and Fohlman (1984). To retrieve the sequence from the mass spectrometric data of the new peptides, a criteria was applied where the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue. Finally the molecule was constructed using CHEMDRAW software and the exact molecular weight, chemical formula and elemental component ratio was determined.

4. RESULTS:

4. 1. BIODIVERSITY:

4. 1. 1. a. Hydrographic parameters of the salt pans:

In Ribandar salt pan during pre-monsoon season, salinity of salt crystalizer ponds ranged from 300 to 305 psu, whereas during post-monsoon salinity was found to be lower (270-275 psu). Agarvado salt pan was found to have less salinity compared to Ribandar, in pre-monsoon the salinity ranged 285-290 psu and whereas in post-monsoon 260-265 psu (Table. 7). In both the salt pan the sediment temperature was about 28–30°C whereas dissolved oxygen content in the overlying water of Ribandar salt pan was higher (2.32-2.55 mg/L) compared to Agarvado (1.82 – 1.98 mg/L). The pH in case of both the salt pan, remained almost constant as in neutral to slight alkaline 7.55 – 7.82.

4. 1. 1. b. Sedimentology analysis:

The distribution of sand, silt and clay showed that clay (%) was more dominant in Agarvado (64%) compared to Ribandar salt pan (58%) whereas content of the silt was found more in Ribandar (15%) compared to Agarvado (10%) (Table. 8). Sand contents in the sediments of the Agarvado and Ribandar 26% and 27% respectively. These results suggest that both these salt pans are not only geographically distant but also varied in their sediment architecture.

Table. 7 Physico chemical parameters of the selected sediment samples of Ribandar and Agarvado

	Samples				
	Pre-monsoon:	Samples were collected in the month of April			
		Salinity (psu)	pH	Temperature (°C)	Dissolved Oxygen in overlying water (mg/lit)
Ribandar	Sediment-1	300	7.64	29.4	2.55
	Sediment-2	305	7.54	29.5	2.45
Agarvado	Sediment-3	285	7.82	30.7	1.93
	Sediment-4	290	7.67	31.5	1.98
	Post-monsoon:	Samples were collected in the month of November			
Ribandar	Sediment-1	270	7.52	30.9	2.32
	Sediment-2	275	7.55	30.5	2.40
Agarvado	Sediment-3	265	7.69	31.4	1.82
	Sediment-4	260	7.63	31.7	1.85

Table. 8 Sediment granulometry analysis of Agarvado and Ribandar salt pan sediments

Sr. No.	Sediment samples	Sand (%)	Silt (%)	Clay (%)
1.	Agarvado salt pan	26 ± 1.2	10 ± 0.8	64 ± 2.3
2.	Ribandar salt pan	27 ± 1	15 ± 1.2	58 ± 1.8

4. 1. 1. c. Total organic carbon (TOC) in sediment:

Estimation of total organic carbon (TOC) in the sediments of Ribandar salt pan showed that during pre-monsoon on average the value ranged from 2.5 to 3.7%. During the post-monsoon the value was found to be higher which ranged from 7.5 to 8.2%. Agarvado salt pan was found in low organic carbon in the sediment; during pre-monsoon season it was found to be 1.7 to 2.8% and during post-monsoon 4.3 to 6%. The variation in the TOC content with varying seasons could be due to the monsoon water bringing the organic matter in the salt pans. Higher TOC in Ribandar sediment reflects that it receives the organic matter from the surrounding mangrove flora.

4. 1. 2. Microbiological parameters:

4. 1. 2. a. Heterotrophic bacteria in pre and post-monsoon season:

The heterotrophic bacterial counts were higher in numbers in the post-monsoon season compared to the pre-monsoon seasons in both Ribandar and Agarvado salt pan. In Ribandar salt pan the heterotrophic counts in pre and post-monsoon season were 465 and 1770×10^3 cfu g⁻¹ respectively. The drastic increase in the CFU counts during the post-monsoon season probably due to the influx of fast growing bacteria from terrestrial ecosystem. Agarvado had relatively lower counts of heterotrophic bacteria, 225 and 1250×10^3 cfu g⁻¹. Fig. 8 shows the variation in the heterotrophic counts of both the salt pans. Difference in the heterotrophic count could be because of the contribution of the organic matter by the mangrove vegetation located surrounding Ribandar salt pan and Agarvado being nutritionally dilute.

4. 1. 2. b. Assessment of soil microbial biomass carbon (SMBC):

Estimation of soil microbial biomass carbon (SMBC) showed the significant difference of microbial flora between two salt pans as well as the seasonal effect on microbial community during pre and post-monsoon seasons. During pre and post-monsoon seasons the SMBC of Ribandar was found to be 694 and 1025 $\mu\text{g C g}^{-1}$ respectively whereas in Agarvado they are 563 and 866 $\mu\text{g C g}^{-1}$. Fig. 9 shows the comparative assessment of SMBC of both the salt pans during pre and post-monsoon seasons. As mentioned earlier the higher microbial population in Ribandar is due higher organic contents in the sediments.

4. 1. 3. Standardization of actinomycetes isolation procedure:

In standardization it was found that overnight treatment with CaCO_3 (1%) to the wet sediments increased the actinomycete counts whereas heat treatment (60°C) to the dissolved sediment (CaCO_3 treated) for 10 mins, very considerably decreases the gram -ve bacterial load. To inhibit the load of other fast growing bacteria *viz.* *Bacillus* sp., Nalidixic acid at a concentration of 25 $\mu\text{g/mL}$ in the culture medium was found to be significant which didn't affect the growth of actinomycetes. Growth of fungi was inhibited on addition of Nystatin (25 $\mu\text{g/mL}$) in the isolation medium. These standardized techniques were used throughout the study for the isolation of actinomycetes.

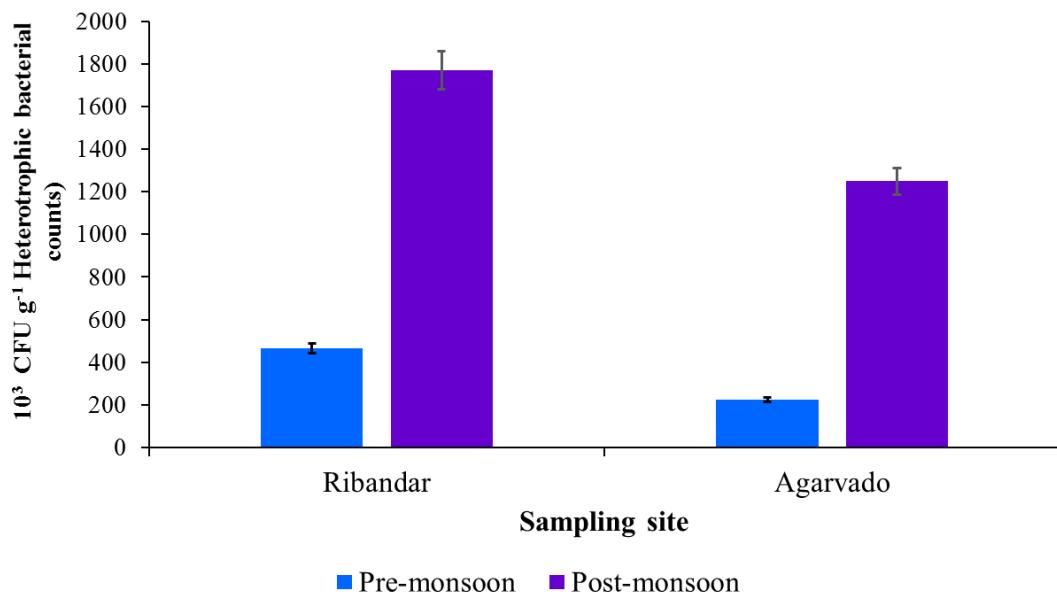


Fig. 8 Heterotrophic bacterial counts in the sediments of Ribandar and Agarvado

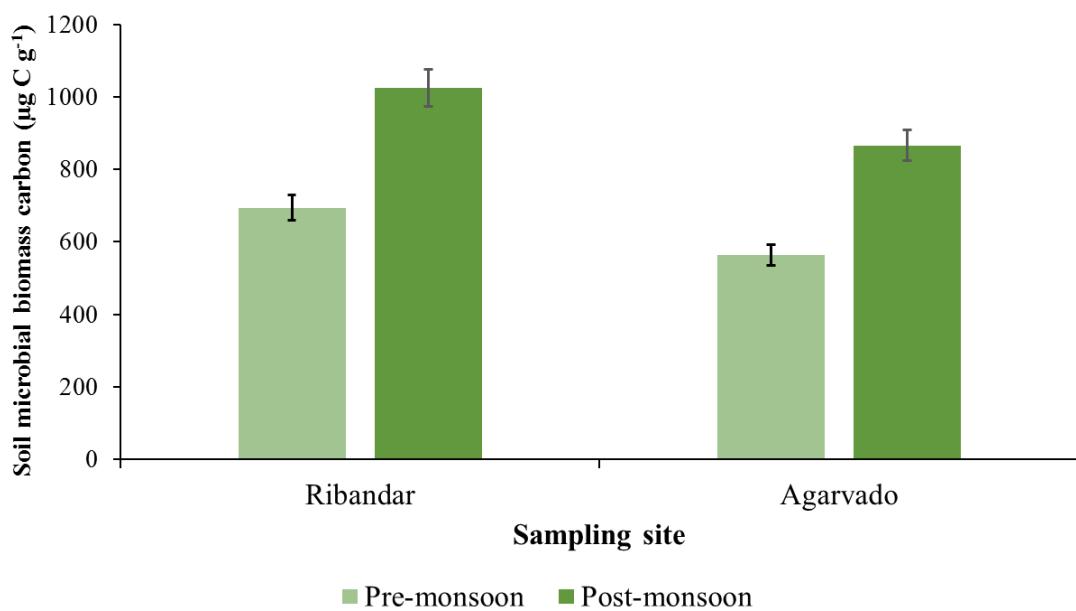


Fig. 9 Variation of SMBC in the sediments of Ribandar and Agarvado salt pan

4. 1. 4. Total heterotrophic and actinomycete CFU counts in pre and post monsoon seasons:

In both the salt pans the heterotrophic and actinomycete CFU counts were high (Fig. 10) in lower salinities (32-50 psu) which is due to the isolation of both indigenous halophilic bacterial population as well as halotolerants, migrated from other ecosystems and got acclimatized in saline environment. Actinomycete CFU counts were ~ 50% of the heterotrophic counts in lower salinities, where as in higher salinities it was found to be ~ 30% suggesting isolation of rare and borderline halophiles. Higher actinomycete CFU counts in post-monsoon season and lower salinities (Fig. 11), suggests the influx of terrestrial strains with monsoon wash off in the salterns. These obsevations indicates the variation in bacterial community structure in both pre and post-monsoon seasons.

4. 1. 5. Percentage of morphologically variant actinomycetes at different levels of salinity:

A total of 500 morphologically variant actinomycetes (Fig. 12) were isolated from both the salterns at 8 different levels of salinities. Percentages were higher in lower salinities viz. 32 psu (44%), 50 psu (27%) and 75 psu (12%) whereas higher salinities have lesser percentages 100 psu (9%), 150 psu (4%), 200 psu (2%), 250 psu (1%) and 300 psu (1%) (Fig. 13). Higher percentage at lower salinities suggest more number of halotolerant actinomycete strains whereas higher salinity resulted in the isolation of moderate to extreme halophilic strains.

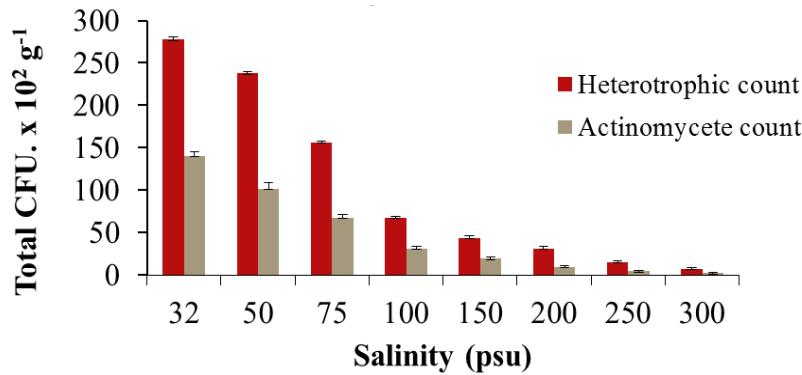


Fig. 10 Comparison of total heterotrophic and actinomycete counts in the sediments

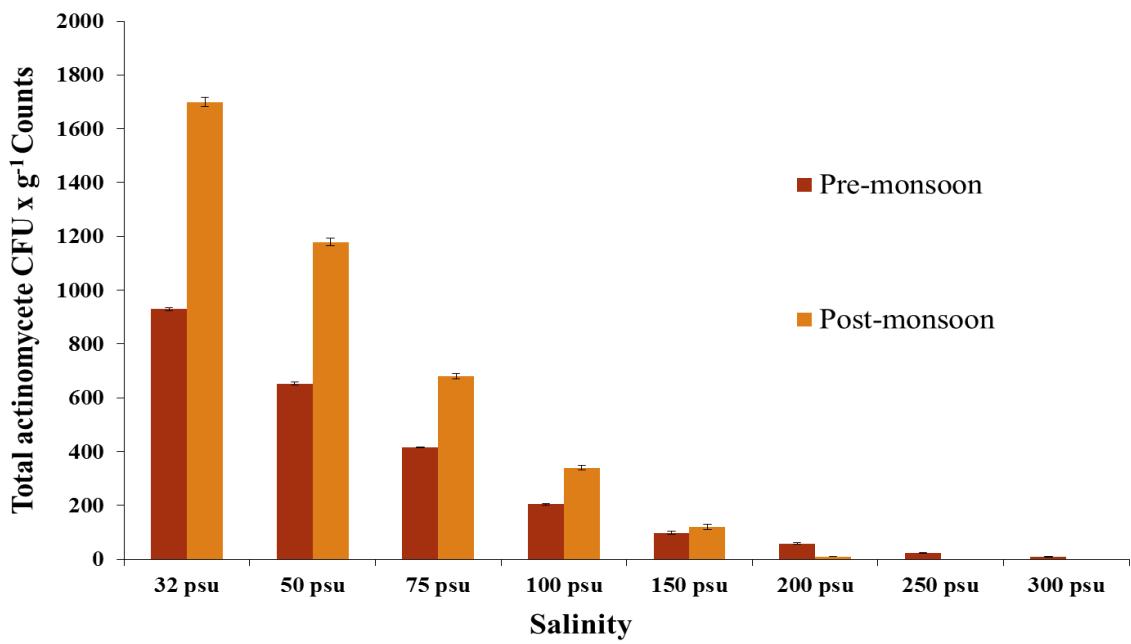


Fig. 11 Comparison of actinomycete CFU counts in pre and post-monsoon seasons



Fig. 12 Actinomycete colonies with diverse morphology on isolation media

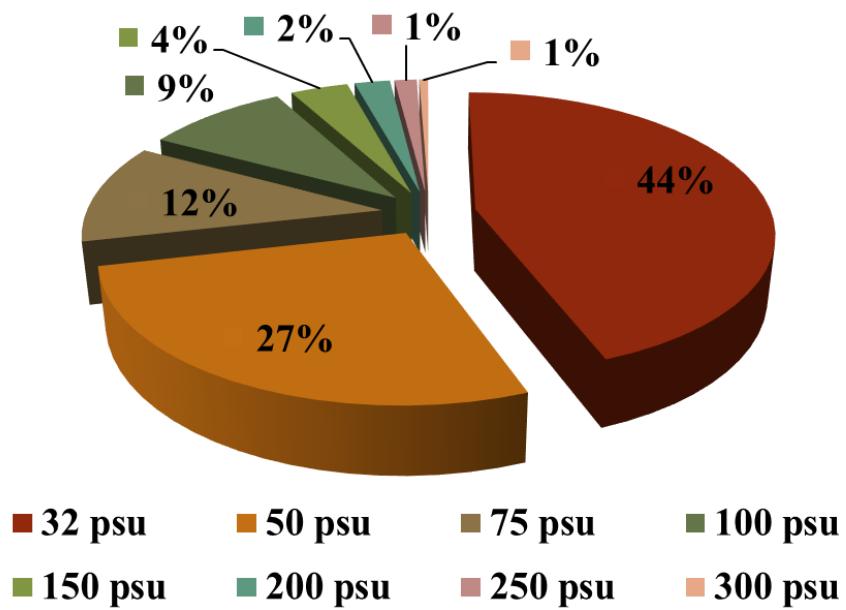


Fig. 13 Percentage of actinomycete isolates obtained at 8 different salinity levels

4. 1. 6. Comparison of various media for isolation of hypersaline actinomycetes:

The number of actinomycete isolates varied considerably in the different media employed for isolation. Fig. 14 shows comparative counts of morphologically variant actinomycetes in the 12 different media used for isolation. R2A agar was found to be the most suitable medium for the isolation of hypersaline actinomycetes from sediment samples. Isolation on 12 different media resulted in the emergence of the actinomycete colonies with varying colour and morphology which helped in the selection of de-replicated isolates. The other media which also supported the growth of significant number of morphologically variant actinomycete colonies were Starch-casein agar, Inorganic salt-starch agar and Yeast-malt extract agar.

4. 1. 7. a. Effect of Salinity on the growth of halotolerant isolates:

Halotolerant isolates viz. SBSK-3, 8, 67, 150 and 356, grew in the saline water (32-50 psu) as well as in absence of salt (0 psu) but their growth decreased with increase in the salinity (10-75 psu) (Fig. 15). Majority of the halotolerant isolated salinity up to 32-75 psu from both Ribandar and Agarvado salt pans. These isolates grew up to 100 psu salt concentration which suggest them to be moderate halotolerants. A total of 380 halotolerant strains were isolated from the water and sediment samples of both the salt pans, belonged to 9 different genera.

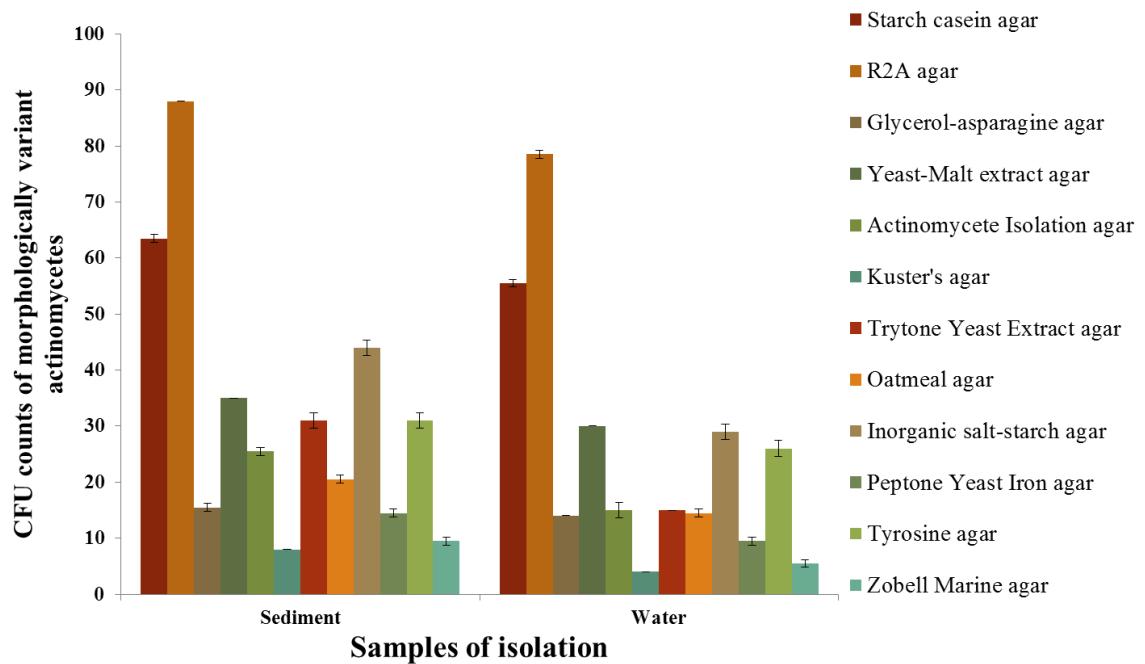


Fig. 14 Comparative assessment of 12 different media used for the isolation of hypersaline actinomycetes

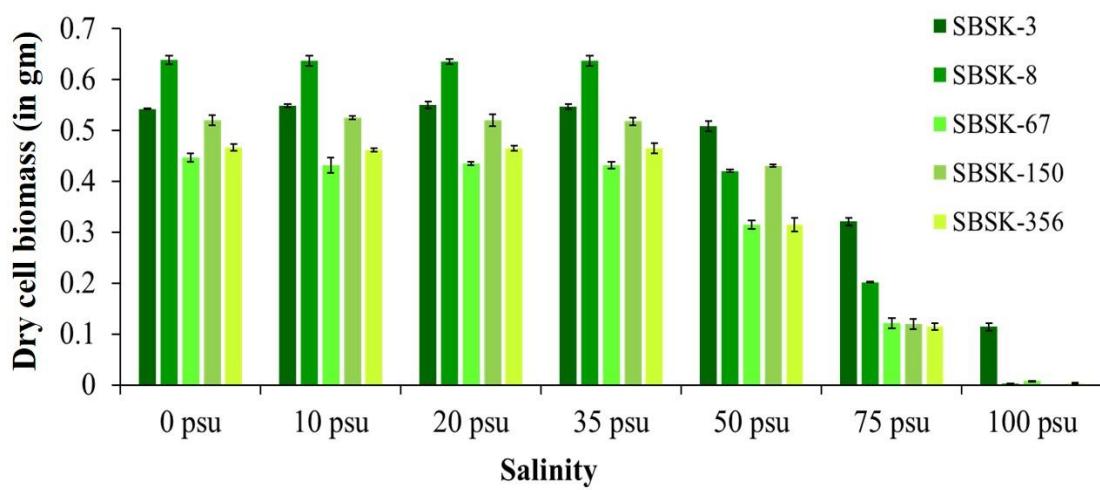


Fig. 15 Graph showing growth profile of 5 halotolerant isolates at various salinities

4. 1. 7. b. Growth of halophilic isolates at various salinities:

A total of 120 halophilic actinomycete isolates belonging to 11 different genera were obtained. Growth of halophilic isolates increased with higher salinity values. Slight to moderate halophiles (SBSK-57, 115 and 120) grew at 20-100 psu with optimum growth at 50 psu salinity (Fig. 16) Extreme halophiles (SBSK-250 and 551) grew up to 150 – 250 psu salinity with optimum growth at 200 psu whereas an extreme borderline halophile SBSKN₅ showed optimum growth at 250 psu and required minimum of 100-150 psu salinity to grow (Fig. 17). Majority of the halophiles were found in the genera *Nocardiopsis*, *Actinomadura*, *Actinopolyspora* and *Streptomonospora* (tentatively identified).

4. 1. 8. Metabolic fingerprinting of hypersaline actinomycetes using BIOLOG microplates:

Utilization profile of 96 carbon substrates aided in differentiating the actinomycete genera even among various species. However, though the halotolerant isolates responded very significantly to BIOLOG but the halophilic isolates didn't give positive results with the same. In halotolerant isolates, these data facilitated grouping various species under the same genera especially of *Streptomyces*, *Micromonospora*, *Micrococcus*, *Kitasatospora* and *Nocardiopsis*.

Fig. 18 shows the carbohydrate utilization profile of strain SBSK-364 on GP2 Microplate. Detailed tabulated results will be included in characterization of individual genera.

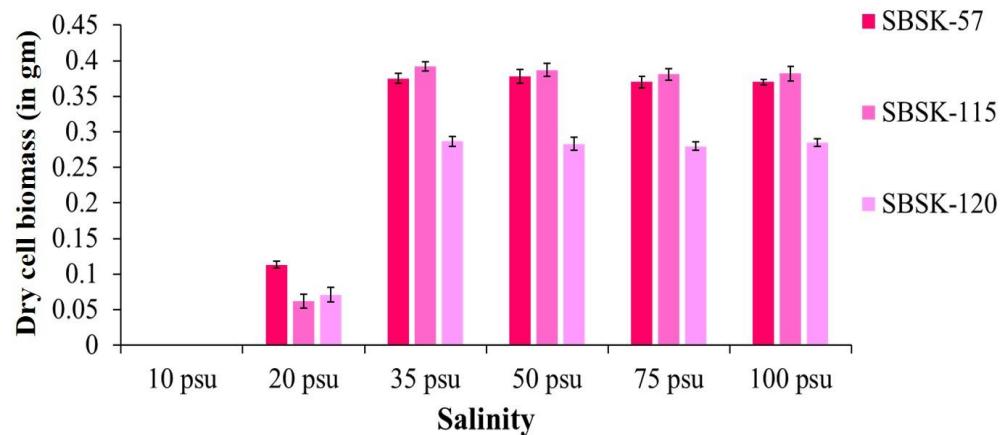


Fig. 16 Graph showing the growth profile of 3 slight halophiles at various salinities

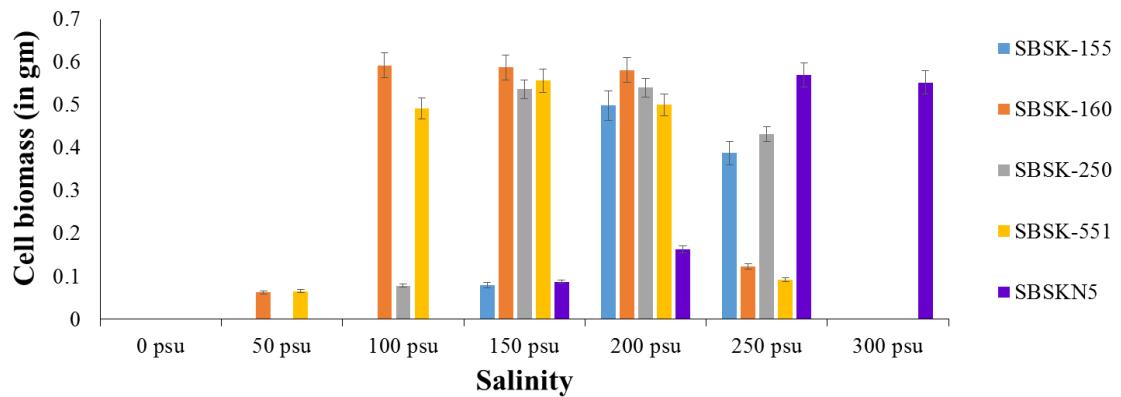
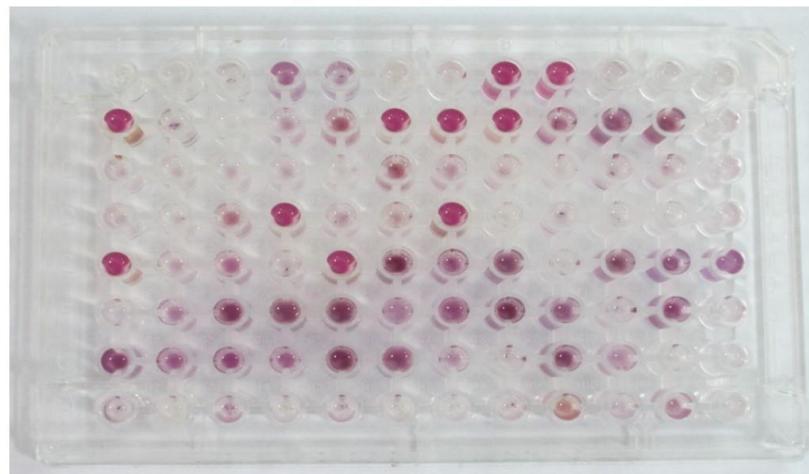


Fig. 17 Graph showing the growth profile of moderate to extreme halophiles at various salinities

A.**B.**

BLANK	α -Cyclodextrin	β -Cyclodextrin	Dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-acetyl-D-glucosamine	N-acetyl-D- β -mannosamine	Amygdalin
L-Arabinose	D-arabitol	Arbutin	D-celllobiose	D-fructose	L-Fucose	D-Galactose	D-galacturonic acid	Gentibiose	D-Gluconic acid	α -D-glucose	m-inositol
α -D-Lactose	Lactulose	Maltose	Maltotriose	D-mannitol	D-mannose	D-Meleziotose	D-Melibiose	α -Methyl-D-galactoside	β -Methyl-D-galactoside	3-methyl-D-glucose	α -Methyl-D-glucoside
β -Methyl-D-glucoside	α -Methyl-D-mannoside	Palatinose	D-psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-sorbitol	Stachyose	Sucrose
D-Tagatose	D-Trehalose	Turanose	Xylitol	D-xylene	Acetic acid	α -Hydroxybutyric acid	β -Hydroxybutyric acid	y-Hydroxybutyric acid	p-Hydroxyphenylacetic acid	α -Ketoglutaric acid	α -Ketovaleric acid
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
L-Alaninanamide	D-Alanine	L-Alanine	L-alanyl-glycine	L-Asparagine	L-glutamate	Glycyl-L-glutamic acid	L-pyroglutamic acid	L-serine	Putrescine	2,3-Butanediol	Glycerol
Adenosine	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-monophosphate	Thymidine-5'-monophosphate	Uridine-5'-monophosphate	D-Fructose-6-Phosphate	α -D-glucose-1-phosphate	D-glucose-6-phosphate	D-L- α -glycerol phosphate

Fig. 18: A. GP2 Microplate showing carbohydrate utilization by isolate SBSK-364; ‘purple colour’ denotes the positive results; B. Carbohydrate utilization chart of GP2 Microplate showing the positive results

4. 1. 9. Molecular characterization of the isolates:

Molecular characterization by 16S rRNA gene sequencing was done for a total of 58 isolates from both the salt pans. Extraction procedure of genomic DNA varied among the isolates viz. in case of non-sporulating coccoid isolates bacterial genomic DNA isolation kit worked well whereas for the sporulating and filamentous isolates fungal genomic DNA isolation protocol was more successful. Genomic DNA profiles of the isolates in 0.8% agarose gel can be seen in Fig. 19 and Fig. 20 shows the amplification 16S rRNA gene (1.5 kb) from the genomic DNA of the isolates by PCR in 1% agarose gel. Further gel purification of these amplified products, followed by sequencing and analysis revealed the identity of the isolates. From Ribandar salt pans 9 different genera were obtained excluding one unidentified isolate which could be a novel species and from Agarvado 12 different genera were obtained. Phylogenetic trees showing the taxonomic relationship of these isolates with their type strains are showing under description of various genera section.

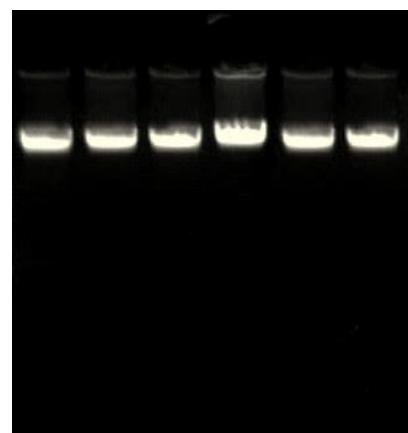


Fig. 19 Genomic DNA profiles of the actinomycete isolates in 0.8% agarose gel in TBE buffer

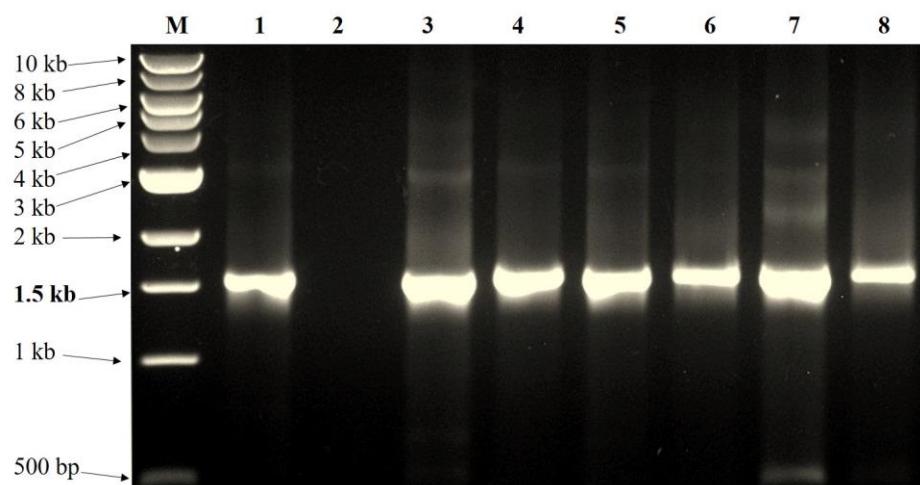


Fig. 20 PCR profile of 16S rRNA gene amplification (1.5 kb) of the actinomycete isolates in 1% agarose gel in TBE buffer; M - DNA ladder (1 kb), 1 - positive control (*E. coli*), 2- negative control, 3 to 8 - actinomycete isolates

4. 1. 11. Abundance of hypersaline actinomycetes in Ribandar and Agarvado salt pans:

Morphological variant actinomycete isolates obtained from both the salt pans were grouped into 13 different clusters based on the colony morphology, light microscopy and salinity requirement for growth and the salient observations are as follows:

4. 1. 11. a. Cluster- I: Genus *Actinomadura*:

Cluster-I includes 15 isolates which show abundantly branched substrate mycelia with no fragmentation (light microscopy). In the aerial mycelia, the short spore chains were present with smooth surface either in ornamented form or as spirals.

Fig. 21 shows the substrate mycelia and spore chains of four different isolates (SBSK-225, SBSK-245, SBSK-520 and SBSK-560). All the strains were found to be halotolerant and tolerated up to 7% (70 psu salinity). Carbohydrate utilization pattern and molecular characterization revealed 6 different species viz. strain SBSK-225 as *Actinomadura algeriensis*, SBSK-245 as *Actinomadura sediminis*, SBSK-340 as *Actinomadura madurae*, SBSK-520 as *Actinomadura namibiensis*, SBSK-560 as *Actinomadura catellatispora* and SBSK-701 as *Actinomadura chokoriensis*. Similarity index of 16S rRNA gene sequences were found to be 98-99%. Table. 9 shows summarizes their isolation details and other physiological properties. These isolates were isolated at different seasons and were discontinuous in their distribution. SBSK-225 (*Actinomadura algeriensis*) were found in Ribandar salt pan in both pre and post-monsoon season, SBSK-245 (*Actinomadura sediminis*) were found in both Ribandar and Agarvado salt pan in both pre and post monsoon season, SBSK-340 (*Actinomadura madurae*) in Ribandar salt pan in post monsoon season, SBSK-520 (*Actinomadura namibiensis*) in Ribandar post-monsoon season, SBSK-560 (*Actinomadura*

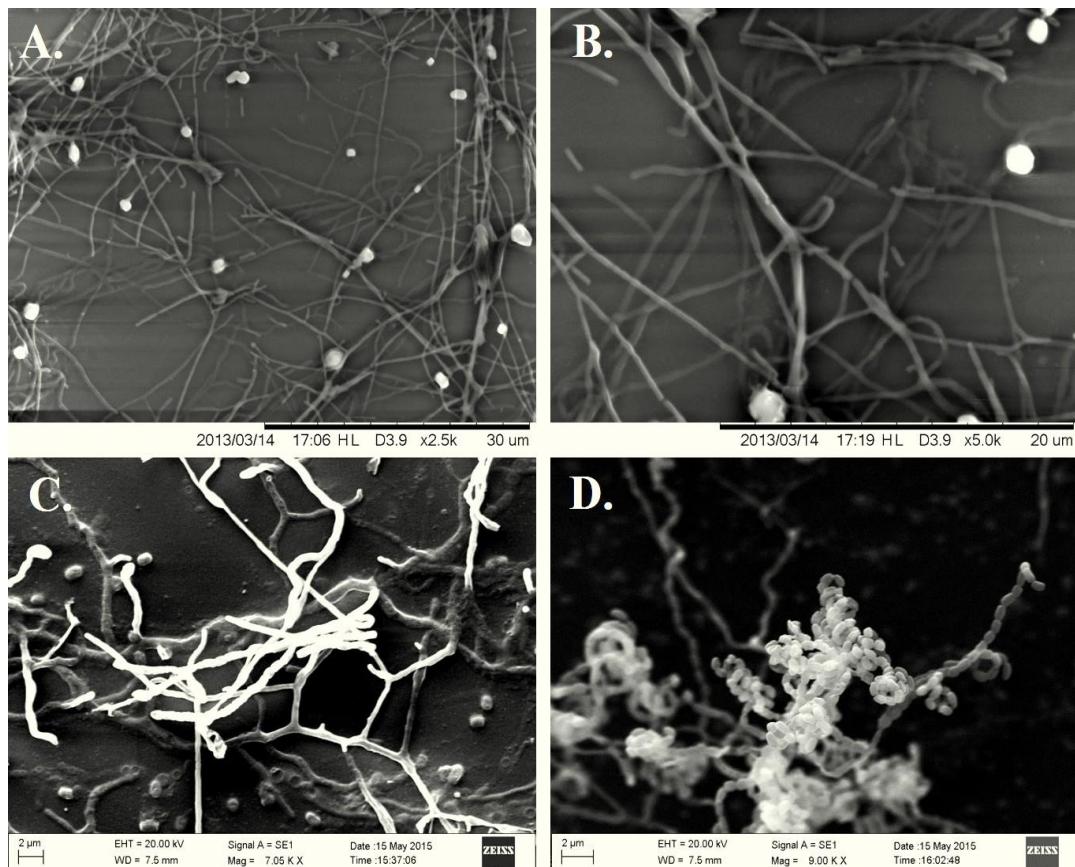


Fig. 21 SEM images: A. and B. Strain SBSK-225 and SBSK-245 showing extensively branched and non-fragmented substrate mycelia attached with salt crystals; C. Short chains of ornamented spores with smooth surface are formed on the aerial mycelia of strain SBSK-560; D. Strain SBSK-520 showing spiral spore chains with smooth surface

Table. 9 Isolates of cluster-I: Isolation details, carbon source utilization, salt tolerance and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	NaCl tolerance
1.	SBSK-225	Ribandar pre and post-monsoon	Yeast extract-malt extract agar & Oatmeal agar and 50 psu	<i>Actinomadura algeriensis</i>	adonitol, arabinose, cellobiose, Tween-80, acetate, pyruvate, succinate, fructose, glucose, maltose, mannitol, rhamnose, ribose, sorbitol, trehalose and xylose	0-7%
2.	SBSK-245	Both Ribandar and Agarvado salt pan in both seasons pre and post monsoon seasons	Zobell marine agar 2216 and 32 psu	<i>Actinomadura sediminis</i>	arabinose, D-fructose, D-galactose, mannitol, L-rhamnose, ribose and D-xylose	0-9%
3.	SBSK-340	Ribandar salt pan post-monsoon season	Tryptone-yeast extract agar and 32 psu	<i>Actinomadura madurae</i>	Glucose, maltose, mannitol, rhamnose, ribose, sorbitol, trehalose and xylose L-arabinose, D-fructose, D-galactose, mannitol and D-xylose	0-3%
4.	SBSK-520	Ribandar, post-monsoon	Actinomycete Isolation Agar and 32 psu	<i>Actinomadura namibiensis</i>	Glucose, arabinose, sucrose, xylose, inositol, mannitol, Rhamnose	0-3%
5.	SBSK-560	Agarvado salt pan, post-monsoon season	Yeast extract-malt extract agar and 32 psu	<i>Actinomadura catellatispora</i>	Glucose, maltose, mannitol, rhamnose, ribose, sorbitol, L-arabinose, Tween-80, acetate, pyruvate, succinate, D-fructose, D-galactose, mannitol and D-xylose	0-4%
6.	SBSK-701	Ribandar salt pan, pre and post-monsoon season	Yeast extract-malt extract agar and 75 psu	<i>Actinomadura chokoriensis</i>	L-arabinose, cellobiose, fructose, glycerol, mannitol, sucrose, xylose	0-10%

catellatispora) in Agarvado salt pan during post-monsoon season and SBSK-701 (*Actinomadura chokoriensis*) in Ribandar salt pan during both pre and post-monsoon season. The strains which were found in both pre and post monsoon seasons viz. SBSK-225, SBSK-245 and SBSK-701, had higher tolerance compared (up to 10%) to the strain which were found only in the post-monsoon season viz. SBSK-340, SBSK-520 and SBSK-560. Molecular characterization by 16S rRNA gene sequencing confirmed the identity of the strains. Phylogenetic tree in Fig. 22 shows the taxonomic relationship of the strains with the type strains and other *Actinomadura* spp. isolated from various environments.

4. 1. 11. b. Cluster- II: Genus *Streptomonospora*:

In cluster-II, a total of 15 isolates were included from Ribandar and 21 isolates from Agarvado. These isolates were obtained on R2A, starch casein and inorganic salt starch agar at extremely high salinity (150 – 200 psu; 15-20% of salt). Single spores with unfragmented substrate mycelia were the characteristics of these isolates. Fig. 23 shows the SEM images of strain SBSK-155 and SBSK-250 suggesting them to belong to genus *Streptomonospora*. Molecular characterization by 16S rRNA sequencing revealed 3 different species viz. *Streptomonospora alba* (SBSK-708), *Streptomonospora salina* (SBSK-155) and *Streptomonospora halophila* (SBSK-250). Table. 10 summarizes the isolation details of the strains and their other physiological properties. Such obligate halophilic actinomycetes species were previously never been reported from Goan salt pans. These isolates being obligate and moderate to extreme halophiles form an integral part of the microbial niche in marine salterns. Fig. 24 shows the taxonomic relationship of the strains with the type strains and other *Streptomonospora* spp. isolated from various environments.

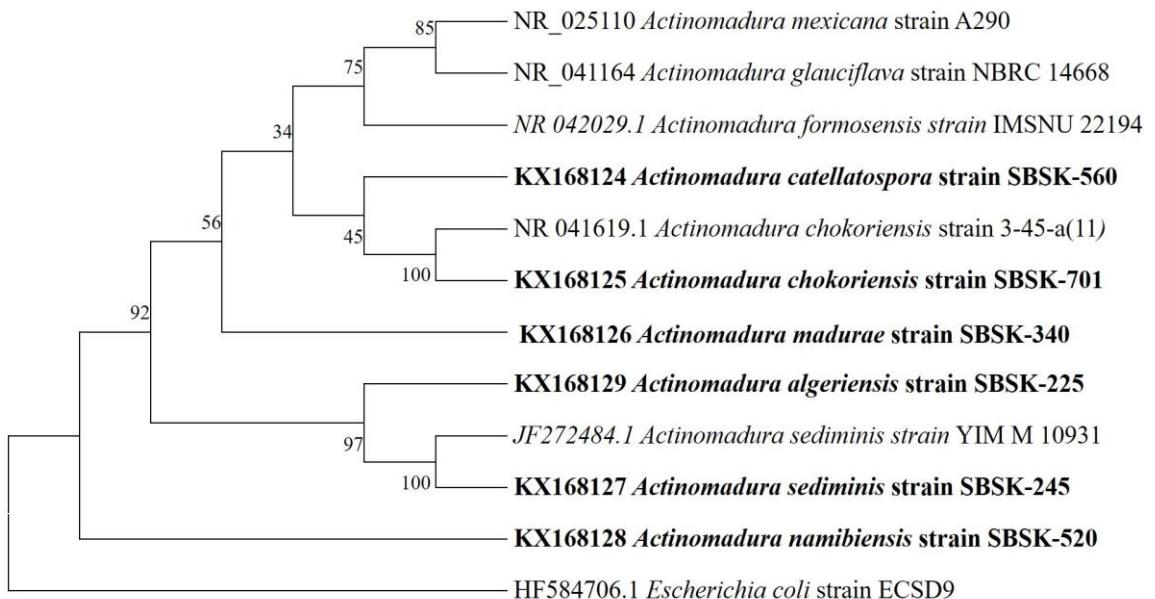


Fig. 22 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Actinomadura*; *Escherichia coli* strain ECSD9 is an out group

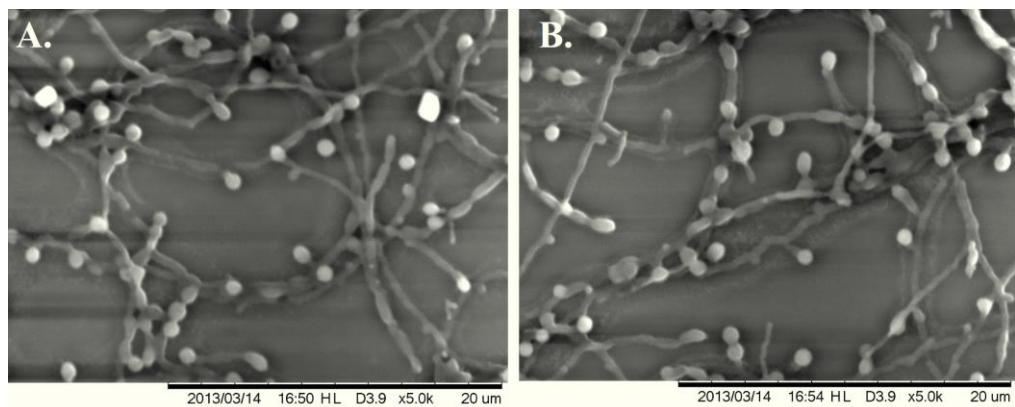


Fig. 23 SEM images: A. and B. Strain SBSK-155 and SBSK-250 showing single spores round to oval in shape on the sporangiophore and unfragmented substrate mycelia; typical characteristic of genus *Streptomonospora*; Cui *et al.* (2001) and Cai *et al.* (2008)

Table. 10 Isolates of cluster-II: Isolation details, carbon source utilization, salt requirement and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	Salt requirement for growth
1.	SBSK-155	Ribandar & Agarvado salt pans, both pre and post-monsoon	R2A and 200 psu	<i>Streptomonospora salina</i>	Glucose, sucrose, maltose, arabinose, rhamnose, starch, glycerol and mannitol	20-25%
2.	SBSK-250	Agarvado salt pan, in both pre and post-monsoon	Inorganic salt starch agar and 150 psu	<i>Streptomonospora halophila</i>	Inositol, mannose, maltose and rhamnose	15-25%
3.	SBSK-708	Ribandar salt pan, both pre and post-monsoon	R2A & SCA and 150 psu	<i>Streptomonospora alba</i>	Glucose, mannose, sucrose and inositol	15-25%

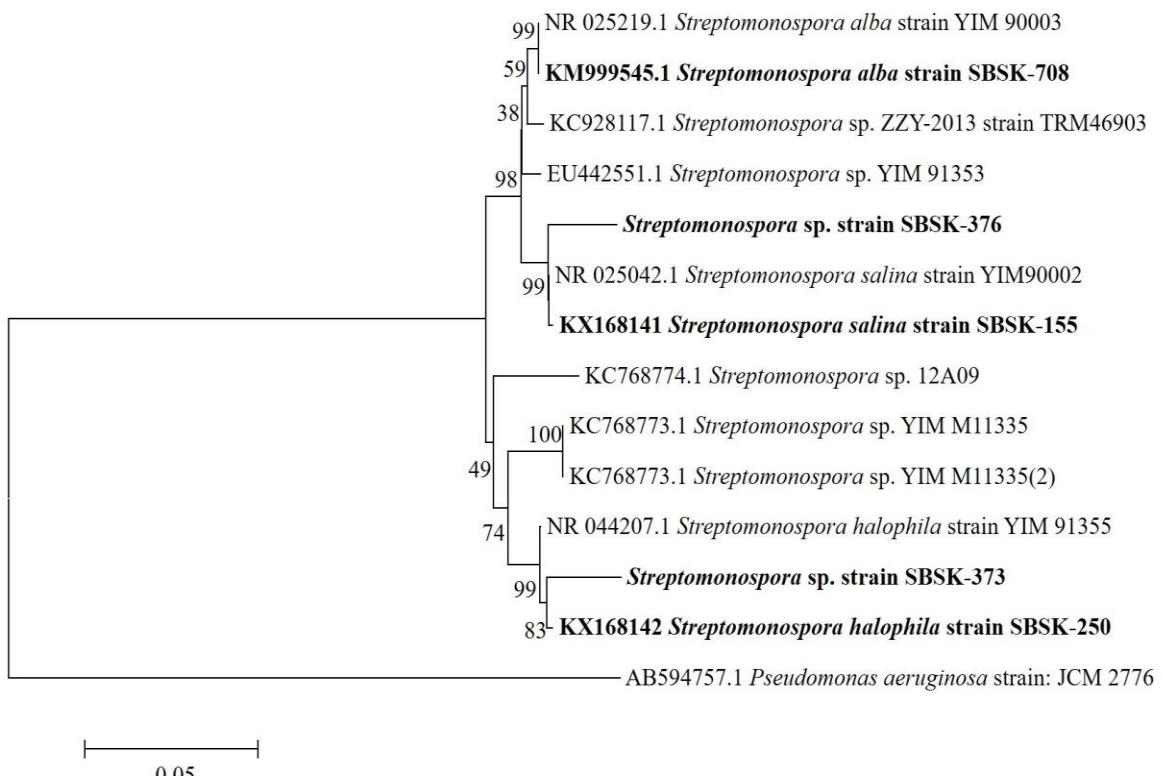


Fig. 24 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Streptomonospora*; *Pseudomonas aeruginosa* strain JCM 2776 is an out group

Strain SBSK-155 (*Streptomonospora salina*) was found in both Ribandar and Agarvado salt pans during both pre and post monsoon seasons whereas strain SBSK-250 (*Streptomonospora halophila*) was found only in Agarvado and SBSK-708 (*Streptomonospora alba*) in Ribandar only in those seasons. This result indicates the difference in the halophilic actinomycete communities in both the salt pans.

4. 1. 11. c. Cluster- III: Genus *Nocardiopsis*:

In cluster-III, a total of 36 strains showing long well developed, fragmented vegetative hyphae and long spore chains borne on aerial hyphae and spores with smooth surface & rod shaped were included (characteristic morphology of genus *Nocardiopsis*, Li *et al.*, 2004). Among the 36 isolates from the sediments of both the salt pans, 24 were found to be halophilic and 12 to be halotolerants. SEM analysis (Fig. 25) and BIOLOG profile revealed 2 halophilic species of *Nocardiopsis* viz. *Nocardiopsis halophila* (SBSK-160) and *Nocardiopsis salina* (SBSK-551) whereas the other one was *Nocardiopsis halotolerans* (SBSK-119). Table. 11 summarizes the isolation details of these three species and their other physiological properties. Both the halophilic strains were found to be moderate halophiles with optimum growth at 10% (100 psu) NaCl concentration and could grow up to 20% (200 psu) whereas the halotolerant strain (SBSK-119) was found to tolerate up to 15% (150 psu) salt concentration. Fig. 26 shows the phylogenetic tree constructed based on the sequences of the 16S rRNA gene depicting taxonomic relationship of these hypersaline strains with the type strains. All the strains were found in both Ribandar and Agarvado salt pans during both pre and post monsoon seasons suggesting them to be indigenous part of the microbial flora of the salt pans.

4. 1. 11. d. Cluster-IV: Genus *Kocuria*:

In Cluster-IV a total of 35 non-sporulating actinomycetes with yellow to red pigmented smooth colonies were included (Fig. 27). Among 35 isolates, 27 of them were isolated on R2A agar at 75-100 psu salt concentration.

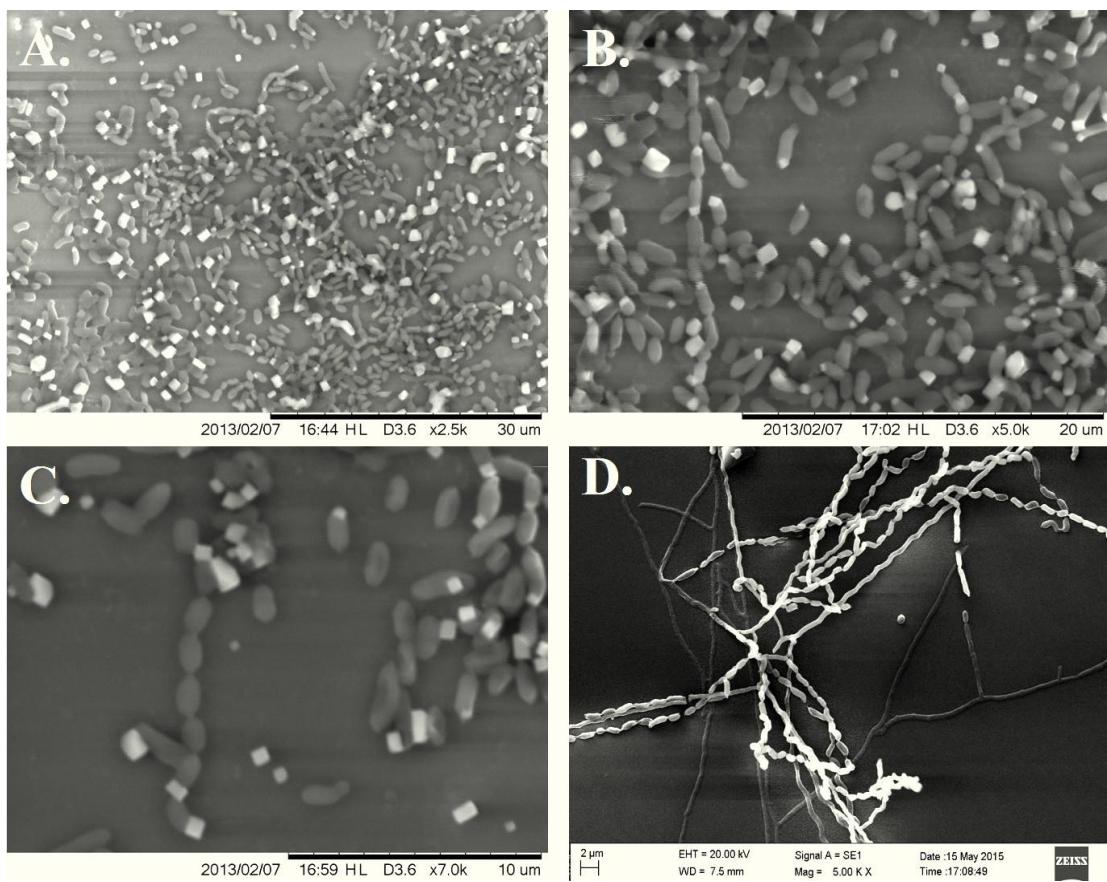


Fig. 25 A. and B. shows the growth of SBSK-160 (*Nocardiopsis halophila*) attached to the salt crystals and long, irregularly branched aerial mycelium with zigzag appearance which is fragmented into elongated spores with smooth surface; C. Strain SBSK-119 (*Nocardiopsis halotolerans*) showing fragmentation of long branched substrate hyphae into non-motile elements; D. Strain SBSK-551 (*Nocardiopsis salina*) showing long well developed and fragmented vegetative hyphae along with long spore chains on aerial hyphae

Table. 11 Isolates of cluster-III: Isolation details, carbon source utilization, salt requirement and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	Salt requirement for growth
1.	SBSK-119	Ribandar & Agarvado salt pans, both pre and post-monsoon	SCA and R2A	<i>Nocardiopsis halotolerans</i>	Glucose, mannose, galactose, sucrose, melibiose and glycerol	0-15%
2.	SBSK-160	Agarvado salt pan, in both pre and post-monsoon	Inorganic salt starch agar (ISP-4)	<i>Nocardiopsis halophila</i>	Inositol, maltose and rhamnose	10-20%
3.	SBSK-551	Ribandar salt pan, both pre and post-monsoon	R2A and Starch casein agar	<i>Nocardiopsis salina</i>	Ribose, sucrose, fructose, raffinose, sodium citrate and sodium acetate	10-20%

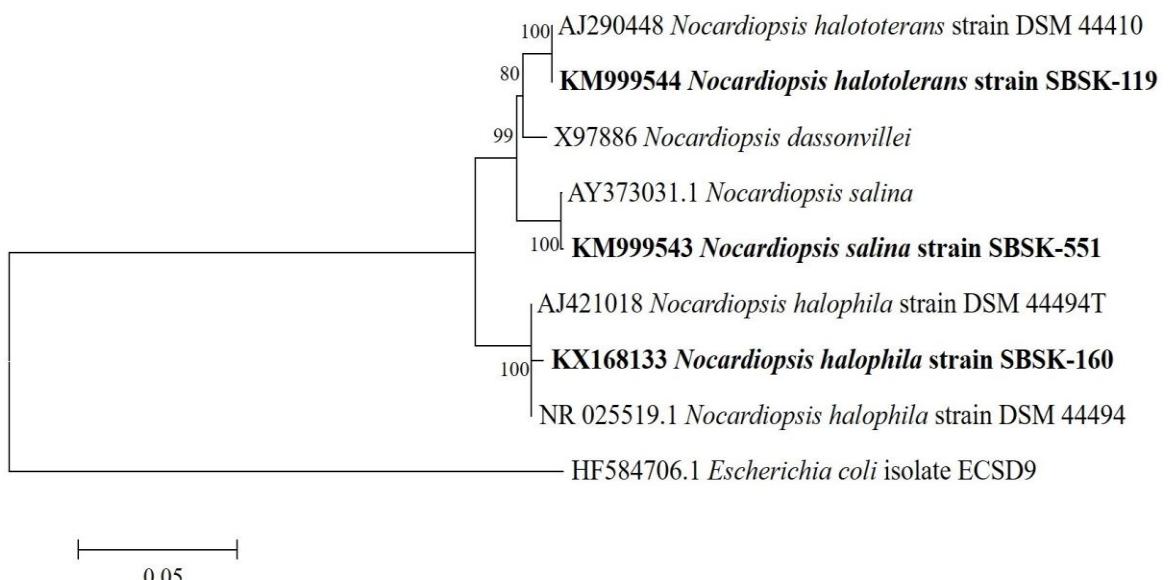


Fig. 26 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Nocardiopsis*; *Escherichia coli* strain ECSD9 is an out group

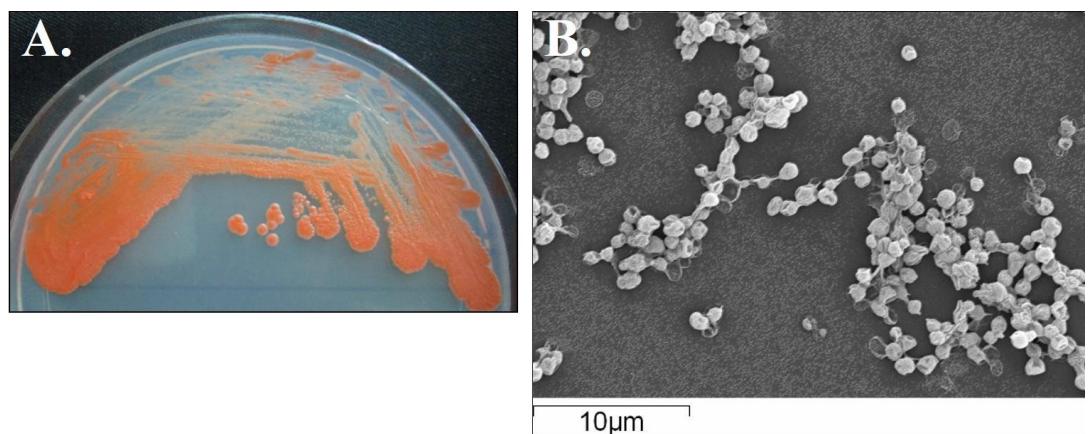


Fig. 27 A. Strain SBSK-325 growing on starch-casein agar (SCA) supplemented with 10% NaCl and B. SEM image of strain SBSK-335 showing coccoid cells with 1-1.5 μm in diameter

BIOLOG profiles and 16S rRNA gene sequencing revealed 5 different species viz. *Kocuria rhizophila* (SBSK-125), *Kocuria palustris* (SBSK-320), *Kocuria marina* (SBSK-325), *Kocuria indica* (SBSK-330) and *Kocuria salsicia* (SBSK-335). The strains varied significantly in their carbon source utilization profiles SBSK-125 and SBSK-330 being utilizers of maximum number of carbon sources (Table. 12). Molecular characterization confirmed the identity of all the 6 strains. Fig. 28 shows the taxonomic position of the all 6 strains in the phylogenetic tree. SBSK-125 (*Kocuria rhizophila*) and SBSK-335 (*Kocuria salsicia*) were found only in post-monsoon seasons and showed comparatively lower tolerance (5-8%) to NaCl which reveals their origin from other ecosystems. Strain SBSK-325 (*Kocuria marina*) which were tolerant up to 15% salt concentration, were isolated from both Ribandar and Agarvado salt pans during both pre and post monsoon seasons, suggesting its hypersaline adaptation and indigenous nature to the marine salt pans.

4. 1. 11. e. Cluster-V: Genus *Streptomyces*:

One of the most predominant genus isolated from the sediments. A total of 267 morphologically variant *Streptomyces* spp. were isolated from Ribandar and Agarvado saltern. Isolates showed variation in the colour and the sporulation pattern on growing on to 7 different *International Streptomyces Project* (ISP) media. The metabolic profile greatly varied in the BIOLOG system (Fig. 29). SEM analysis showed varying architecture of mycelial filaments and spore chain patterns (Fig. 30). Majority of the isolates were isolated on R2A & SCA media at salinity (32-75 psu salinity) and were found to be halotolerants. Table. 13 summarizes their carbon source utilization and salt tolerance profiles. Among the 12 selected isolates, 5 were found to be slight to moderate halophiles (grew in presence of 1-10% or 10-100 psu salt) viz. SBSK-170, SBSK-230, SBSK-233, SBSK-439 and SBSK-635.

Table. 12 Isolates of cluster-IV: Isolation details, carbon source utilization, salt tolerance and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	NaCl tolerance
1.	SBSK-125	Agarvado salt pan in post-monsoon season	R2A agar and 32 psu	<i>Kocuria rhizophila</i>	Mannose, saccharose, adonitol, L-arabinose, fructose, L-fucose, glucose, turanose, xylitol, methyl- pyruvate, glucuronamide, dextrin, glycogen, Tween 40, Tween 80 and N-acetyl-D-glucosamine	0-8%
2.	SBSK-320	Ribandar salt pan in both pre and post-monsoon seasons	R2A agar and 75 psu	<i>Kocuria palustris</i>	D-glucose, D-fructose, galactose, lactose and saccharose, adonitol, glucuronamide, inosine, Tween 40 and Tween 80	0-15%
3.	SBSK-325	Ribandar & Agarvado salt pans in both pre and post-monsoon seasons	SCA agar and 100 psu	<i>Kocuria marina</i>	Glucose, lactose, mannose and sucrose, L-fucose	0-20%
4.	SBSK-330	Ribandar salt pan in post-monsoon season	R2A agar and 50 psu	<i>Kocuria indica</i>	Cellobiose, D-galactose, D-glucose, glycogen, D-fructose, inositol, cupric acid, D-mannitol, D-mannose, melibiose, melezitose, D-ribose, L-rhamnose, salicin, phenylacetic acid, trisodium citrate, trehalose and turanose	0-15%
5.	SBSK-335	Agarvado salt pan in post-monsoon season	SCA agar and 32 psu	<i>Kocuria salsicia</i>	Starch, urea, gelatin, D-glucose, D-mannose, maltose, potassium gluconate, adipic acid, L-malic acid, trisodium citrate and phenylacetic acid	0-5%

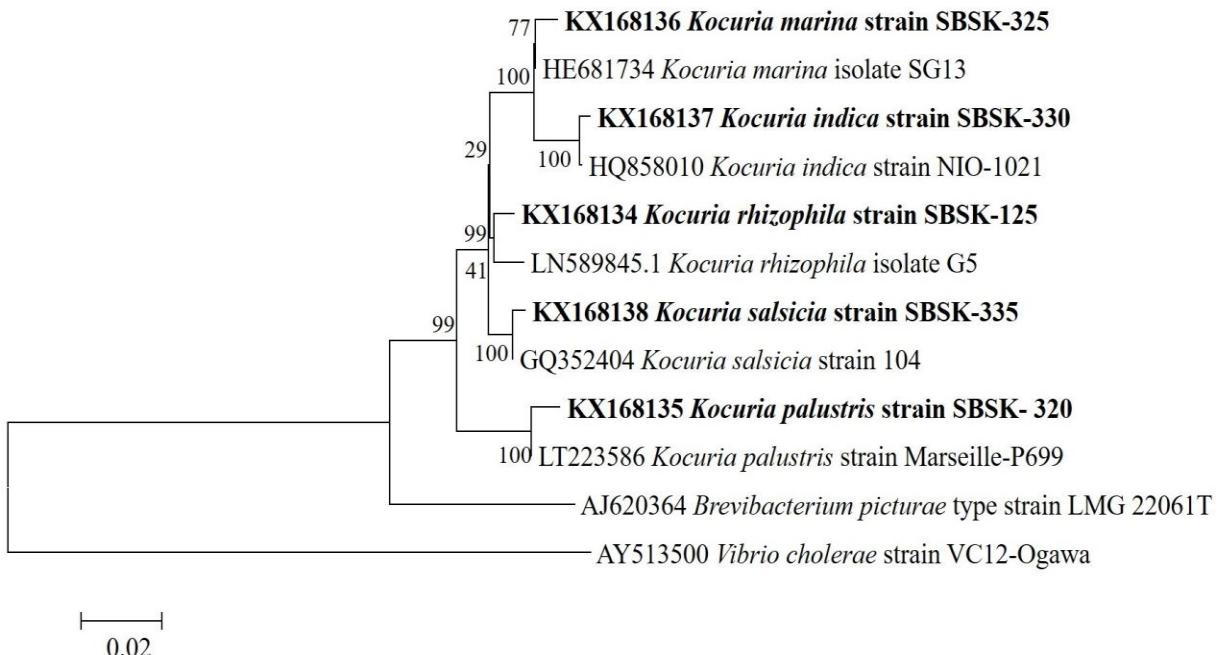


Fig. 28 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Kocuria*; *Vibrio cholera* strain VC12-Ogawa is an out group

BLANK	α -Cyclodextrin	β -Cyclodextrin	Dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-acetyl-D-glucosamine	N-acetyl-D- β -mannosamine	Amygdalin
L-Arabinose	D-arabitol	Arbutin	D-cellobiose	D-fructose	L-Fucose	D-Galactose	D-galacturonic acid	Gentiobiose	D-Gluconic acid	α-D-glucose	m-inositol
α -D-Lactose	Lactulose	Maltose	Maltotriose	D-mannitol	D-mannose	D-Melezitose	D-Melibiose	α -Methyl-D-galactoside	β -Methyl-D-galactoside	3-methyl-D-glucose	α -Methyl-D-glucoside
β -Methyl-D-glucoside	α -Methyl-D-mannoside	Palatinose	D-psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-sorbitol	Stachyose	Sucrose
D-Tagatose	D-Trehalose	Turanose	Xylitol	D-xylose	Acetic acid	α -Hydroxybutyric acid	β -Hydroxybutyric acid	γ -Hydroxybutyric acid	p-Hydroxyphenylacetic acid	α -Ketoglutaric acid	α -Ketovaleric acid
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
L-Alaninamide	D-Alanine	L-Alanine	L-alanyl-glycine	L-Asparagine	L-glutamate	Glycyl-L-glutamic acid	L-pyroglutamic acid	L-serine	Putrescine	2,3-Butanediol	Glycerol
Adenosine	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-monophosphate	Thymidine-5'-monophosphate	Uridine-5'-monophosphate	D-Fructose-6-Phosphate	α -D-glucose-1-phosphate	D-glucose-6-phosphate	D-L-a-glycerol phosphate

Fig. 29 BIOLOG carbohydrate utilization profile of halotolerant strain SBSK-8 (*Streptomyces* sp.); highlighted cells represent the utilization of the carbon sources

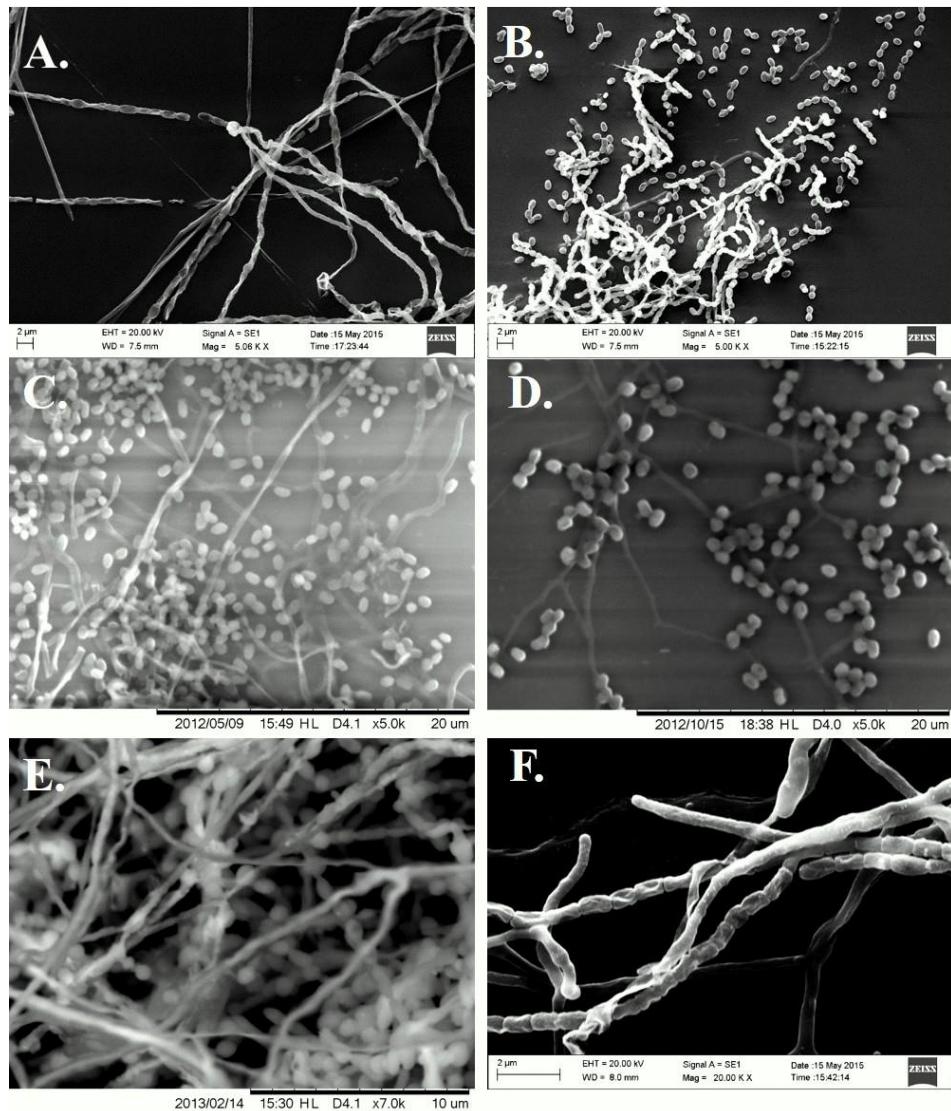


Fig. 30 SEM images: A. Aerial mycelia showing cylindrical spores and spore chains of SBSK-1, B. SBSK-165 showing spiral spore chains with smooth surface, C. Long fragmented substrate mycelia and spore chains of strain SBSK-439, D. Branched substrate mycelia and spore chains and oval shaped free spores of strain SBSK-8, E. Strain SBSK-285 showing unbranched substrate mycelia and aerial hyphae differentiating into straight to spiral chains of oval to round shaped spores, F. Strain SBSK-230 showing rectiflexible aerial mycelia differentiated into long bead like chains

Table. 13 Isolates of cluster-V: Isolation details, carbon source utilization, salt tolerance and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	NaCl requirement/tolerance
1.	SBSK-1	Ribandar salt pan in both pre and post-monsoon seasons	SCA and 50 psu	<i>Streptomyces sparsus</i>	glucose, galactose, rhamnose, arabinose, xylose, raffinose, starch, ribose, inositol, mannitol, glycine, histidine, methionine and asparagine	0-15%
2.	SBSK-275	Ribandar salt pan in both pre & post-monsoon seasons and Agarvado salt pan in post-monsoon season	R2A and 50 psu	<i>Streptomyces sporocinereus</i>	Starch, mannitol, glucose, fructose, inositol, arabinose, xylose, sucrose, glycerol,	0-5%
3.	SBSK-150	Ribandar & Agarvado salt pans in both pre and post-monsoon seasons	SCA and 32 psu	<i>Streptomyces radiopugnans</i>	Glucose, fructose, mannitol, starch, xylose, arabinose, sucrose, inositol and xylose	0-4%
4.	SBSK-165	Ribandar salt pan in post-monsoon season	R2A and 50 psu	<i>Streptomyces klenkii</i>	Starch, Tween-40 and Tween-80 adonitol, mannitol and sucrose	0-10%
5.	SBSK-170	Ribandar & Agarvado salt pans in both pre and post-monsoon seasons	SCA and 100 psu	<i>Streptomyces marinus</i>	Fructose, mannitol, sucrose, glucose, arabinose and xylose	3-9%
6.	SBSK-285	Ribandar salt pans in both pre and post-monsoon seasons	R2A and 32 psu	<i>Streptomyces nanhaiensis</i>	Acetate, cellobiose, fructose, fucose, galactose, glucose, maltose, sucrose and xylose	0-7.5%
7.	SBSK-439	Ribandar salt pan in both pre & post-monsoon seasons	SCA and 75 psu	<i>Streptomyces haliclonae</i>	Fructose, mannitol, sucrose, glucose, raffinose, sorbitol and myo-inosito	2-9%

8.	SBSK-635	Ribandar salt pan in post-monsoon season	SCA and 100 psu	<i>Streptomyces tateyamensis</i>	Mannitol, sucrose, glucose, sorbitol and inositol	2-10%
9.	SBSK-230	Agarvado salt pans in both pre and post-monsoon seasons	R2A and 100 psu	<i>Streptomyces oceanii</i>	Cellobiose, fructose, galactose, glucose, inositol, lactose, mannitol, L-rhamnose, ribose, sodium citrate and xylitol	3-10%
10.	SBSK-233	Agarvado salt pans in post-monsoon season	ISP-2 and 75 psu	<i>Streptomyces qinglanensis</i>	Raffinose, inositol, arabinose, sucrose, glucose, mannitol, cellobiose, galactose, melibiose, trehalose, xylose, fructose and mannose	1-3%
11.	SBSK-219	Ribandar salt pan in post-monsoon season	R2A and 32 psu	<i>Streptomyces specialis</i>	N-acetyl-glucosamine, glucose, gluconate, ribose, sucrose, adonitol, arabitol, sorbitol, myo-inositol, propionate, glutarate, butyrate, isobutyrate, isovaleric acid, L-arginine and L-asparagine.	0-5%

Molecular characterization confirmed the isolates to belong to 12 different species viz. *Streptomyces sparsus* (SBSK-1), *Streptomyces* sp. (SBSK-8), *Streptomyces radiopugnans* (SBSK-150), *Streptomyces klenkii* (SBSK-118), *Streptomyces specialis* (SBSK-219), *Streptomyces sporocinereus* (SBSK-275), *Streptomyces nanhiensis* (SBSK-285), *Streptomyces haliclonae* (SBSK-439), *Streptomyces oceanii* (SBSK-601), *Streptomyces quinglaensis* (SBSK-627), *Streptomyces marinus* (SBSK-656) and *Streptomyces tateyamensis* (SBSK-635). Similarity index ranged from 99-100%.

Fig. 31 shows the taxonomic relationship of the isolates with their type strains, all the similar species are clustering together whereas the outgroup sequences are branched out of the tree. The halotolerant strains (SBSK-1, 8, 118, 150, 219, 275, 627 and 656) were found mainly during the post-monsoon seasons whereas the halophilic ones (SBSK-170, 230, 233, 439 and 635) were seen in both pre and post monsoon seasons. This seasonal variation could be because of halotolerant being originated in the terrestrial ecosystems and have been carried to the salt pans with monsoon run off whereas these slight to moderate halophiles represented the indigenous members of the salt pans.

4. 1. 11. f. Cluster-VI: Genus *Micromonospora*:

Cluster-VI includes, a total of 42 isolates with no aerial mycelia and single small smooth spores on to branched substrate mycelia as a characteristic morphology. The colonies were orange to dark brown in colour. Light microscopy and SEM analysis revealed the detailed structural architecture (Fig. 32). Table. 14 summarizes the phenotypic characteristics of 5 strains which showed significant variations.

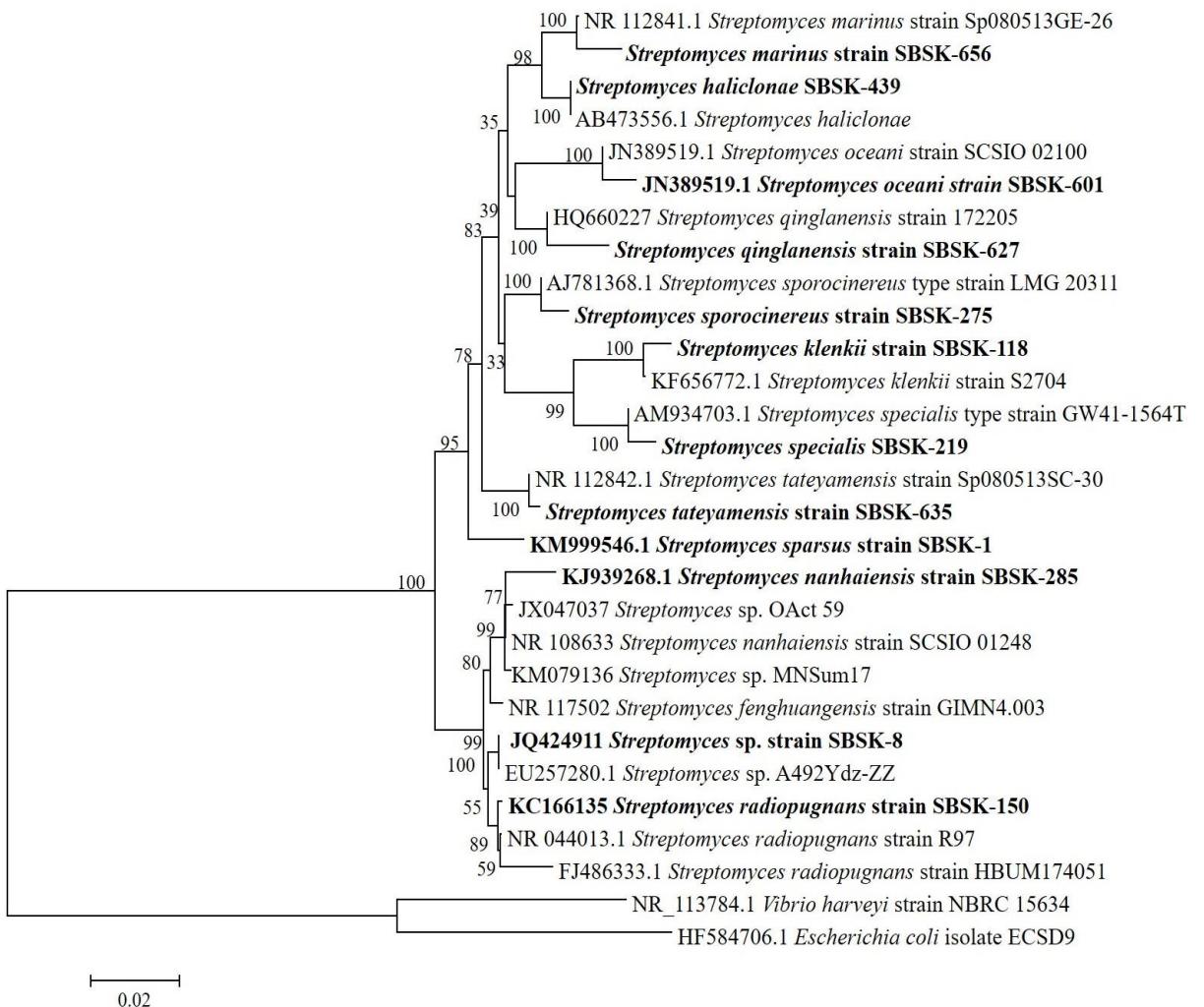


Fig. 31 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Streptomyces*; *Escherichia coli* strain ECSD and *Vibrio harveyi* strain NBRC 15634 are out groups

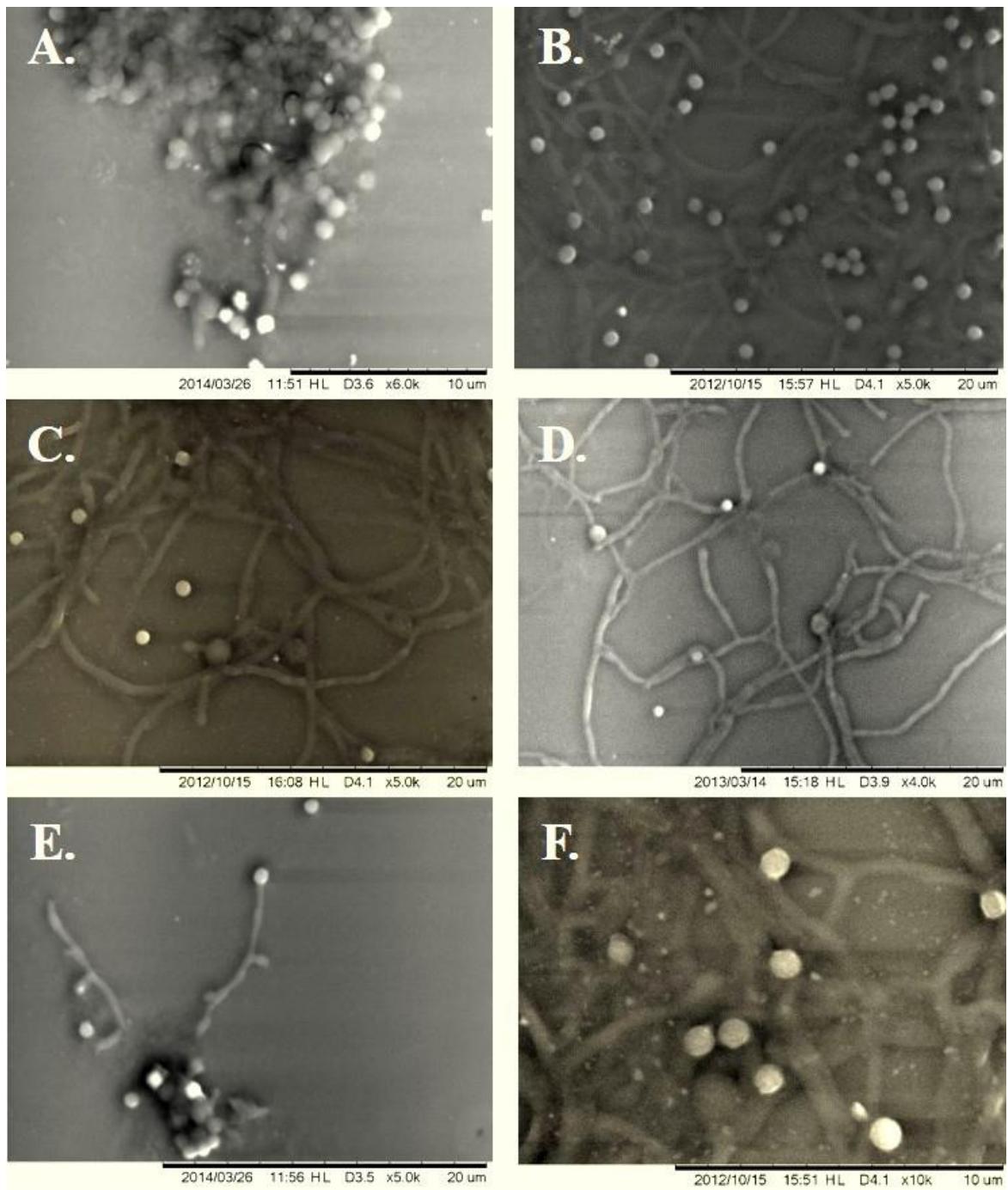


Fig. 32 SEM images: A. Single colony of strain SBSK-356 growing on R2A agar; B. Strain SBSK-359 showing smooth round spores with extensively branched substrate mycelia; C. and D. Single spores on the substrate hyphae of strain SBSK-378 and SBSK-568; E. SBSK-358 showing single spore on the tip of branched hyphae; F. Branched hyphae and spores of strain SBSK-233

Table. 14 Isolates of cluster-IV: Isolation details, carbon source utilization, salt tolerance and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	NaCl tolerance
1.	SBSK-233	Agarvado salt pan in post-monsoon season	R2A agar and 32 psu	<i>Micromonospora carbonacea</i>	Mannose, saccharose, adonitol, L-arabinose, fructose, L-fucose, glucose, turanose, xylitol, methyl-pyruvate, glucuronamide, dextrin, glycogen, Tween 40, Tween 80 and N-acetyl-D-glucosamine	0-8%
2.	SBSK-366	Ribandar salt pan in both pre and post-monsoon seasons	R2A agar and 75 psu	<i>Micromonospora coxensis</i>	D-glucose, D-fructose, galactose, lactose and saccharose, adonitol, glucuronamide, inosine, Tween 40 and Tween 80	0-10%
3.	SBSK- 358	Ribandar & Agarvado salt pans in both pre and post-monsoon seasons	SCA agar and 50 psu	<i>Micromonospora marina</i>	Glucose, lactose, mannose and sucrose, L-fucose	0-10%
4.	SBSK- 356	Ribandar salt pan in post-monsoon season	Inorganic salt starch agar and 32 psu	<i>Micromonospora aurantiaca</i>	Cellobiose, D-galactose, D-glucose, glycogen, D-fructose, inositol, cupric acid, D-mannitol, D-mannose, melibiose, melezitose, D-ribose, L-rhamnose, salicin, phenylacetic acid, trisodium citrate, trehalose and turanose	0-5%
5.	SBSK-568	Agarvado salt pan in post-monsoon season	SCA agar and 32 psu	<i>Micromonospora haikouensis</i>	Starch, urea, gelatin, D-glucose, D-mannose, maltose, potassium gluconate, adipic acid, L-malic acid, trisodium citrate and phenylacetic acid	0-5%

All the isolates were halotolerants and could tolerate up to 75 psu of salinity. Though appeared similar in morphology with light microscopy and SEM, metabolic fingerprinting (Fig. 33) and molecular characterization revealed that these 42 isolates represented 9 different species viz. *Micromonospora carbonacea* (SBSK-233), *Micromonospora halophytica* (SBSK-333), *Micromonospora echinaurantiaca* (SBSK-585), *Micromonospora marina* (SBSK-358), *Micromonospora coxensis* (SBSK-366), *Micromonospora krabiensis* (SBSK-359), *Micromonospora haikouensis* (SBSK-568) and *Micromonospora wenchangensis* (SBSK-378). After *Streptomyces*, the most predominant genera in the sediments of marine salt pans. From previous reports on marine actinomycetes, “*Micromonospora*” is known to adopt in the marine environments. Fig. 34 shows the taxonomic relationship of the strains with their type strains. Similarity index in 16S rRNA gene sequences were 99-100%.

4. 1. 11. g. Cluster-VII: Genus *Kitasatospora*:

Growth was seen with prominent sporulation, aerial and substrate mycelia. Colouration: White to pinkish aerial mycelia and off-white substrate mycelia. Substrate and aerial mycelia were extensively branched and with long chains of spores. Aerial mycelia developed into spore chains with spores of smooth surface and cylindrical shape. A total of 23 strains were isolated from both salt pans. For the first time genus *Kitasatospora* is being reported from salt pans and all the strains were halotolerants. Fig. 35 shows the characteristic morphology of the substrate and aerial mycelia of genus *Kitasatospora* in 3 selected strains. BIOLOG carbohydrate utilization profile (Table. 15) and 16S rRNA gene sequencing (Fig. 36 and 37) revealed 3 different species viz. *Kitasatospora mediocidica* (SBSK-227), *Kitasatospora kazusensis* (SBSK-430), *Kitasatospora atroaurantiaca* (SBSK-567).

BLANK	α -Cyclodextrin	β -Cyclodextrin	Dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-acetyl-D-glucosamine	N-acetyl-D- β -mannosamine	Amygdalin
L-Arabinose	D-arabitol	Arbutin	D-cellulose	D-fructose	L-Fucose	D-Galactose	D-gallacturonic acid	Gentiobiose	D-Gluconic acid	α-D-glucose	m-inositol
α -D-Lactose	Lactulose	Maltose	Maltotriose	D-mannitol	D-mannose	D-Meleziotose	D-Melibiose	α -Methyl-D-galactoside	β -Methyl-D-galactoside	3-methyl-D-glucose	α -Methyl-D-glucoside
β -Methyl-D-glucoside	α -Methyl-D-mannoside	Palatinose	D-psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-sorbitol	Stachyose	Sucrose
D-Tagatose	D-Trehalose	Turanose	Xylitol	D-xylose	Acetic acid	α -Hydroxybutyric acid	β-Hydroxybutyric acid	γ-Hydroxybutyric acid	p-Hydroxyphenylacetic acid	α-Ketoglutaric acid	α-Ketovaleric acid
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
L-Alaninamide	D-Alanine	L-Alanine	L-alanyl-glycine	L-Asparagine	L-glutamate	Glycyl-L-glutamic acid	L-pyroglutamic acid	L-serine	Putrescine	2,3-Butanediol	Glycerol
Adenosine	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-monophosphate	Thymidine-5'-monophosphate	Uridine-5'-monophosphate	D-Fructose-6-Phosphate	α -D-glucose-1-phosphate	D-glucose-6-phosphate	D-L- α -glycerol phosphate

Fig. 33 BIOLOG carbohydrate utilization profile of halotolerant strain SBSK-568 (*Micromonospora* sp.); highlighted cells represent the utilization of the carbon sources

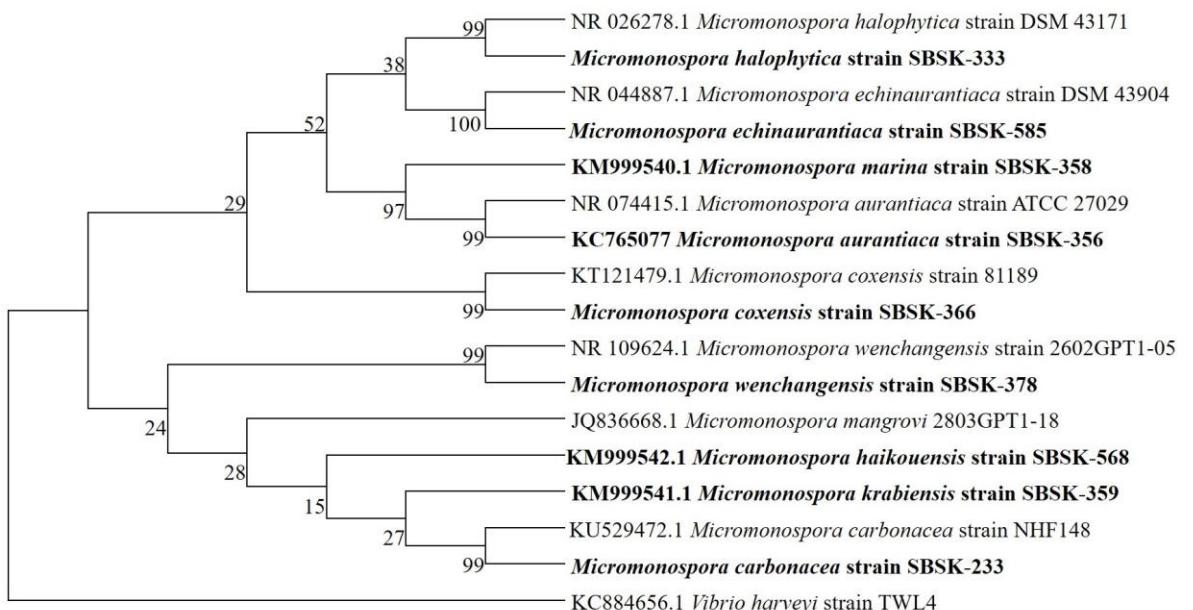


Fig. 34 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Micromonospora*; *Vibrio harveyi* strain TWL4 is an out group

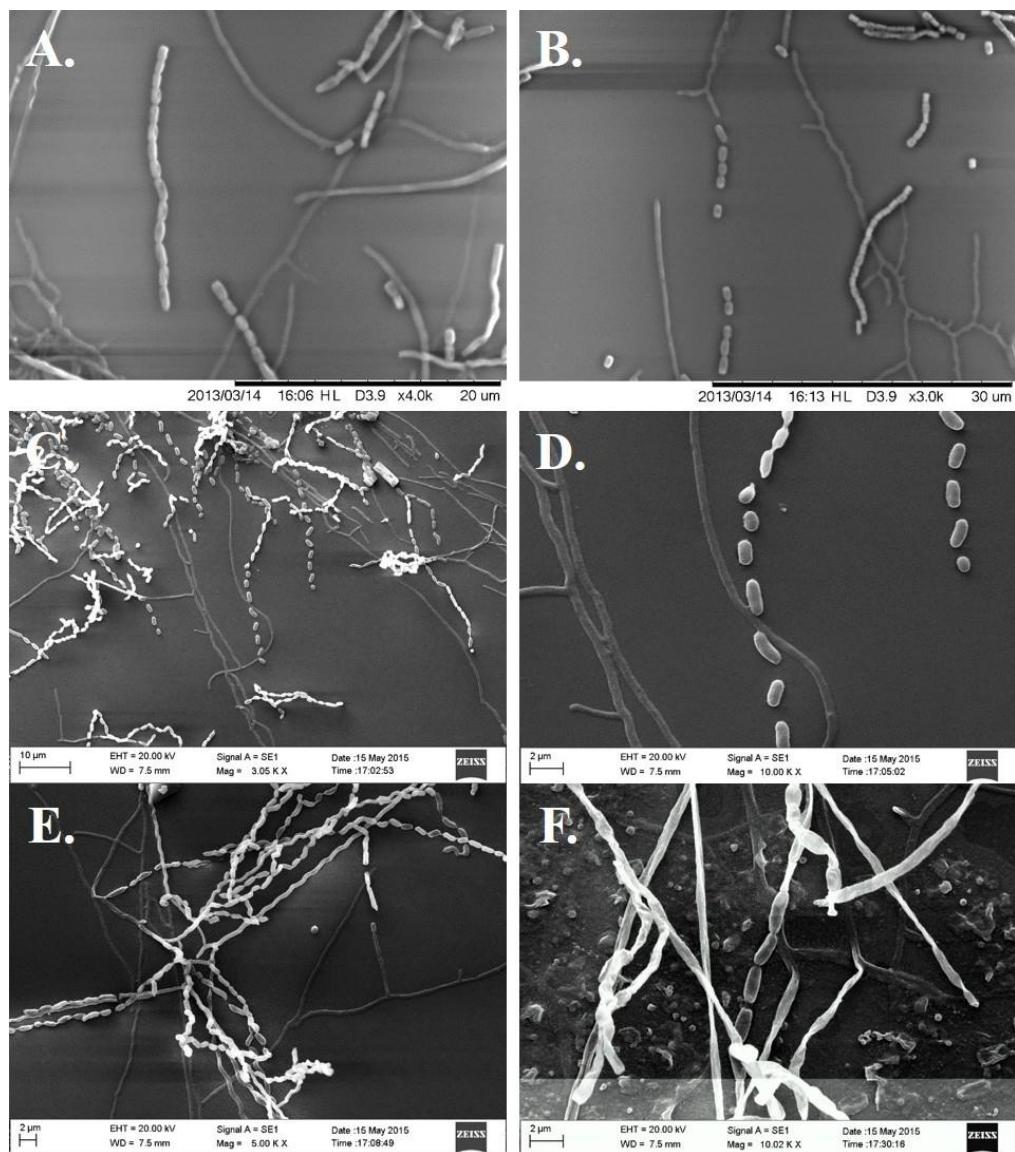


Fig. 35 SEM images: A and B. Long rod shaped, smooth surfaced, straight and slightly flexious spore chains of SBSK-567, C and D. Aerial hyphae with smooth surfaced spores with short open coils of strain SBSK-227, E and F. Branched substrate mycelia and aerial hyphae fragmenting into cylindrical spore chains of strain SBSK-430

Table. 15 Isolates of cluster-VII: Isolation details, carbon source utilization, salt requirement and species level identification

Sr. No.	Isolate Codes	Isolation source and season	Isolation media and salinity	Species	Carbon sources utilized	Salt requirement for growth
1.	SBSK-567	Ribandar & Agarvado salt pans, both pre and post-monsoon	R2A and 50 psu	<i>Kitasatospora atroaurantiaca</i>	Glucose, galactose, arabinose, xylose	0-3%
2.	SBSK-430	Agarvado salt pan, in both pre and post-monsoon	Inorganic salt starch agar and 32 psu	<i>Kitasatospora kazusanensis</i>	Inositol, maltose and rhamnose	0-5%
3.	SBSK-227	Ribandar salt pan, both pre and post-monsoon	R2A and 50 psu	<i>Kitasatospora Mediocidica</i>	Ribose, sucrose, fructose, raffinose, sodium citrate and sodium acetate	0-7.5%

BLANK	α -Cyclodextrin	β -Cyclodextrin	Dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-acetyl-D-glucosamine	N-acetyl-D- β -mannosamine	Amygdalin
L-Arabinose	D-arabitol	Arbutin	D-cellobiose	D-fructose	L-Fucose	D-Galactose	D-gallacturonic acid	Gentiobiose	D-Gluconic acid	α -D-glucose	m-inositol
α -D-Lactose	Lactulose	Maltose	Maltotriose	D-mannitol	D-mannose	D-Meleziotose	D-Melibiose	α -Methyl-D-galactoside	β -Methyl-D-galactoside	3-methyl-D-glucose	α -Methyl-D-glucoside
β -Methyl-D-glucoside	α -Methyl-D-mannoside	Palatinose	D-psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-sorbitol	Stachyose	Sucrose
D-Tagatose	D-Trehalose	Turanose	Xylitol	D-xylene	Acetic acid	α -Hydroxybutyric acid	β -Hydroxybutyric acid	γ -Hydroxybutyric acid	p-Hydroxyphenylacetic acid	α -Ketoglutaric acid	α -Ketovaleric acid
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	Succinic acid	succinic acid	N-acetyl-L-glutamic acid
L-Alaninamide	D-Alanine	L-Alanine	L-alanyl-glycine	L-Asparagine	L-glutamate	Glycyl-L-glutamic acid	L-pyroglutamic acid	L-serine	Putrescine	2,3-Butanediol	Glycerol
Adenosine	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-monophosphate	Thymidine-5'-monophosphate	Uridine-5'-monophosphate	D-Fructose-6-Phosphate	α -D-glucose-1-phosphate	D-glucose-6-phosphate	D-L- α -glycerol phosphate

Fig. 36 BIOLOG carbohydrate utilization profile of halotolerant strain SBSK-430

(*Kitasatospora* sp.); highlighted cells represent the utilization of the carbon sources

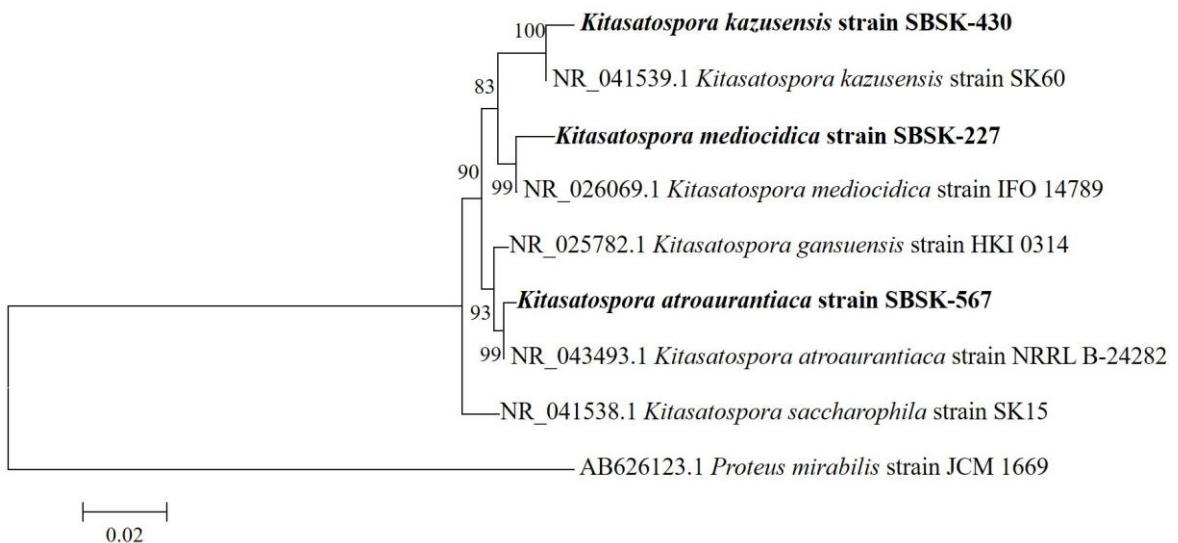


Fig. 37 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Kitasatospora*; *Proteus mirabilis* strain JCM1660 is an out group

4. 1. 11. h. Cluster-VIII: Genus *Rhodococcus*:

Typical non-sporulating, reddish colony. Cells are rod shaped in logarithmic phase and coccoid in stationary phase. Fig. 38 shows the reddish colony of strain SBS-297 growing on R2A agar and a mature colony in SEM. A total of 15 *Rhodococcus* strains

were isolated from both the salt pans. Majorly were found to be halotolerants and few had a requirement of sea water (35 psu) for optimum growth suggesting their marine adaptation. Characterization by BIOLOG and 16S rRNA gene sequencing revealed three major spp. viz. *Rhodococcus marinonascens* (SBSK-297), *Rhodococcus nanhaiensis* (SBSK-651) and *Rhodococcus opacus* (SBSK-111).

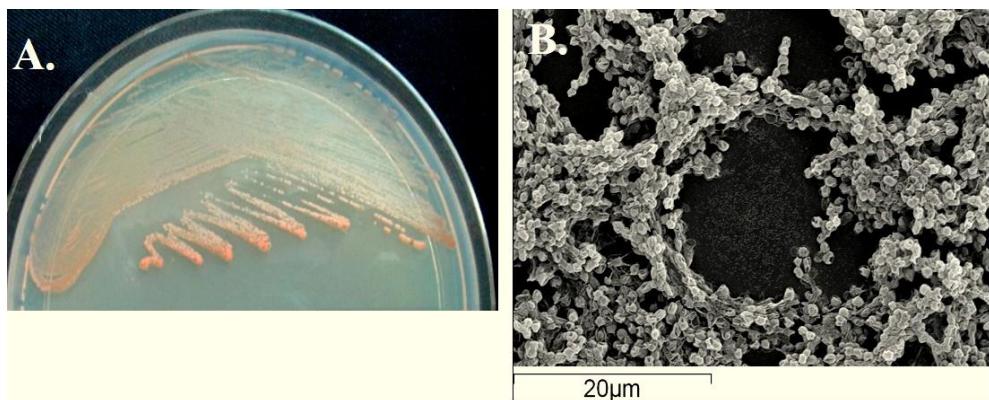


Fig. 38 Colony morphology and SEM of strain SBSK-297

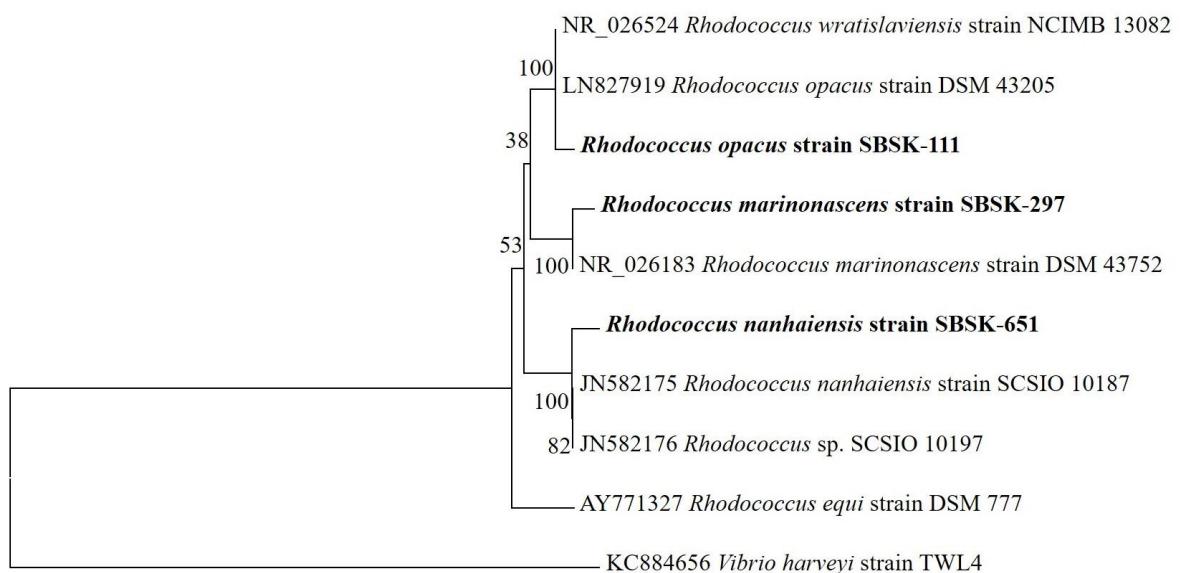


Fig. 39 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Rhodococcus*; *Vibrio harveyi* strain TWL4 is an out group

4. 1. 11. i. Cluster-IX: Genus *Micrococcus*:

Cluster-IX represented the non-sporulating actinomycete strains which grew as coccoid cells in clusters (Fig. 40). Hypersaline *Micrococcus* has never been reported earlier. Only 5 strains were isolated; 3 from Ribandar and 2 from Agarvado. All the strains were halotolerants and could tolerate up to 50 psu salinity only. From the 5 isolates, two species were identified viz. *Micrococcus luteus* (SBSK-527) and *Micrococcus yunnanensis* (SBSK-636) (Fig. 41).

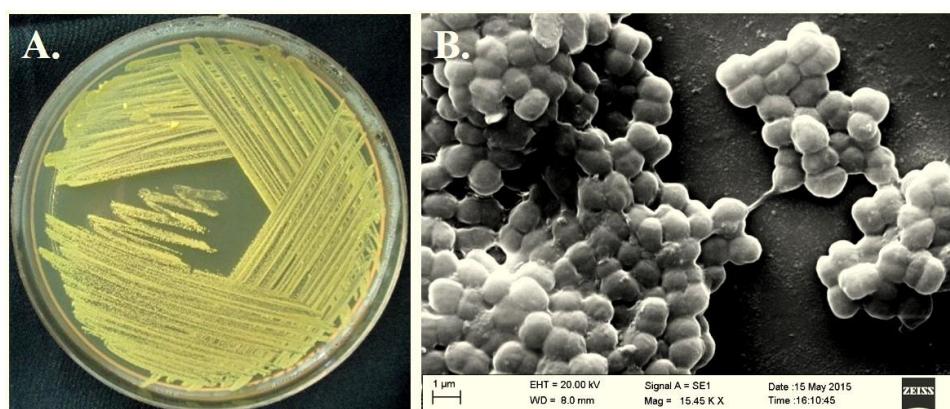


Fig. 40 Colony morphology and SEM of strain SBSK-527

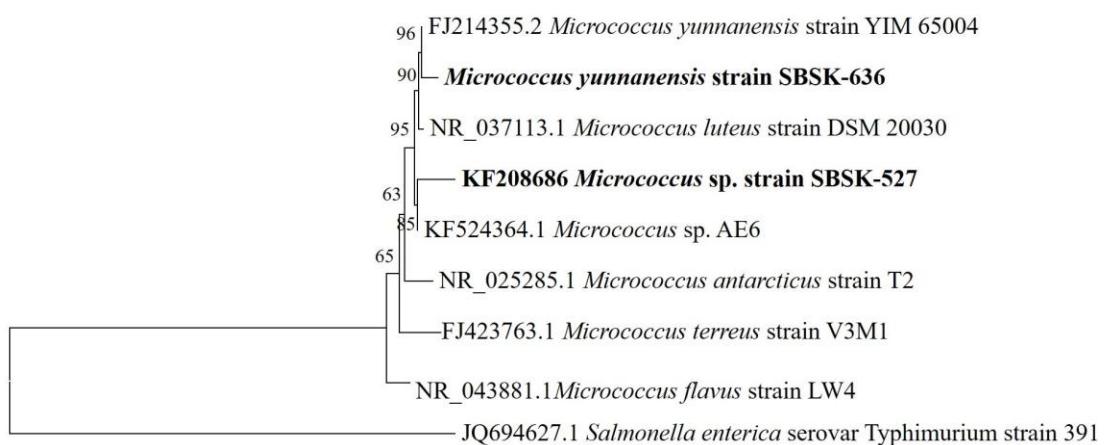


Fig. 41 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Micrococcus*; *Salmonella enterica* serovar Typhimurium strain 391 is an out group

4. 1. 11. j. Cluster-X: Genus *Gordonia*:

The colonies appeared orange in colour, non-sporulating with rough surface and irregular margins. Light microscopy showed branching of substrate mycelial hyphae to rod shaped and coccoid structures. Isolated only from Agarvado salterns but 3 halotolerant strains showing the rare presence of this particular genera in marine salterns. All the three strains were identified to be *Gordonia paraffinivorans*. Fig. 43 shows the taxonomic relationship of the strain SBSK-255 with the other type strains of genus *Gordonia*.

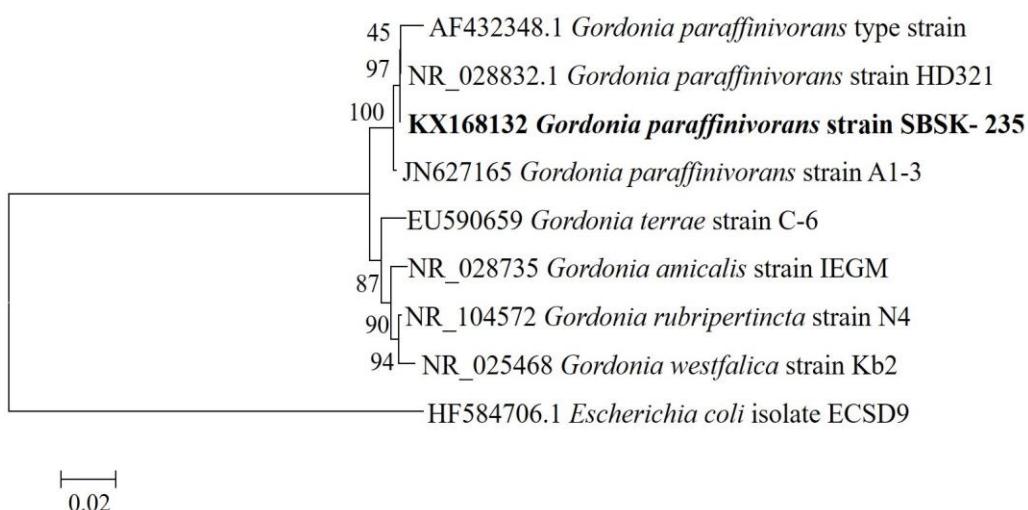


Fig. 43 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Gordonia*; *Escherichia coli* isolate ECSD9 is an out group

4. 1. 11. k. Cluster-XI: Genus *Brevibacterium*:

Cluster-XI included the strains which appeared to be non-sporulating, white, circular and opaque colonies. Light microscopy and SEM analysis showed smooth rod shaped cells. A total of 4 strains were isolated from Agarvado salterns on peptone-yeast

extract iron agar with 50 psu salinity. All the four strains were found to be moderate halotolerant since showed growth in broth media without salt and could tolerate up to 50-75 psu salinity. BIOLOG profile revealed them to belong to two different species. Strain SBSK-230 utilized cyclodextrin, N-acetyl-D-glucosamine, L-fucose, D-gluconic acid, myo-inositol, lactulose, mannitol, methyl b-D-galactoside, methyl a-D-glucoside, mannose, salicin, trehalose, turanose, D-malic acid, succinic acid, D-alanine, L-alanyl glycine, putrescine, uridine and Tween-80. The other strain SBSK-404 utilized malonate, L-proline, L-tyrosine, L-serine, D-glucose, D-galactose, fructose, mannose, rhamnose, D-xylose, D-cellobiose, melibiose, D-raffinose, adonitol, dulcitol, maltose, lactose, D-ribose, trehalose, sucrose and inositol. These results showed clear difference in the species level. Molecular characterization by 16S rRNA gene sequencing and phylogenetic analysis confirmed that SBSK-230 to be *Brevibacterium album* and SBSK-404 to be *Brevibacterium oceanii* since showed 99% similarity in the 16S rRNA gene sequence. The GenBank accession numbers are KX168130 and KX168131.

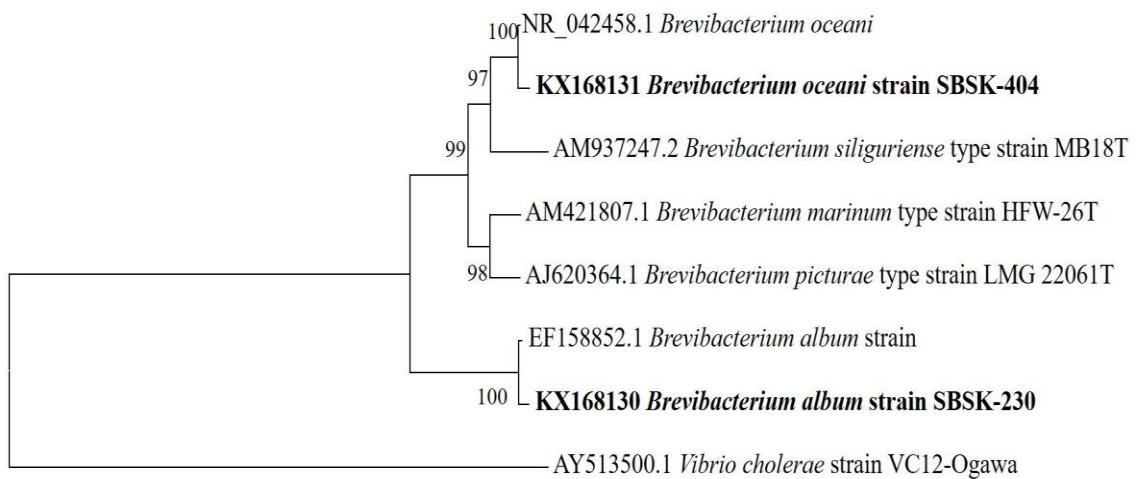


Fig. 44 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Brevibacterium*; *Vibrio cholerae* strain VC12-Ogawa is an out group

4. 1. 11. I. Cluster-XII: Genus *Arthrobacter*:

Cluster-XII includes the non-sporulating, off-white colony forming strains. The isolates were found to be rod shaped and could tolerate up to 100 psu salt concentration. Strains belonged to two species viz. *Arthrobacter pigmenti* (SBSK-240) and *Arthrobacter subterraneus* (SBSK-401) (Fig. 45 and 46).

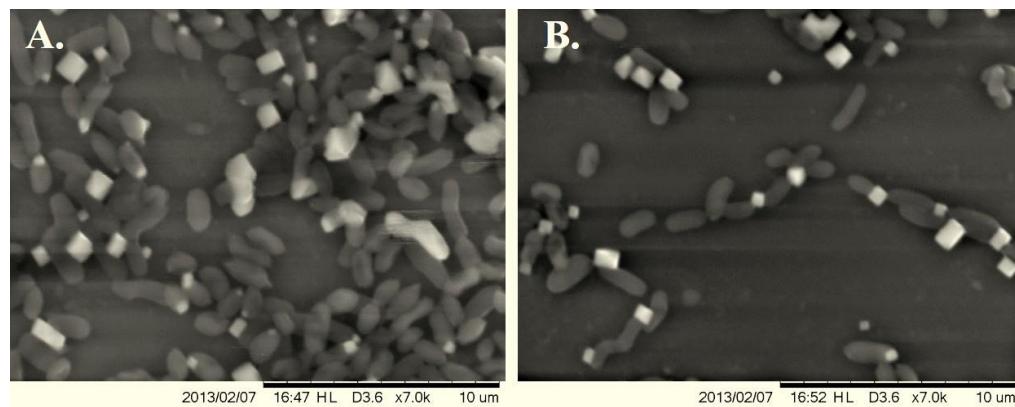


Fig. 45 SEM of strain SBSK-401, rod shaped cells growing attached to salt crystals

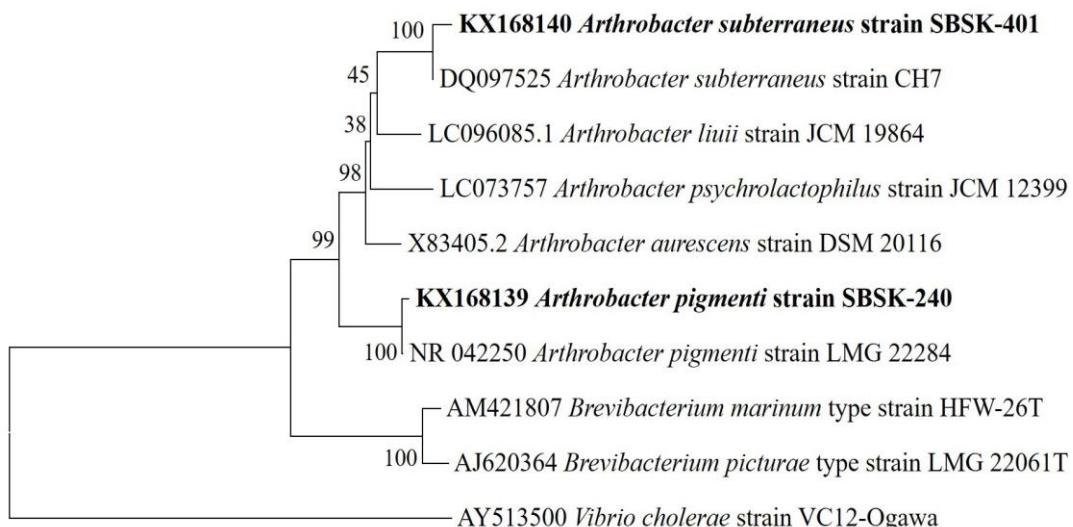


Fig. 46 Phylogenetic tree constructed by Neighbour joining method and Tamura-Nei model showing the taxonomic relationship of the isolates with the type strains of the genus *Arthrobacter*; *Vibrio cholerae* strain VC12-Ogawa is an out group

4. 1. 11. m. Cluster-XIII: Unclassified isolate SBSKN₅:

This cluster includes a single extremely halophilic strain SBSKN₅, which didn't resemble the conventionally reported actinomycete strains. This strain was isolated from the sediment of Ribandar salt pan during the pre-monsoon. Isolation media was R2A and with 250 psu salt concentration. Grows solely in presence of 250 – 300 psu crude natural salt. Optimized growth medium for liquid culture is ½ strength nutrient broth at 37°C and pH 7 (Fig. 48), however for sporulation in the solid media only R2A is suitable. No growth in presence of synthetic NaCl (25%) in optimized broth media. “Defined media” optimized for growth in broth was inorganic salt-starch agar base with 24% NaCl + 1% KCl + 0.5% MgCl₂.

Detailed SEM analysis showed substrate mycelia is branched and bears ‘sporangiophore’, aerial mycelia consisted of smooth round spores which originates from substrate mycelia and the strain grows attached to the salt crystals. Based on detailed SEM analysis and phenotypic as well as metabolic characteristics it was concluded to belong to genus “*Actinopolyspora*”. The isolate didn't show any positive result with BIOLOG microplate assay but showed prominent growth in the broth media prepared with basal salt solution of ISP-4, 25% natural sea salt and single carbon source. Molecular characterization by 16S rRNA gene sequencing, BLAST and EzTaxon server search showed the strain to show only 97% similarity with reported type strain *Actinopolyspora algeriensis* strain H19 and 96% similarity with other hypersaline *Actinopolyspora* strains viz. *Actinopolyspora saharensis* strain H32 and *Actinopolyspora xinjiangensis* strain YIM 90829. Table. 16 the comparative assessment of phenotypic and metabolic characteristics of strain SBSKN₅ and the other type strains of the genus *Actinopolyspora*. Fig. 50 shows the phylogenetic tree and taxonomic relationship of the isolate with the type strains.

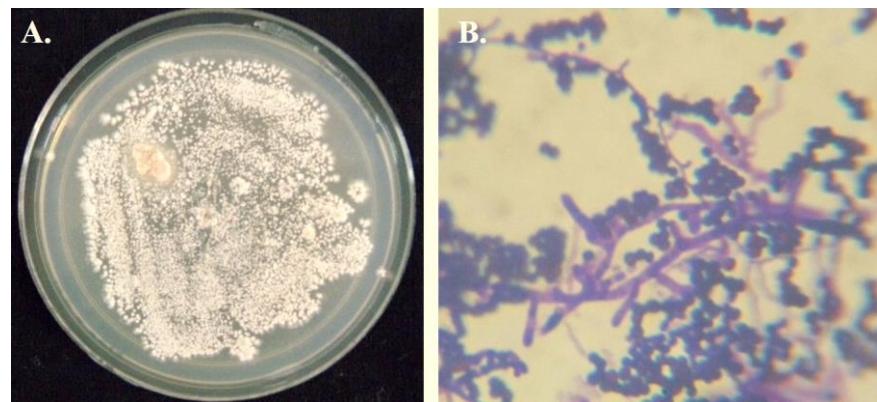


Fig. 47 A. Colony morphology of isolate SBSKN₅ on R2A agar with 250 psu salt; B. Light microscopy (100X) showing the presence of branched mycelial filaments and smooth round spores attached

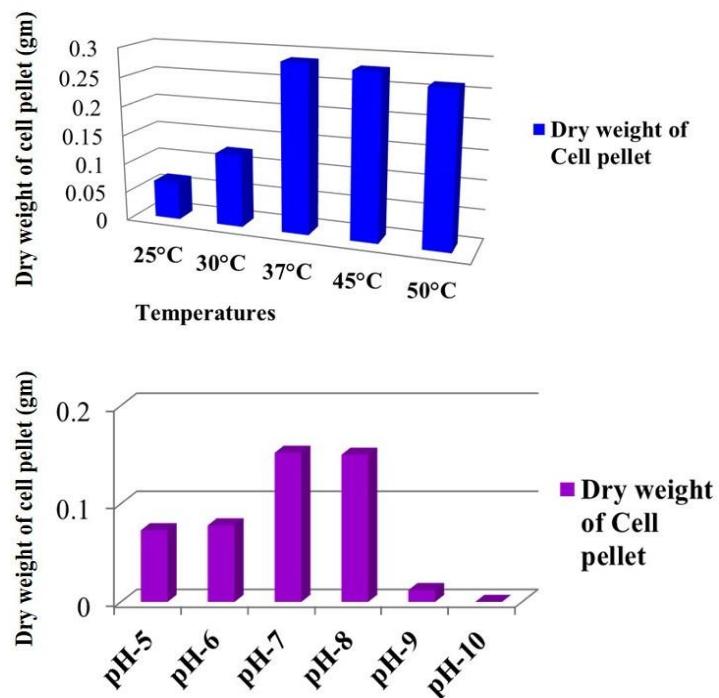


Fig. 48 Growth of isolate SBSKN₅ at various pH and temperatures

Table. 16 Cluster-XIII: Strain SBSKN₅ and the type strains comparative assessment of phenotypic and metabolic characters

Sr. No.	Strains	Isolation source	Isolation media and salinity	Growth temperature	Carbon sources utilized	NaCl requirement
1.	SBSKN ₅	Ribandar salt pan in pre-monsoon season	R2A agar at 250 psu	37°C	Starch, glucose, cellobiose, fructose, mannose, trehalose, xylose and galactose	25-30%
2.	<i>Actinopolyspora Algeriensis</i> sp. nov. [Meklat <i>et al.</i> , 2012]	saline soil sample of Bamendil palm grove, Ouargla province, South Algerian Sahara	Humic acid-vitamin agar	30°C	Arabinose, cellobiose, erythritol, fructose, galactose, glucose, glycerol, lactose, inositol, mannose, raffinose, trehalose, sucrose and xylose	7-30%
3.	<i>Actinopolyspora alba</i> sp. nov. [Tang <i>et al.</i> , 2011]	Baicheng salt field of Xinjiang province, North-west China	Cellulose casein multi-salt agar	37°C	Tween-40, fructose, galactose, glucose and aesculin	10-25%
4.	<i>Actinopolyspora erythrea</i> sp. nov. [Tang <i>et al.</i> , 2011]	Salt field of Xinjiang province, North-west China	Cellulose casein multi-salt agar	37°C	Aesculin, casein, dextrin, starch and Tweens 40 and Tween 80	10-25%
5.	<i>Actinopolyspora xinjiangensis</i> sp. nov. [Guan <i>et al.</i> , 2013]	Salt lake of Xinjiang province, China	GW3 agar	37°C	Glucose, arabinose, xylose, fructose and galactose	10-15%

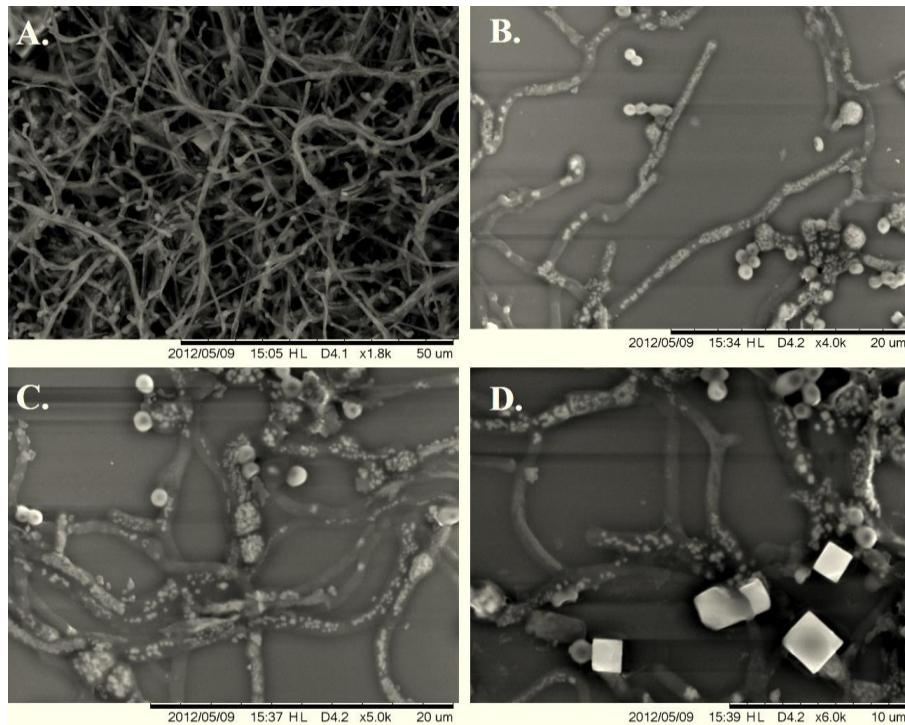


Fig. 49 SEM images of strain SBSKN₅, Grown on R2A agar with 250 psu salt: A. Mature colony showing mycelial filaments bearing sporangiophore; B. Spore arrangement on the sporangiophores on top of mycelial filaments; C. Single spores appearing from the sporangiophore; D. Strain SBSKN₅ growing attached to the salt crystals at saturating salt concentration

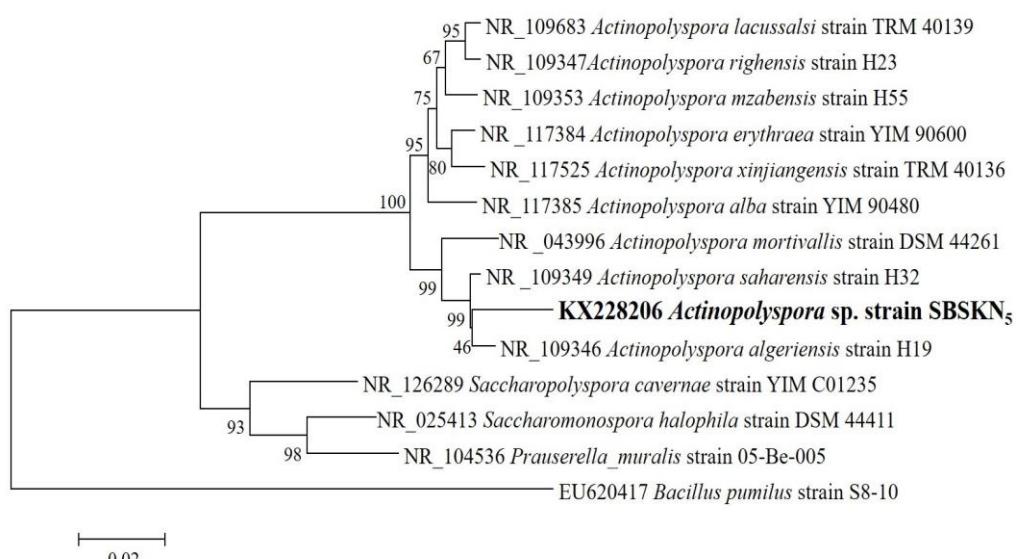


Fig. 50 Phylogenetic tree constructed using neighbor-joining method (Tamura-Nei model, 5 gamma parameter) based on 16S rRNA gene sequences showing the position of strain ‘SBSKN5’ along with its closest type strains

All these observations suggest that extreme borderline halophilic strain SBSKN₅ could be a novel species under the genus ‘*Actinopolyspora*’.

4. 1. 12. Distribution of various genera in both the salt pans:

From the data obtained, Ribandar salt pan was found to harbor 9 different genera and *Streptomyctete* and rare actinomycete percentage was 34% and 76% respectively. Fig. 51 shows the distribution and the percentage of 9 different genera. Among the rare actinomycetes *Micromonospora* was found to be the most pre-dominant member which constituted 24% of the total number of isolates followed by *Nocardiopsis* and *Kocuria* 14% and 9% respectively. Other 5 groups viz. *Actinomadura*, *Streptomonospora*, *Rhodococcus*, *Micrococcus* and *Kitasatospora* constituted lower percentages ($\geq 5\%$).

In Agarvado salt pan 12 different genera of actinomycetes were found and percentage of *Streptomyctete* and rare actinomycetes were 23% and 77% respectively. Fig. 52 shows the distribution and percentage of various genera in Agarvado salt pan. The *Streptomyces* spp. (23%) were found to be less as compared to Ribandar salt pan (34%). Three rare actinomycete genera viz. *Gordonia* (2%), *Brevibacterium* (4%) and *Arthrobacter* (3%) were found only in Agarvado salt pan. Among the rare actinomycetes, *Micromonospora* (15%) and *Nocardiopsis* (14%) were higher in number compared to the other groups whereas the remaining genera constituted 39% of the total isolates. From Fig. 51 and 52, it is prominent that Agarvado salt pan is more diverse in its actinomycete population as compared to Ribandar salt pan.

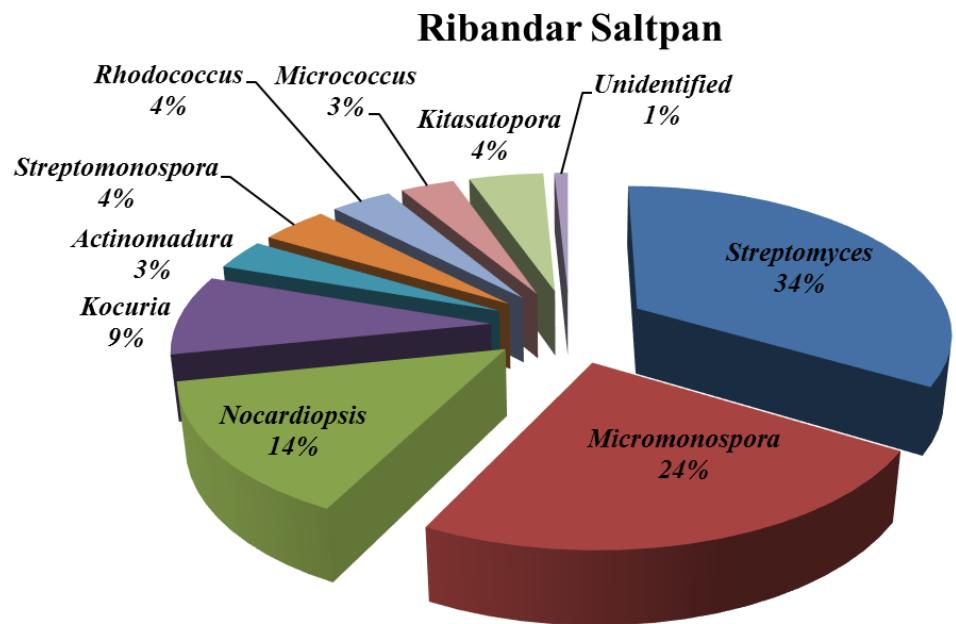


Fig. 51 Pie chart showing percentage of 9 different genera obtained from Ribandar salt pan

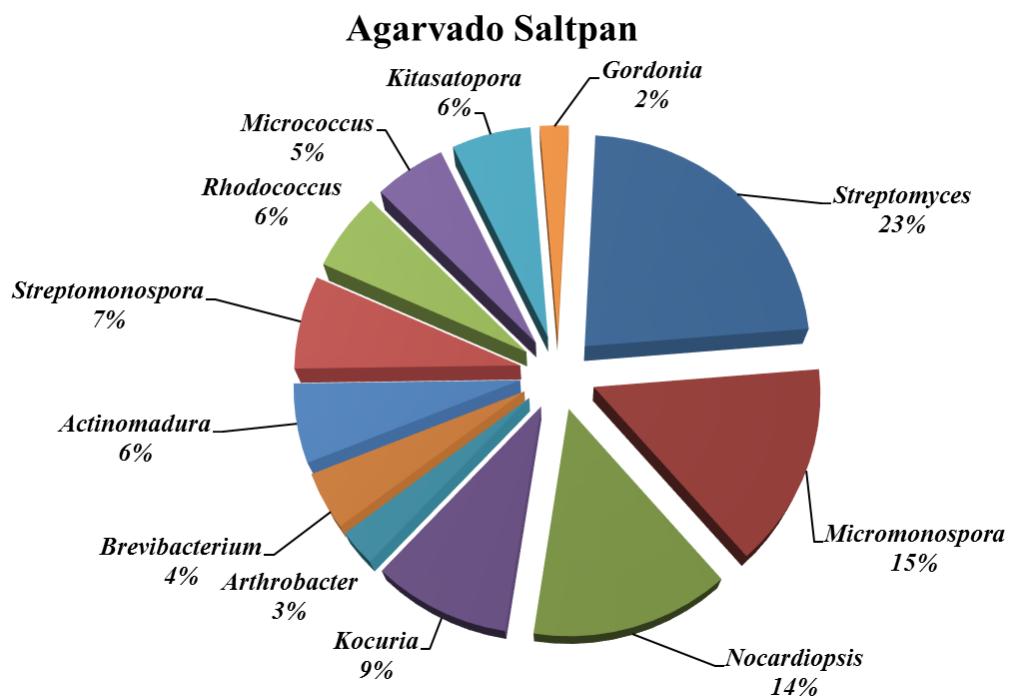


Fig. 52 Pie chart showing percentage of 12 different genera obtained from Agarvado salt pan

However, the actinomycete population was found to be significantly affected by the seasonal variation. Diversity indexes were calculated to represent the species diversity in both the salt pans during pre and post-monsoon seasons.

4. 1. 13. Diversity indices of actinomycete species of Ribandar and Agarvado salt pans:

It is evident from different diversity indices that the actinomycete species diversity was highly abundant in both Ribandar and Agarvado salt pans during the post-monsoon season ($H = 1.454, 1.448$ and $1/D = 28.455, 27.972$) relative to pre-monsoon ($H = 1.313, 1.292$ and $1/D = 22.05, 21.541$) (Table. 17 and 19). The evenness of the community structure was higher in post-monsoon season as compared to pre-monsoon in SHE value analysis (Table. 18 and 20). Descriptive statistics values confirms the same (Table. 21 and 22).

Table. 17 Shannon and Simpson's diversity index values for species richness in both pre and post-monsoon seasons for Ribandar salt pan

Diversity indices	Pre-monsoon	Post-monsoon
Shannon H' Log Base 10	1.313	1.454
Shannon Hmax Log Base 10	1.38	1.544
Shannon J'	0.952	0.942
Simpsons Diversity (D)	0.045	0.035
Simpson Diversity (1/D)	22.05	28.455

Table. 18 SHE analysis values of actinomycetes species diversity for both pre and post-monsoon seasons of Ribandar salt pan

Seasons	N	S	LnS	H	LnE	LnE/LnS
Pre-monsoon	99	24	3.18	3.02	-0.15	-0.05
Post-monsoon	250	36	3.58	3.3	-0.29	-0.08

Table. 19 Shannon and Simpson's diversity index values for species richness both pre and post-monsoon seasons for Agarvado salt pan

Diversity indices	Pre-monsoon	Post-monsoon
Shannon H' Log Base 10	1.292	1.448
Shannon Hmax Log Base 10	1.362	1.531
Shannon J'	0.949	0.945
Simpsons Diversity (D)	0.046	0.036
Simpson Diversity (1/D)	21.541	27.972

Table. 20 SHE analysis values of actinomycetes species diversity for both pre and post-monsoon seasons of Agarvado salt pan

Seasons	N	S	LnS	H	LnE	LnE/LnS
Pre-monsoon	73	23	3.14	2.97	-0.16	-0.05
Post-monsoon	192	34	3.53	3.29	-0.24	-0.07

Table. 21 Descriptive statistics depicting the species diversity of actinomycetes at Ribandar salt pan

Seasons	Mean Individuals	Variance	Standard Deviation	Standard Error	Total Individuals	Total Species	Minimum	Maximum	Mean Confidence Interval
Pre-monsoon	2.75	7.621	2.761	0.46	99	24	0	10	2.49
Post-monsoon	4.194	8.961	2.994	0.499	151	35	0	14	2.927

Table. 22 Descriptive statistics depicting the species diversity of actinomycetes at Agarvado salt pan

Seasons	Mean Individuals	Variance	Standard Deviation	Standard Error	Total Individuals	Total Species	Minimum	Maximum	Mean Confidence Interval
Pre-monsoon	2.147	4.857	2.204	0.378	73	23	0	10	1.633
Post-monsoon	3.5	6.197	2.489	0.427	119	34	1	14	2.083

4. 2. BIOPROSPECTING

4. 2. 1. Primary screening of actinomycete isolates for anti-microbial activity:

In the primary screening using cell free extracellular secondary metabolites (Fig. 53 and 54), among the 500 isolates, 132 showed anti-microbial activity against various pathogens tested. 71% isolates showed production of antibacterial, 20% showed anti-fungal and 9% showed both antibacterial and antifungal metabolites. Among 132 isolates, 55 isolates were *Streptomyces* spp. Other 77 isolates were rare actinomycetes, constituted of 10 different genera viz. *Actinomadura* (2), *Nocardiopsis* (15), *Brevibacterium* (2), *Gordonia* (1), *Kocuria* (13), *Kitasatospora* (5), *Micrococcus* (4), *Micromonospora* (30), *Rhodococcus* (3), and *Streptomonospora* (2) (Fig. 55 and 56). Among the rare actinomycete isolates, genera *Micromonospora* and *Nocardiopsis* showed the maximum antagonistic activity whereas obligate halophiles *Streptomonospora* and *Actinomadura* produced lesser anti-microbial metabolites.

4. 2. 2. Secondary screening and selection of the potential isolates:

Among of the positive isolates from the primary screening, 10 isolates belonging to 5 different genera viz. *Streptomyces*, *Micromonospora*, *Nocardiopsis*, *Kocuria* and *Kitasatospora* showed consistent and maximum activity against the test pathogens.

Table. 22. A shows the antibiotic resistance and susceptibility profile of the bacterial pathogens tested. Table. 23 summarizes the activity profile of these isolates against various clinical and agricultural pathogens. These isolates produced maximum amount of anti-microbial metabolites after 2 weeks of incubation and activity remained constant in multiple replicates, hence chosen for further studies.



Fig. 53 Cell free metabolites of various isolates showing anti-bacterial activity against A. *Escherichia coli*, B. *Proteus mirabilis* and C. *Salmonella typhimurium*



Fig. 54 Anti-fungal activity of the cell free metabolites of various isolates against A. *Aspergillus niger*, B. *Penicillium* sp., C. *Curvularia lunata*

Majority of the *Streptomyces* spp. (both halotolerant and halophilic) viz. SBSK-3, SBSK-8 and SBSK-57 showed anti-bacterial activity towards Gram negative bacteria. Rare actinomycete isolates viz. SBSK-67, SBSK-120 (*Kocuria* spp.), SBSK-115 (*Nocardiopsis* sp.) and SBSK-356 (*Micromonospora* sp.) were showed specific anti-bacterial activity towards a single bacterial pathogen each. One *Micromonospora* sp. from the Agarvado salt pan, strain SBSK-364 showed a significant broad spectrum of anti-bacterial activity against 8 Gram positive and Gram negative bacterial pathogens viz. *Staphylococcus aureus*, *Staphylococcus citreus*, *Aeromonas hydrophila*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhi*, *Salmonella typhimurium* and *Vibrio cholera*. Three selected halotolerant isolates viz. SBSK-200 (*Streptomyces* sp.), SBSK-376

(*Kitasatospora* sp.) and SBSK-527 (*Micrococcus* sp.) showed promising anti-fungal activity against either two or three pathogenic fungi. Isolate SBSK-364 (*Micromonospora* sp.) showed a broad range anti-fungal activity against four phyto and human pathogenic fungi viz. *Aspergillus niger*, *Aspergillus fumigatus*, *Curvularia lunata* and *Penicillium* sp.

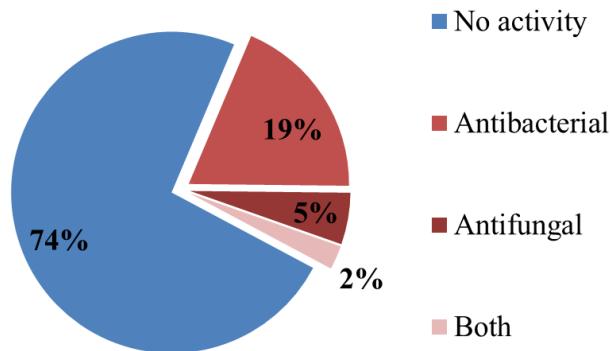


Fig. 55 Graph showing the percentage of isolates showing anti-microbial activity

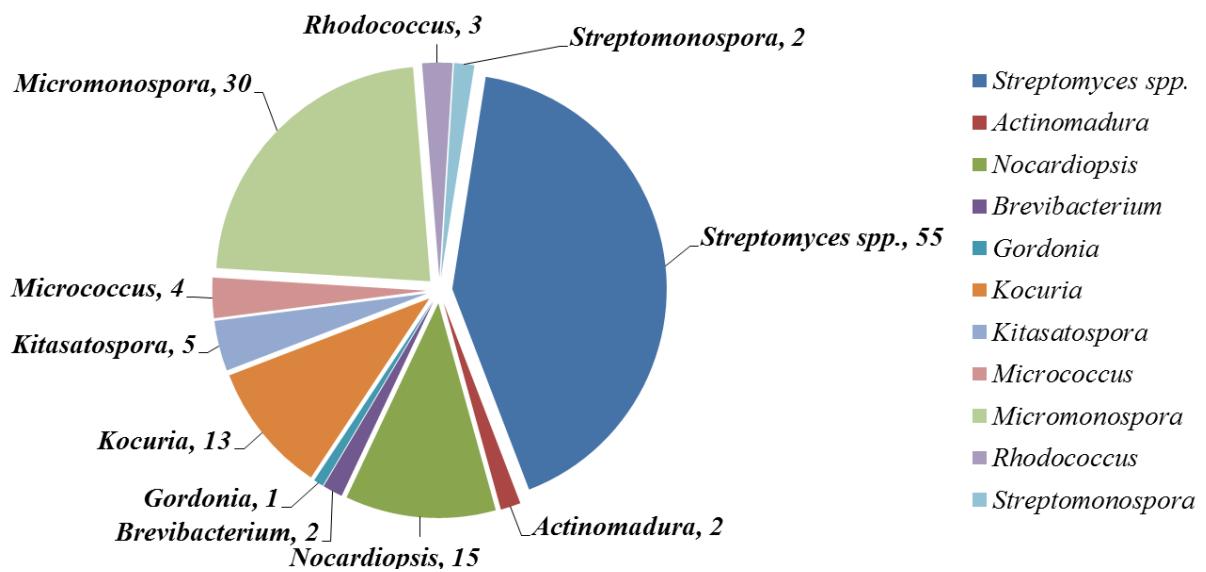


Fig. 56 Graph showing the number of positive isolates from various genera both *Streptomycete* and rare actinomycete genera

4. 2. BIOPROSPECTING

4. 2. 1. Primary screening of actinomycete isolates for anti-microbial activity:

In the primary screening using cell free extracellular secondary metabolites (Fig. 53 and 54), among the 500 isolates, 132 showed anti-microbial activity against various pathogens tested. 71% isolates showed production of antibacterial, 20% showed anti-fungal and 9% showed both antibacterial and antifungal metabolites. Among 132 isolates, 55 isolates were *Streptomyces* spp. Other 77 isolates were rare actinomycetes, constituted of 10 different genera viz. *Actinomadura* (2), *Nocardiopsis* (15), *Brevibacterium* (2), *Gordonia* (1), *Kocuria* (13), *Kitasatospora* (5), *Micrococcus* (4), *Micromonospora* (30), *Rhodococcus* (3), and *Streptomonospora* (2) (Fig. 55 and 56). Among the rare actinomycete isolates, genera *Micromonospora* and *Nocardiopsis* showed the maximum antagonistic activity whereas obligate halophiles *Streptomonospora* and *Actinomadura* produced lesser anti-microbial metabolites.

4. 2. 2. Secondary screening and selection of the potential isolates:

Among of the positive isolates from the primary screening, 10 isolates belonging to 5 different genera viz. *Streptomyces*, *Micromonospora*, *Nocardiopsis*, *Kocuria* and *Kitasatospora* showed consistent and maximum activity against the test pathogens.

Table. 22. A shows the antibiotic resistance and susceptibility profile of the bacterial pathogens tested. Table. 23 summarizes the activity profile of these isolates against various clinical and agricultural pathogens. These isolates produced maximum amount of anti-microbial metabolites after 2 weeks of incubation and activity remained constant in multiple replicates, hence chosen for further studies.



Fig. 53 Cell free metabolites of various isolates showing anti-bacterial activity against A. *Escherichia coli*, B. *Proteus mirabilis* and C. *Salmonella typhimurium*



Fig. 54 Anti-fungal activity of the cell free metabolites of various isolates against A. *Aspergillus niger*, B. *Penicillium* sp., C. *Curvularia lunata*

Majority of the *Streptomyces* spp. (both halotolerant and halophilic) viz. SBSK-3, SBSK-8 and SBSK-57 showed anti-bacterial activity towards Gram negative bacteria. Rare actinomycete isolates viz. SBSK-67, SBSK-120 (*Kocuria* spp.), SBSK-115 (*Nocardiopsis* sp.) and SBSK-356 (*Micromonospora* sp.) were showed specific anti-bacterial activity towards a single bacterial pathogen each. One *Micromonospora* sp. from the Agarvado salt pan, strain SBSK-364 showed a significant broad spectrum of anti-bacterial activity against 8 Gram positive and Gram negative bacterial pathogens viz. *Staphylococcus aureus*, *Staphylococcus citreus*, *Aeromonas hydrophila*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhi*, *Salmonella typhimurium* and *Vibrio cholera*. Three selected halotolerant isolates viz. SBSK-200 (*Streptomyces* sp.), SBSK-376

Table 23: Selected halophilic and halotolerant isolates showing anti-microbial activity against clinical and agricultural pathogens

Sr. No.	Isolate codes	Genera	Isolation source	Nature	Name of the pathogens
1.	SBSK-3	<i>Streptomyces</i>	Ribandar salt pan	Halotolerant	<i>Escherichia coli, Proteus vulgaris, Salmonella typhi & Salmonella typhimurium</i>
2.	SBSK-8	<i>Streptomyces</i>	Ribandar salt pan	Halotolerant	-do-
3.	SBSK-57	<i>Streptomyces</i>	Ribandar salt pan	Halophilic	-do-
4.	SBSK-67	<i>Kocuria</i>	Ribandar salt pan	Halotolerant	<i>Staphylococcus aureus</i>
5.	SBSK-115	<i>Nocardiopsis</i>	Ribandar salt pan	Halophilic	<i>Staphylococcus citreus</i>
6.	SBSK-120	<i>Kocuria</i>	Ribandar salt pan	Halophilic	<i>Staphylococcus aureus</i>
7.	SBSK-356	<i>Micromonospora</i>	Ribandar salt pan	Halotolerant	<i>Vibrio cholera</i>
8.	SBSK-200	<i>Streptomyces</i>	Ribandar saltern	Halotolerant	<i>Aspergillus niger, Curvularia lunata & Penicillium sp.</i>
9.	SBSK-364	<i>Micromonospora</i>	Agarvado salt pan	Halotolerant	<i>Escherichia coli, Proteus vulgaris, Vibrio cholera, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Staphylococcus citreus, Aeromonas hydrophila, Aspergillus niger, Aspergillus fumigatus, Curvularia lunata & Penicillium sp.</i>
10	SBSK-285	<i>Streptomyces</i> sp.	Ribandar salt pan	Halotolerant	<i>Curvularia lunata & Penicillium sp.</i>
11.	SBSK-376	<i>Kitasatospora</i>	Agarvado salt pan	Halotolerant	<i>Curvularia lunata & Penicillium sp.</i>
12.	SBSK-527	<i>Micrococcus</i>	Agarvado salt pan	Halotolerant	<i>Aspergillus niger, Curvularia lunata & Penicillium sp.</i>

(*Kitasatospora* sp.) and SBSK-527 (*Micrococcus* sp.) showed promising anti-fungal activity against either two or three pathogenic fungi. Isolate SBSK-364 (*Micromonospora* sp.) showed a broad range anti-fungal activity against four phyto and human pathogenic fungi viz. *Aspergillus niger*, *Aspergillus fumigatus*, *Curvularia lunata* and *Penicillium* sp.

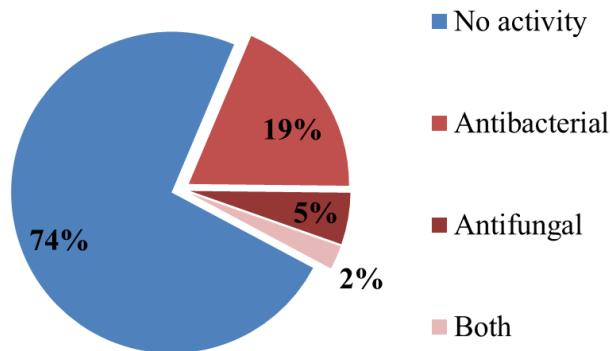


Fig. 55 Graph showing the percentage of isolates showing anti-microbial activity

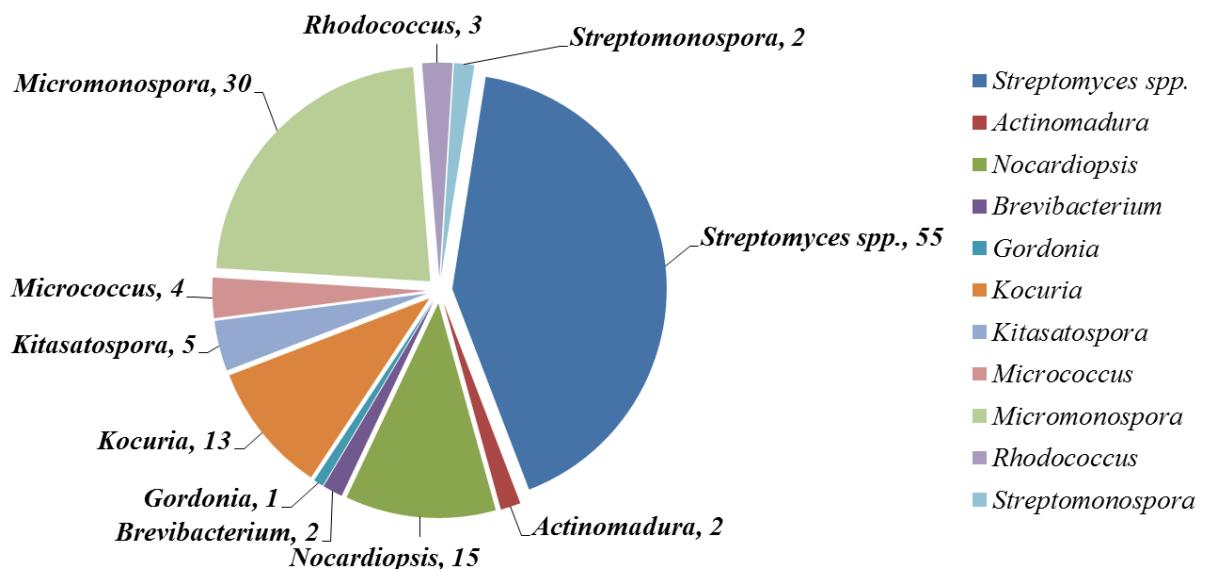


Fig. 56 Graph showing the number of positive isolates from various genera both *Streptomycete* and rare actinomycete genera

4. 2. 3. Effect of salinity on the production of anti-microbial compound:

Salinity was found to have a profound effect on the production of anti-microbial compounds. Assessment on 8 selected isolates from Ribandar salt pan at increasing levels (0-100 psu) of salinities, showed significant variation production of antibiotic (Fig. 57 and Fig. 58). The production of anti-bacterial compounds in halotolerant isolates (SBSK-3, SBSK-8, SBSK-67, SBSK-150 and SBSK-356) varied at different salinities, maximum production was at 35 psu. Increase in the production of anti-bacterial metabolites at 35 psu in five halotolerant isolates viz. three *Streptomyces* sp. (SBSK-3, SBSK-8 & SBSK-150) and one *Micromonospora* sp. (SBSK-356) and one *Kocuria* sp. (SBSK-67) can be seen in (Fig. 57). Maximum production of antibiotic in halophilic isolates viz SBSK-57 (*Streptomyces* sp.), SBSK-115 (*Nocardiopsis* sp.) and SBSK-120 (*Kocuria* sp.) was also observed at 35 psu however SBSK-120 and SBSK-57 produced almost half of the antibiotic at 20 psu and no production below 20 psu. SBSK-115 did not show any antibiotic production at salinities below 35 psu but produced the metabolite up to 50 psu. SBSK-120 & SBSK-57 similarly produced the antibacterial compound up to 75 psu (Fig. 58). It was found that the halotolerant *Streptomyces* sp. strain SBSK-8 and *Micromonospora* sp. strain SBSK-356 produced comparatively higher amount of antibacterial compound in presence of sea water (35 psu). At 35 psu and above, halophilic *Nocardiopsis* sp. strain SBSK-115 and *Kocuria* sp. strain SBSK-120, was consistent in the production of anti-bacterial metabolite and which was produced during the stationary phase. Thus in case of both halotolerant and halophilic isolates, 35 psu (sea water salinity) was found to be a consistent factor to trigger the production of anti-microbial compound.

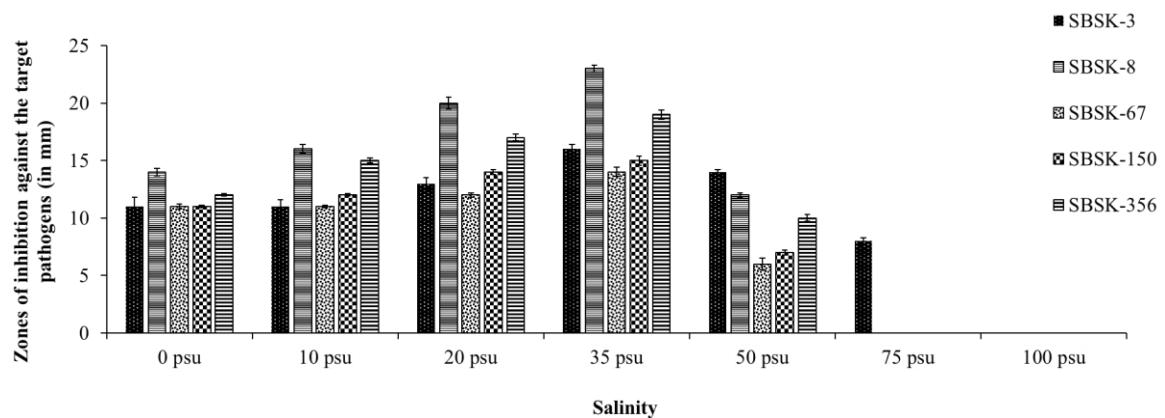


Fig. 57 Antibiotic production of 5 selected halotolerant actinomycetes at various salinities against test pathogens

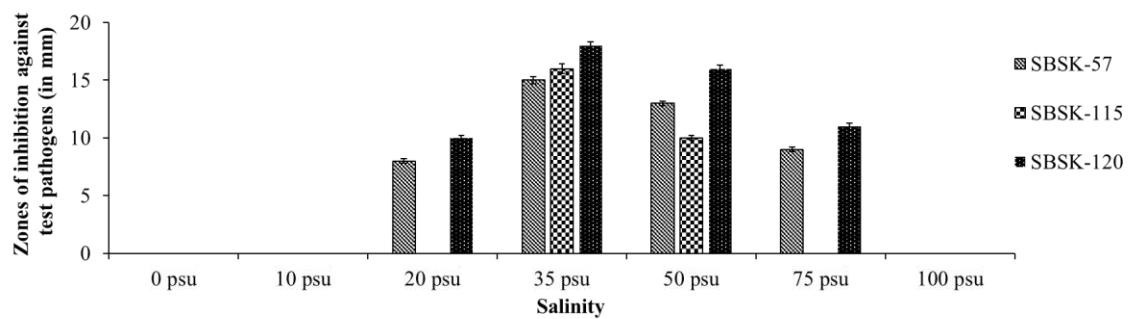


Fig. 58 Antibiotic production of 3 selected halophilic actinomycetes at various salinities against test pathogens

4. 2. 4. Characterization of potential strains:**4. 2. 4. a. Microscopy:**

Eight selected potential strains were identified by microscopy to belong to four different genera viz. *Kocuria*, *Micromonospora*, *Nocardiopsis* and *Streptomyces*. Fig. 59 shows their morphological architecture in SEM analysis. Four isolates SBSK-3, SBSK-8, SBSK-57 and SBSK-150 showed long spirally coiled chains of spores; filamentous substrate mycelia and aerial mycelia as oval shaped spores as chains and free spores; rod shaped spores arranged in a chain & branched substrate mycelia; round and smooth abundant spores with segmented substrate mycelia respectively, which are the typical features of various strains of genus *Streptomyces* hence identified as *Streptomyces* spp. Isolate SBSK-67 and SBSK-120 showed coccoid cells which are in pairs or clusters, each cell is of 1-1.5 μm in diameter and non-spore forming; suggesting them to belong to genus *Kocuria*. Strain SBSK-115 was found to bear aerial mycelia as spherical smooth rod shaped spores; characteristic of genus *Nocardiopsis*. Strain SBSK-356 had extensively branched, non-fragmented hyphae along with single, non-motile and smooth spores; typical characters of genus *Micromonospora*.

4. 2. 4. b. Molecular characterization of the selected isolates of Ribandar saltern:

Molecular characterization through 16S rRNA gene sequencing and phylogenetic analysis revealed the identity and relation with the counter parts. The taxonomic position of the 8 potential isolates of Ribandar salt pan with their closest relatives in the phylogenetic tree and *Bacillus subtilis* strain BCRC 10058 as an out group is shown in Fig. 60.

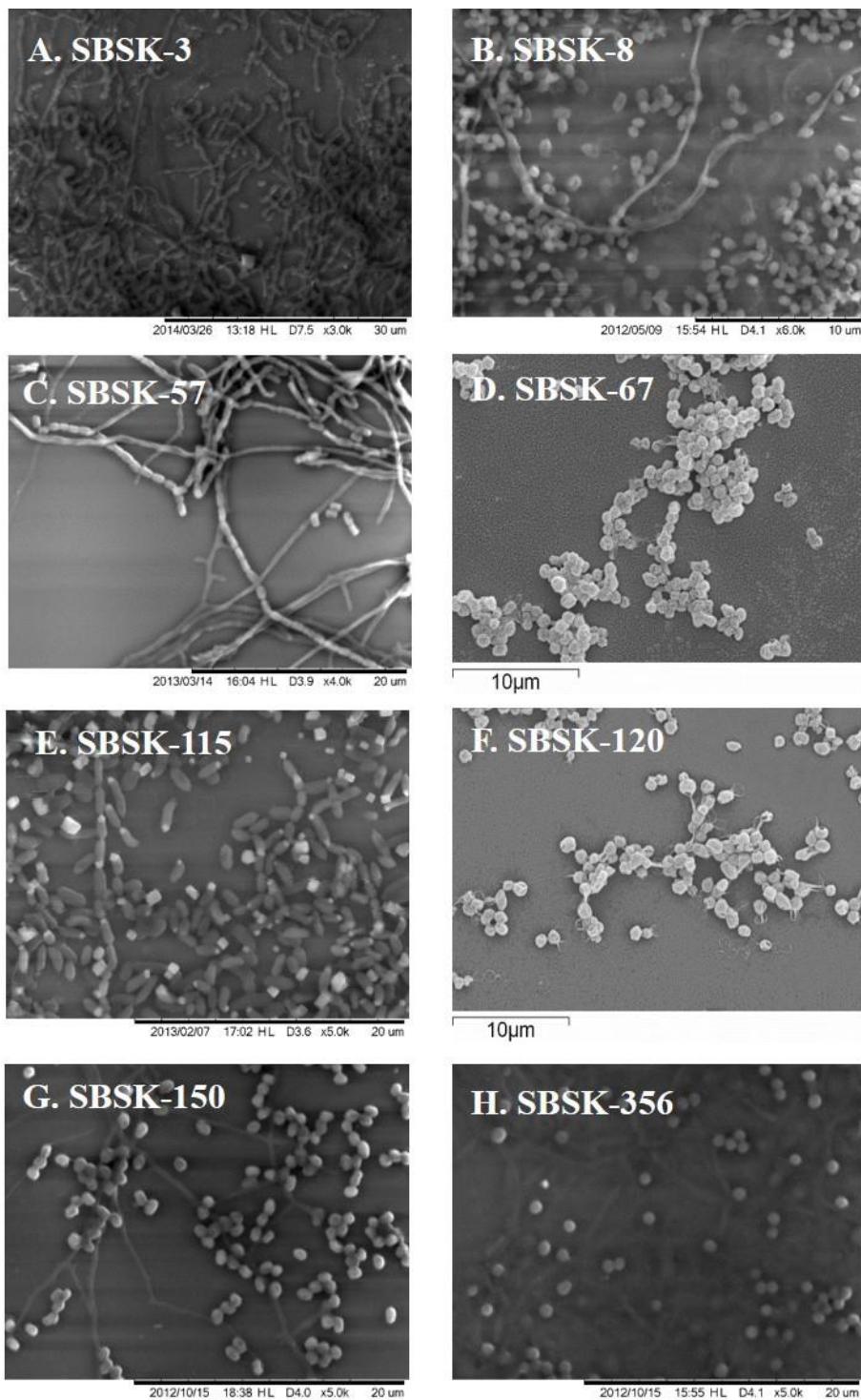


Fig. 59 SEM images: A. SBSK-3, B. SBSK-8, C. SBSK-57 and G. SBSK-150 being *Streptomyces* spp. D. SBSK-67 (*Kocuria* sp.) E. SBSK-115 (*Nocardiopsis* sp.) F. SBSK-120 (*Kocuria* sp.) H. SBSK-356 (*Micromonospora* sp.)

Halotolerant *Kocuria palustris*, strain SBSK-67 showed 99% similarity with a) *Kocuria palustris* strain S62, isolated from a marine sponge of South China Sea and b) *Kocuria palustris* sp. nov. strain TAGA27 which was isolated from a rhizoplane of the narrow-leaved cattail (*Typha angustifolia*) [Kovács *et al.*, 1999]. Halophilic *Kocuria* sp., strain SBSK-120 showed 97% similarity and clustered with *Kocuria halotolerance* sp. nov. strain YIM 90716 isolated from saline soil of Xinjiang Province, China [Tang *et al.*, 2009] and *Kocuria* sp. strain WJ15 isolated from infected citrus. Halophilic *Nocardiopsis* sp strain SBSK-115 had a similarity index of 98% with *Nocardiopsis halotolerans* sp. nov. (DSM 44410) isolated from salt marsh soil of Kuwait [Al-Zarban *et al.*, 2002] and with *Nocardiopsis* spp. (CNP-966_SD01 and CNR923 PL04) isolated from the marine sediment of California coast.

Halotolerant *Micromonospora* sp., strain SBSK-356 clustered with multiple strains of *Micromonospora* spp. (201101, 162225, CGMC 4.1278) isolated from the marine sediments of China and *Micromonospora coxensis* sp. nov. from the sandy soil of Bangladesh [Ara *et al.*, 2007] with a maximum similarity index of 98%. Halotolerant *Streptomyces radiopugnans* strain SBSK-3, showed maximum similarity (99%) and clustered with *Streptomyces radiopugnans* (HBUM174999 and HBUM174201) isolated from China. Halotolerant *Streptomyces* sp., SBSK-8 showed 99% similarity with *Streptomyces* sp. A391 Ydz-Qz isolated from the Yellow Sea, China and with *Streptomyces* sp. WBF11 which was isolated sea bed of Wenhai, China whereas halophilic *Streptomyces* sp. SBSK-57 showed 99% similarity to *Streptomyces* sp. ZZY-2013 strain TRM46710.1 isolated from a salty beach of Tarim Basin, China. Halotolerant *Streptomyces sporocinereus* SBSK-150 showed 99% similarity with type strain *Streptomyces sporocinereus* (BCRC 100766) and clustered together.

4. 2. 5. Assessment of sequential solvent extracted anti-microbial compound(s):

The activity of the concentrated solvent extracts of 5 selected isolates of Ribandar saltern viz. SBSK-8 (*Streptomyces* sp.), SBSK-115 (*Nocardiopsis* sp.), SBSK-120 (*Kocuria* sp.) and SBSK-356 (*Micromonospora* sp.) in six solvent systems is shown in Table 24. These metabolites being extracellularly produced can be easily extracted using organic solvents and the compound(s) can be concentrated by evaporation in their active forms. Different actinomycetes isolates showed a variation in the activity profile with respect to the organic solvent used for extraction of anti-bacterial compounds which suggests that these potential metabolites produced, have inter-generic variations in their chemical signatures. The concentrated residue of ethyl acetate extract of isolate *Streptomyces* sp. (SBSK-8) showed highest activity against three Gram negative bacteria viz. *Salmonella typhimurium*, *Escherichia coli* and *Proteus vulgaris* with zones of inhibition 31 mm, 32 mm and 29 mm respectively. Chloroform extract of *Micromonospora* sp (SBSK-356) against *Vibrio cholera* exhibited a zone of inhibition of 35 mm (Fig. 61) suggesting the two compounds to be peptides, fatty acids or amino acids. The concentrated butanol extract of *Nocardiopsis* sp. (SBSK-115) gave 35 mm zone of inhibition against *Staphylococcus citreus*, suggesting that the active metabolite is either a fatty acid or a peptide whereas butanol extracts of *Streptomyces* sp. (SBSK-8) showed with three Gram negative bacteria. The concentrated residue of hexane extract of the culture supernatant of isolate SBSK-120 (*Kocuria* sp.) has shown the highest activity against *Staphylococcus aureus* (28 mm) and suggests the active metabolite could be an ester of fatty acid. The ethyl acetate extract of SBSK-285 showed maximum 20 mm and 18 mm

Table. 24 Activity profile of partially purified (sequential solvent extraction) anti-microbial metabolites of the selected isolates:

Sr. No.	Culture code	Genera	Solvents	Test pathogens (Bacteria and fungi)	Respective inhibition zone* (in mm)
1.	SBSK-8	<i>Streptomyces</i> sp.	Di-ethyl ether	<i>Salmonella typhimurium</i>	18 ± 1.15
				<i>Proteus vulgaris</i>	16 ± 0.62
				<i>Escherichia coli</i>	18 ± 0.59
			Ethyl acetate	<i>Salmonella typhimurium</i>	31 ± 0.58
				<i>Proteus vulgaris</i>	29 ± 1.56
				<i>Escherichia coli</i>	28 ± 1.12
			Butanol	<i>Salmonella typhimurium</i>	19 ± 0.58
				<i>Proteus vulgaris</i>	20 ± 1.13
				<i>Escherichia coli</i>	17 ± 0.72
2.	SBSK-115	<i>Nocardiopsis</i> sp.	Di-ethyl ether	<i>Staphylococcus citreus</i>	16 ± 0.58
			Ethyl acetate	<i>Staphylococcus citreus</i>	20 ± 1.73
			Butanol	<i>Staphylococcus citreus</i>	35 ± 1.15
3.	SBSK-120	<i>Kocuria</i> sp.	Petroleum ether	<i>Staphylococcus aureus</i>	14 ± 1.53
			Hexane	<i>Staphylococcus aureus</i>	28 ± 1.0
			Chloroform	<i>Staphylococcus aureus</i>	11 ± 1.16
4.	SBSK-356	<i>Micromonospora</i> sp.	Petroleum ether	<i>Vibrio cholerae</i>	17 ± 1.53
			Chloroform	<i>Vibrio cholerae</i>	34 ± 2.3
5.	SBSK-285	<i>Streptomyces</i> sp.	Ethyl acetate	<i>Curvularia lunata</i>	20 ± 1.00
				<i>Penicillium</i> sp.	18 ± 0.50

*Mean data ± SD; the disc size was 5 mm; maximum inhibition zones: bold.

zones of inhibition against *Curvularia lunata* and *Penicillium* sp. respectively. However, the active metabolite(s) from the culture supernatants of the respective isolates were not very specific to the organic solvents. Fig. 62 shows the activity of the solvent extracted concentrated residue of isolate SBSK-8, the active metabolite component was found three different solvents viz. butanol, Di-ethyl ether and ethyl acetate with 14 mm, 20 mm and 24 mm zones of inhibition respectively, suggesting multiple metabolites contributing for the anti-microbial activity.



Fig. 61 Activity of concentrated anti-bacterial metabolites extracted from the culture supernatant in their respective organic solvents against clinically significant pathogenic bacteria: A. Chloroform extract from isolate SBSK-356 against *Vibrio cholerae*, B. Butanol extract from isolate SBSK-115 against *Staphylococcus citreus*, C. Hexane extract of SBSK-120 against *Staphylococcus aureus*, D. Ethyl acetate extract from isolate SBSK-8 against *Salomonella typhimurium*

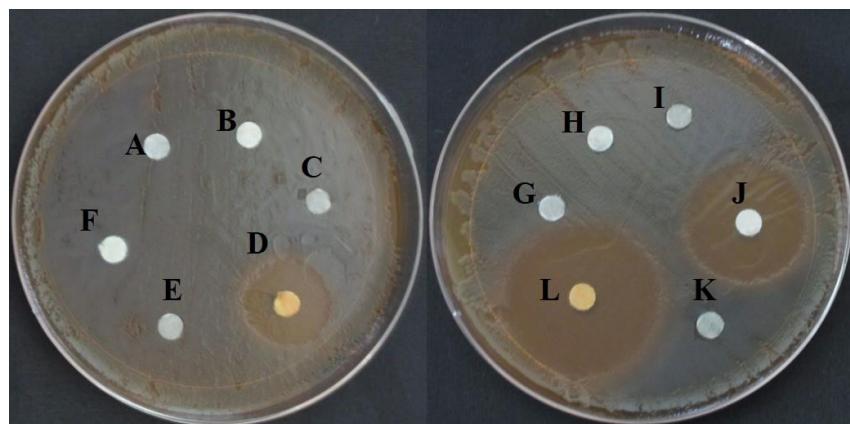


Fig. 62 Activity profile of concentrated anti-bacterial metabolites of isolate SBSK-8 against *Salmonella typhimurium*, A & B. Hexane (control & extract), C & D. Butanol (control & extract), E & F. Petroleum ether (control & extract), G & H. Chloroform (control & extract), I & J. Di-ethyl ether (control & extract) and K & L. Ethyl acetate (control & extract)

These observations of halophilic and halotolerant actinomycetes inhibiting multiple pathogens and the partial purification of anti-bacterial metabolites, have been published as a full length experimental paper in Journal of Bioscience and Bioengineering. The paper highlighted on *Streptomyces* as well as rare actinomycetes viz. *Micromonospora*, *Nocardiopsis* and *Kocuria*, as producers of antibiotics.



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Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites

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Marine salterns are estuarine ecosystems in Goa, receiving inputs from riverine and marine waters. The Salinity fluctuates between 0 and 300 psu which makes it a conducive niche for salt tolerant and salt loving *Actinomycetales*. Halotolerant and halophilic *Actinomycetales* producing anti-bacterial metabolites were studied from crystallizer pond sediments of Ribandar saltern, Goa. Three media viz. Starch casein, R2A and Inorganic salt starch agar at four different salinities (35, 50, 75 and 100 psu) were used for isolation. R2A agar at 35 psu was the most preferred by hypersaline actinomycetes. The dominant group was halotolerant *Streptomyces* spp. others being rare actinomycetes viz. *Nocardiopsis*, *Micromonospora* and *Kocuria* spp. More than 50% of the isolates showed anti-bacterial activity against one or more of the fifteen human pathogens tested. Eight strains from 4 genera showed consistent anti-bacterial activity and

4. 2. 6. Production of anti-microbial metabolite by strain SBSK-364 from Agarvado salt pan:

Isolate SBSK-364 from the Agarvado salt pan was selected for further characterization and anti-microbial compound extraction, for its broad spectrum of anti-microbial activity and also to obtain a specific a ‘single anti-microbial metabolite’ component and its structural elucidation. Cell free metabolite preparation from the culture broth of the strain SBSK-364 was found to inhibit the growth of 7 bacterial and 5 fungal pathogens. Table. 23 summarizes the activity profile assessed by disc diffusion assay.

4. 2. 7. Taxonomic characterization of the strain SBSK-364:

Colonies of strain SBSK-364 appeared orange in colour on the isolation media (R2A agar at 50 psu salinity) (Fig. 63) and on maturation it turned dark orange to brownish. It showed growth only in selective media viz. R2A agar, Inorganic Salt starch agar (ISP-4), Yeast extract-malt extract agar (ISP-2) and Tyrosine agar (ISP-7). Substrate mycelia was prominent but no aerial mycelia with was observed. Light microscopy and SEM analysis branched substrate mycelia bearing single spores. Spores were spherical, approx. 900 nm to 1 μm in size and smooth on the surface (Fig. 63). Morphological architecture and SEM analysis suggested the isolate to belong to genus *Micromonospora* sp. Molecular characterization by 16S rRNA gene sequencing revealed the isolate to be *Micromonospora marina* (GenBank accession ID: KU183028). The isolate showed maximum similarity (99%) with *Micromonospora marina* strain JSM1-1 and JSM1-3. Fig. 64 shows the taxonomic relationship of the strain SBSK-364 with other type strains and various isolates of the genus *Micromonospora*.

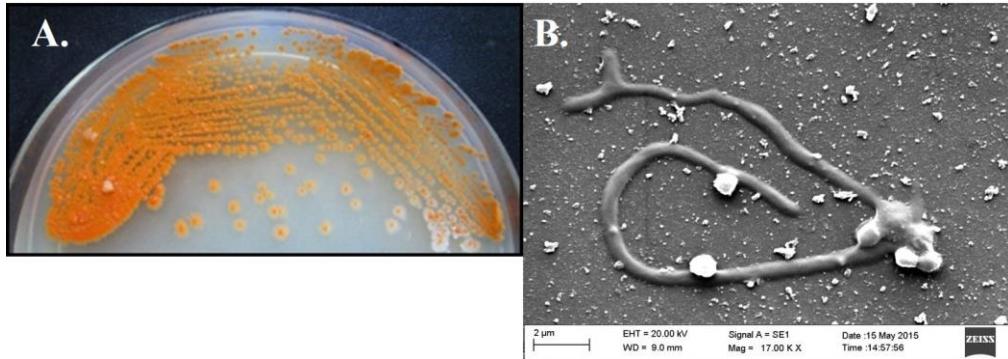


Fig. 63 A. Strain SBSK-364 growing on R2A agar supplemented with sea water, B. SEM analysis of the isolate SBSK-364, showing branched substrate mycelia and single spores appearing from the same, suggests the typical characteristic of genus *Micromonospora*

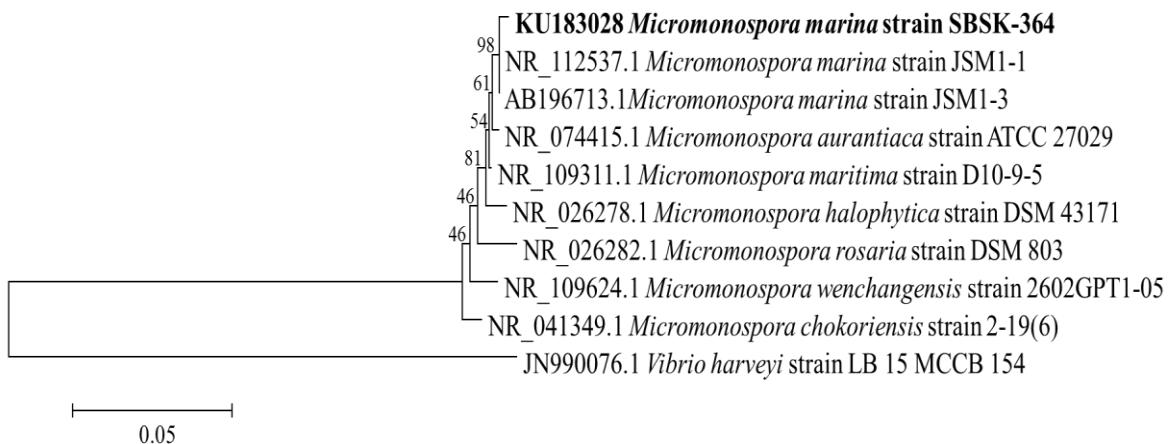


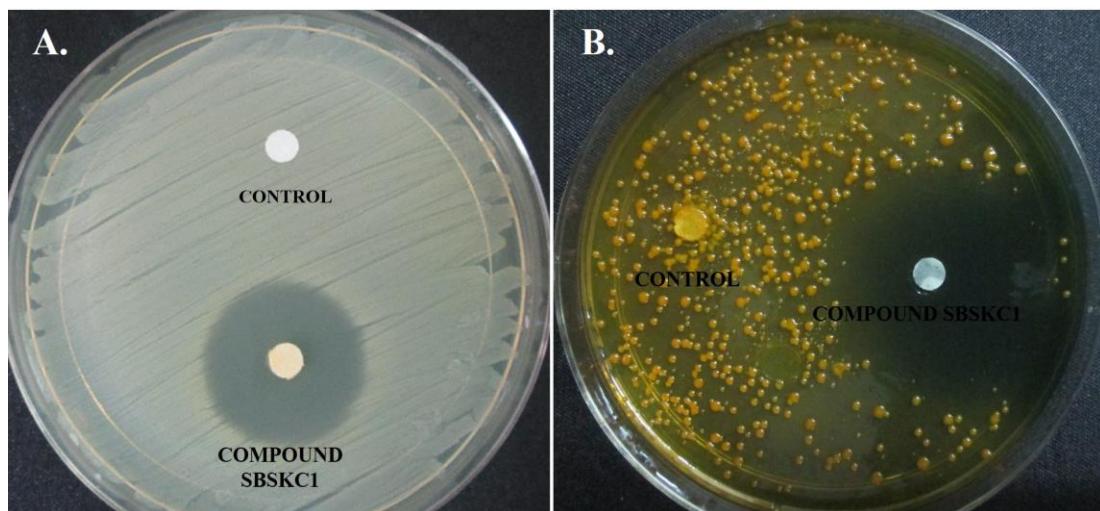
Fig. 64 Phylogenetic tree constructed by showing the taxonomic relationship of candidate strain SBSK-364 with other type strains of the same genus; *Vibrio harveyi* strain LB 15 MCCB 154 is an out group

4. 2. 8. Characterization of the anti-microbial compound:

Sequential solvent extraction and bioassay of the culture broth of SBSK-364 didn't show any activity. Compound "SBSKC1" was extracted using Dia-ion resin and methanol, followed by concentration by using rotary vapor evaporator; Table. 25 shows the activity

Table. 25: Activity profile of the cell free metabolite of strain SBSK-364 and Compound 'SBSKC1'

Sr. No.	Test pathogens (Bacteria and fungi)	Zone of inhibition (in mm)	
		Cell free metabolite	SBSKC1 (2 mg/ml)
1.	<i>Salmonella typhimurium</i>	15 ± 0.58	25 ± 2.64
2.	<i>Salmonella typhi</i>	15 ± 1.27	24 ± 1.39
3.	<i>Staphylococcus aureus</i>	18 ± 1.74	28 ± 1.00
4.	<i>Staphylococcus citreus</i>	17 ± 1.42	26 ± 1.58
5.	<i>Escherichia coli</i>	14 ± 1.73	22 ± 1.53
6.	<i>Proteus vulgaris</i>	15 ± 1.00	25 ± 0.65
7.	<i>Vibrio cholerae</i>	14 ± 1.42	25 ± 0.58
8.	<i>Aspergillus niger</i>	16 ± 0.67	26 ± 1.42
9.	<i>Curvularia lunata</i>	16 ± 1.53	29 ± 0.67
10.	<i>Penicillium sp.</i>	15 ± 0.58	28 ± 0.65
11.	<i>Aspergillus fumigatus</i>	14 ± 1.74	26 ± 2.42
12.	<i>Rhizopus stolonifer</i>	12 ± 1.00	25 ± 1.62

**Fig. 65** Compound SBSKC1 showing zones of inhibition against A. *Staphylococcus aureus*, B. *Vibrio Cholera*

profile. It was found to be soluble only in methanol and water hence the compound didn't get extracted in solvent extraction. SBSKC1 gave positive reaction to Ninhydrin in TLC and FTIR data confirmed the presence of peptide groups. The compound was found to be stable up to 70°C and pH 4-9.

4. 2. 9. TLC and FTIR analysis of 'SBSKC1':

Compound 'SBSKC1' initially showed a single spot when TLC was done with Methanol: Water (1:1) as mobile phase but with mobile phase; Butanol: Water: Acetic acid, TLC showed the presence of two major components as two prominent spots (Fig. 66). All the three spots positive reaction with ninhydrine suggesting the compound to be 'peptide in nature. However, they gave negative reaction with Rhodamine B, which suggests the absence of the lipid groups. The FTIR spectra recorded in KBr of the compound 'SBSKC1' confirmed the presence of peptide groups (Fig. 67). The peak at 3277 cm⁻¹ indicated the presence of a -OH/-NH group. The signal at 2970 cm⁻¹ was attributed to the CH stretching of alkyl (-R) group. The peak at 1658 cm⁻¹ attributed to the carbonyl moiety of amide (CO-NH₂) group. This signal indicates the presence of peptide group and the other signal at 1138 cm⁻¹ is due to the 'C-O' stretch.

4. 2. 10. Minimum Inhibitory Concentration (MIC) of compound SBSKC1:

Compound SBSKC1 was found to inhibit both Gram positive and Gram negative bacteria. The MIC for *Staphylococcus aureus* was found to be 2 µg/mL whereas for *Salmonella typhimurium*, *Escherichia coli* and *Proteus vulgaris* it ranged 1-1.5 µg/mL. For fungi the MIC was higher than bacteria, lowest MIC was found for 1.5 µg/mL whereas for *Aspergillus niger* and *Penicillium* sp. it was 5 and 3.5 µg/mL respectively.

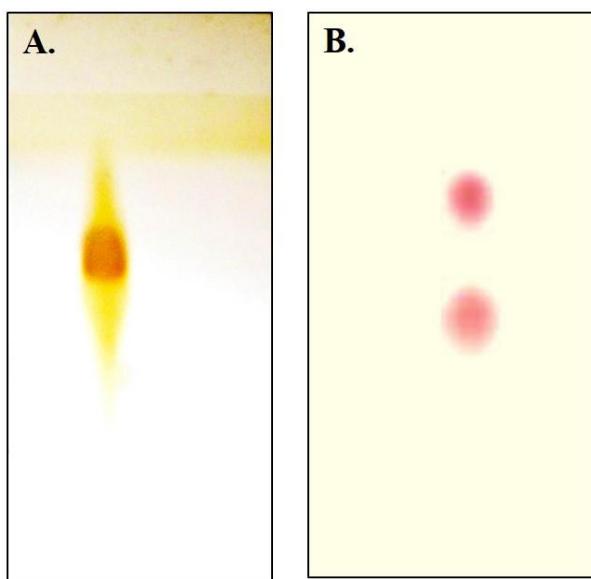


Fig. 66 Compound SBSKC1 on TLC: A. Solvent system- Methanol: Water (1:1) and detection by iodine vapour; B. Solvent system- Butanol: Acetic acid: Water (6:1:2) and detection by ninhydrine reagent

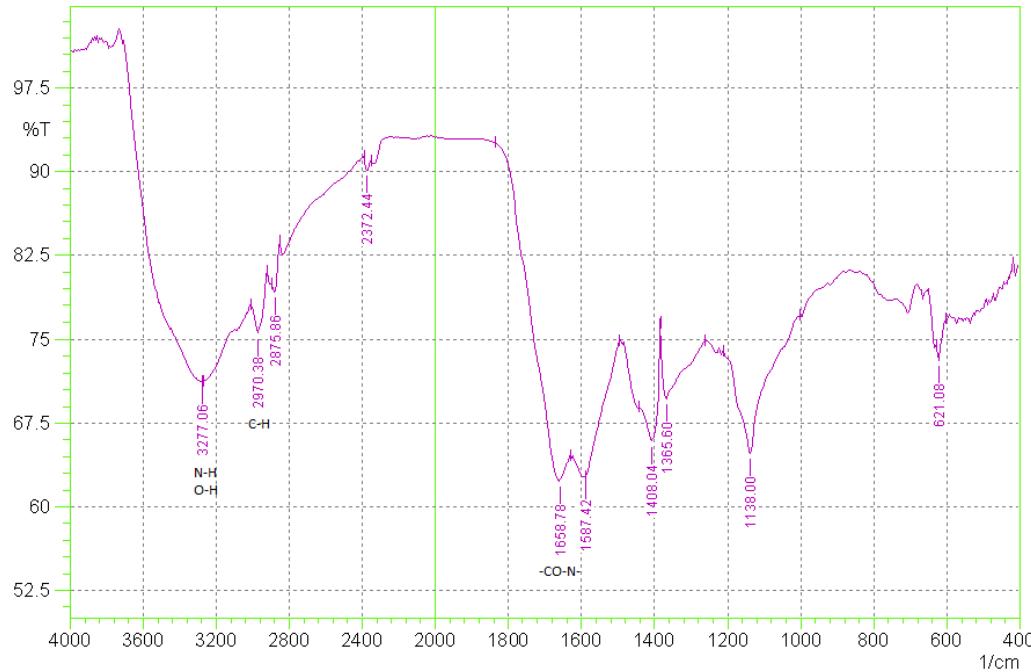


Fig. 67 FTIR profile of compound ‘SBSKC1’ showing the peptide groups

4. 2. 11. LC/MS and Q-ToF MS/MS analysis:

The purified compound from MeOH extract was found to contain 3 components i.e. mixture of peptides, which were indicated by TLC profile using ninhydrine as spraying agent (mobile phase Butanol: Water: Acetic acid). We didn't succeed in purifying these individual components and hence we had used ESI-MS for the characterizations of compounds present in partially purified mixture. In recent years, tandem mass spectrometry (MS/MS) has become a powerful technique for the sequencing of peptides and small proteins. In the present study, the use of the ESI-MS/MS technique to characterize linear peptides present in *Micromonospora marina strain*, has been discussed. The fragmentation pattern for determining the sequence of the peptide is represented in Fig. 68-69. The tandem mass spectrometry technique used in this study is one of the popular, faster characterization techniques and does not require a highly purified material for detection of minor peptides in given mixtures.

4. 2. 12. Structural elucidation of SBSKC1:

ESI-TIC in positive mode showed the presence of two different peptide as major compounds in SBSKC1 fraction suggesting the compound to be a “synergistic mixture of peptides” contributing to its bioactivity (Fig. 68). Two novel peptides viz. ‘Peptide-1’ and ‘Peptide-2’ were characterized in SBSKC1 fraction. The fragment ion peaks are labeled according to the nomenclature proposed by Roepstorff and Fohlman (1984).

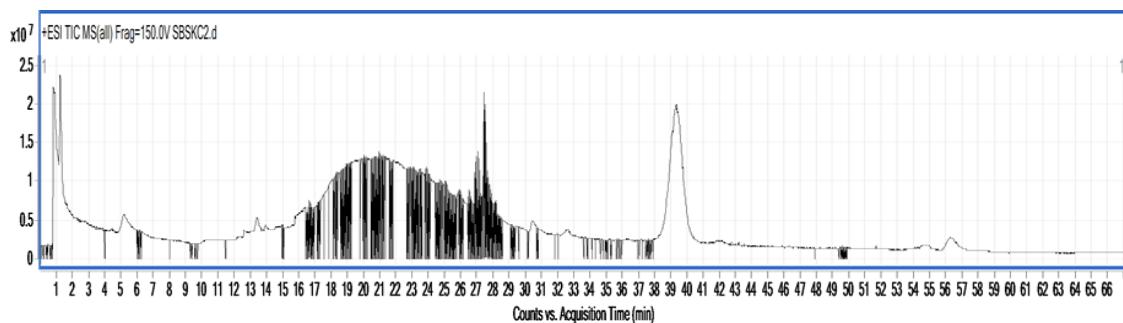


Fig. 68 ESI-TIC profile from the LC-MS/MS of the compound 'SBSKC1'

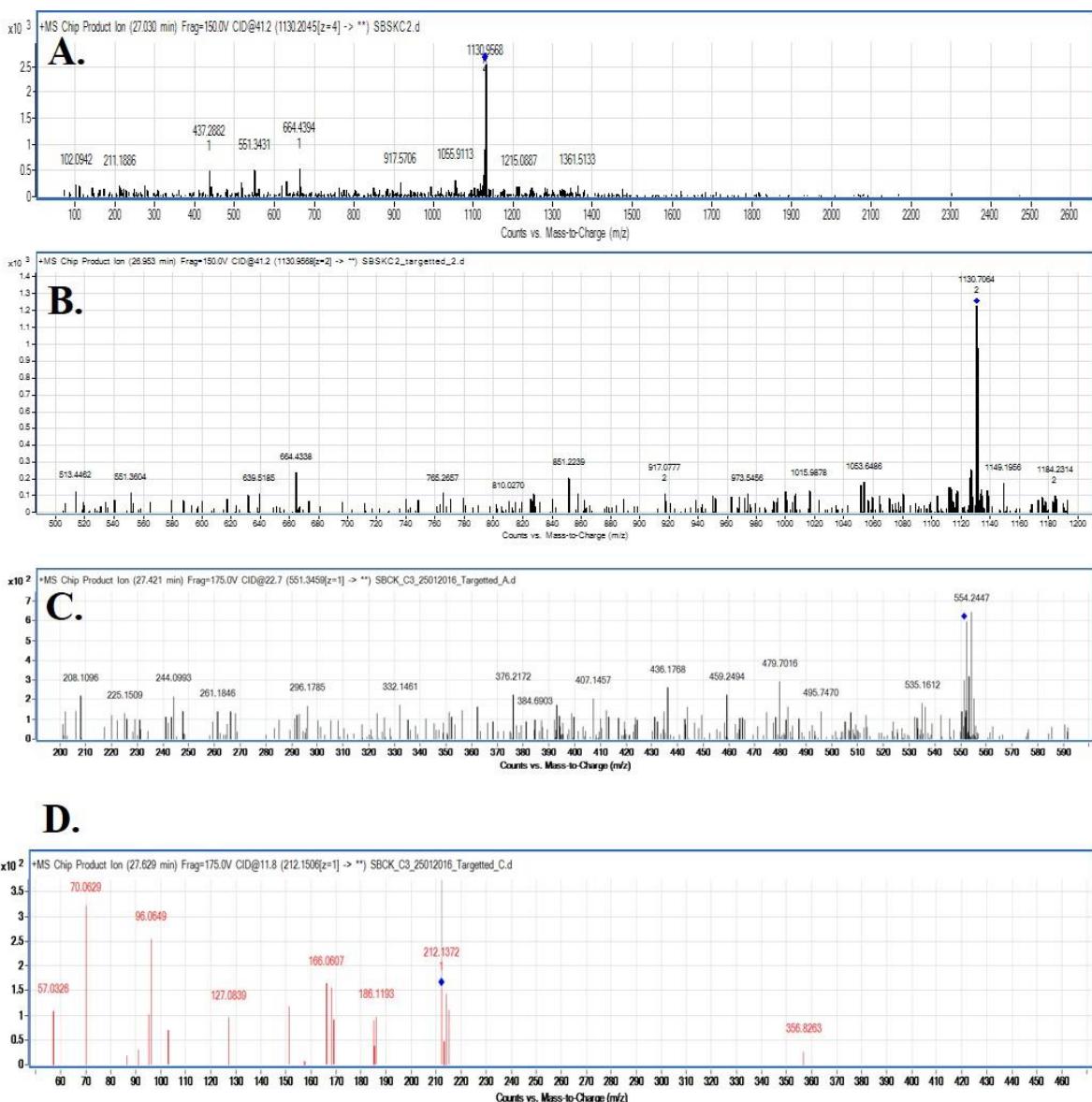


Fig. 69 A. ESI-MS/MS spectra of 'Peptide-1', B. MS/MS at m/z 1130, C. MS/MS at m/z 551 and D. MS/MS at m/z 221

To retrieve a sequence from the mass spectrometric data of the new peptides, we applied the criteria that the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue. ESI-MS of peptide (**1**) yielded m/z 1363 for the $[M+H]^+$ ion (Fig. 70), indicating a molecular formula of $C_{59}H_{96}N_{16}O_{21}$.

ESI-MS data in positive mode for peptide 1

m/z	221	437	551	638	765	852	1015	1130	1216	1362
differences	226	114	87	127	87	163	115	85	146	
aa residue	Leu+Leu	Asn	Ser	Lys	Ser	Tyr	Asp	Ser	Lys+18	

The lowest m/z = 221
 $221 = 114 + 97$
= Asn+Pro as N-terminal

The peptide sequence from N-terminal as follows:
H-Asn-Pro-Leu-Leu-Asn-Ser-Lys-Ser-Tyr-Asp-Ser-Lys-OH

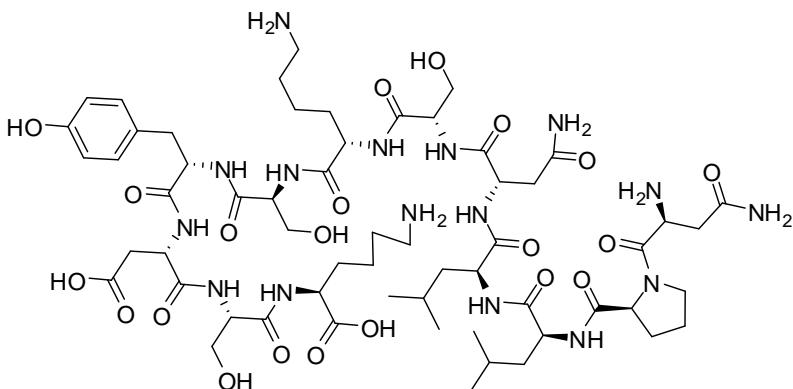


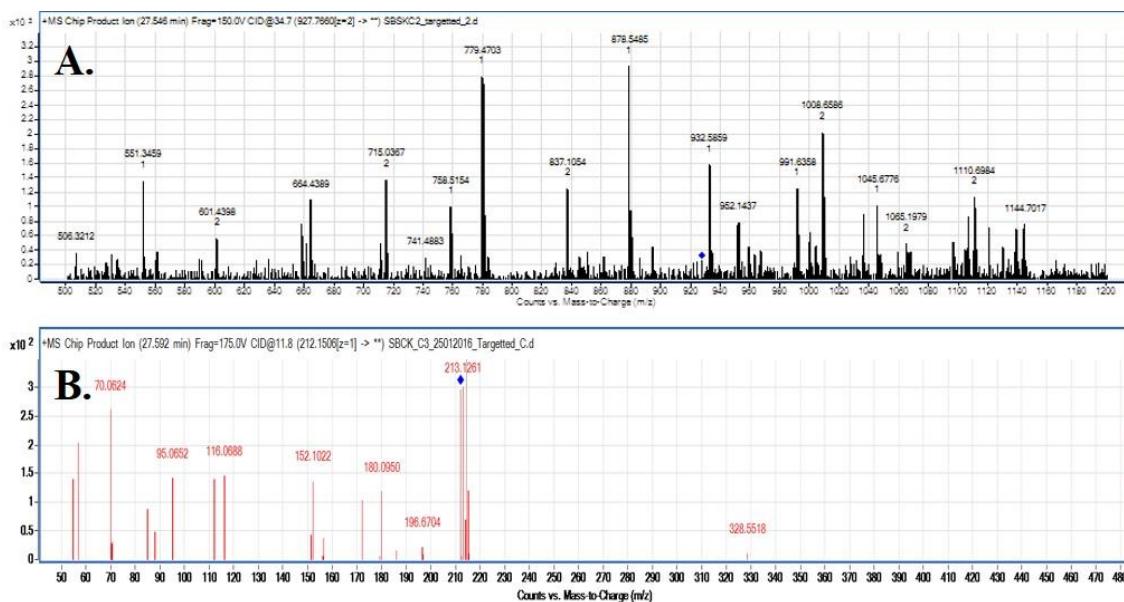
Fig. 70 Structure of ‘peptide-1’ along with ESI-MS data and the conventional order of sequence from *N*-terminal of peptide

The amino acid residues of peptide bonds were attributed based on tandem mass spectroscopy. The conventional order from *N*-terminal of peptide **1** could be H-Asn-Pro-Leu-Leu-Asn-Ser-Lys-Ser-Tyr-Asp-Ser-Lys-OH based sequential loss of amino acid as 221 (Asparagine+Proline), 226 (Leucine+Leucine), 114, 87 127 87, 163, 115, 85, 146 (Lys+18). In this case MS/MS at m/z 221 indicated presence of Asn-Pro fragment. The

characteristic mass peak at m/z 70 due to ammonium ion of proline along with m/z 97 whereas peak at m/z 114 is due to Asparagine. However m/z 226 is attributed to Leu-Leu or Ile-Ile or Leu-Ile fragments. Since this peptide was separated by LC-ESI MS, determination of the configuration of the amino acids by using degradation studies was not done.

ESI-MS of Peptide (**2**) yielded m/z 1066 for the $[M+H]^+$ ion (Fig. 71), indicating a molecular formula of **C₄₅H₆₇N₁₂O₁₆S**. The constituents in the peptidic head group of **2** and their connectivity were initially deduced by tandem mass spectrometry. A sequential loss of 113, 115, 57, 115, 113, 192 and 147 and fragmentation was attributed to an amino acid sequence of peptide **2** as Isoleucine, Aspartate, Glycine, Aspartate, Leucine, Serine+Cystein, and Phenylalanine from the N-terminal (Fig. 72). The lowest mass corresponding to m/z 212 is attributed for Glycine-Histidine-OH (212-18 = 194) attached to carboxylate terminus of peptide and was further confirm by MS/MS experiment at m/z 212.

In the present study, a name was proposed as ‘**Micromonosporopeptide**’, for this broad spectrum anti-microbial compound and ‘synergistic mixture of peptides’ comprising two novel peptides.

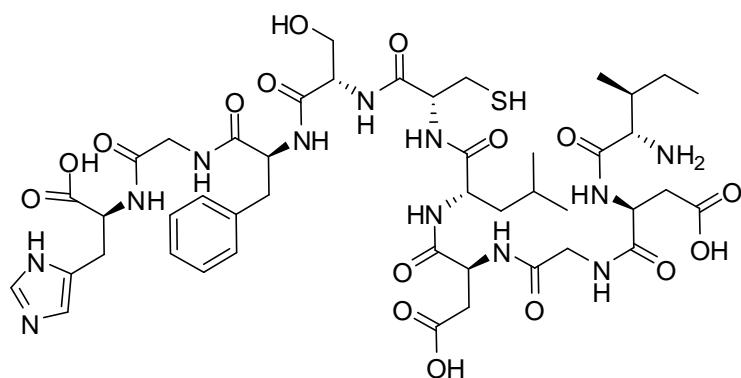
**Fig. 71** A. ESI-MS of 'Peptide-2' and B. MS/MS at m/z 212**ESI-MS data in positive mode**

m/z	212	359	551	664	779	837	952	1065
differences	147	192	113	115	58	115	113	
aa residue	Phe	Cys+Ser	Leu	Asp	Gly	Asp		Ile

The lowest m/z is 212
 $212 - 18 = 194 = 137 + 57$
 Therefore C-terminal is HO-His-Gly-

The peptide sequence from N-terminal is as follows:

H-Ile-Asp-Gly-Asp-Leu-Cys-Ser-Phe-Gly-His-OH

**Fig. 72:** Structure of peptide 2 along with ESI-MS data and the conventional order of sequence from *N*-terminal of peptide

5. DISCUSSION:

5. 1. PART-I: BIODIVERSITY

5. 1. 1. Heterotrophic bacteria and actinomycetes in Ribandar and Agarvado salt pans:

Ribandar and Agarvado salt pans not only varied in their geographical locations but also in various physico-chemical parameters which affects the microbial community structure. The salinity and dissolved oxygen content was found to be lower in Agarvado salt pan (280 psu and 1.8 mg/L) compared to Ribandar (300 psu and 2.5 mg/L) even in salt manufacturing season. The total organic carbon (TOC) content was found to be high too in Ribandar (7.5 to 8.2%), whereas Agarvado sediment had 4.3 to 6%, which in turn explains the reason of higher heterotrophic bacterial counts in Ribandar (1770×10^3 cfu g⁻¹) compared to Agarvado (1250×10^3 cfu g⁻¹). In case of both the salt pans, post-monsoon season showed higher bacterial counts and also higher soil microbial biomass carbon, which is probably due the entry of the terrestrial and fresh water strains into the salt pans with the monsoon run off.

In the comparison of pre-monsoon and post-monsoon seasons the actinomycete counts in the lower salinities are higher during post-monsoon season, which suggests the isolation of halotolerant strains originated from other environments. Pre-monsoon seasons have higher salinities, which restricts the growth of halotolerants and supports the growth of the halophilic strains. The marine environment is dilute in nutrient contents. In our study majority of the halophilic strains are isolated in R2A, starch casein and inorganic salt starch agar media,

which is low in nutrient content compared to the other media, suggesting these organisms to be indigenous population of marine salt pans.

5. 1. 2. Metabolic profiling and molecular characterization of hypersaline actinomycetes:

In the carbohydrate utilization profiles assessed by BIOLOG (for halotolerant isolates) as well as individual substrate supplementation (for halophilic isolates), there was a significant variation among the halophilic and halotolerant isolates. Moderate to extreme halophilic isolates viz. *Nocardiopsis halophila* (SBSK-160), *Nocardiopsis salina* (SBSK-551), *Streptomonospora salina* (SBSK-155), *Streptomonospora halophila* (SBSK-250), *Streptomonospora alba* (SBSK-708) etc. were found to utilize less number of carbon sources compared to halotolerants viz. *Actinomadura algeriensis* (SBSK-225), *Actinomadura catellatispora* (SBSK-560), *Kocuria rhizophila* (SBSK-125), *Kocuria indica* (SBSK-330) etc. This could be explained by exclusive adaptation of the halophilic strains to the environment of salt pans, since the marine environment is nutritionally dilute in nature whereas halotolerant isolates, normally originating from terrestrial environments, is acclimatized to the habitats of nutrient abundance. Tripathi *et al.* (2011) also showed a similar observation in the BIOLOG profiles of the various *Streptomyces* spp. and reported that this could be a reflection of the *in-situ* ecology since some of the substrate usage not only reveals the metabolic activity of the isolates but also the nutrient abundance of the specific isolation environments.

Bioinformatic analysis of the 16S rRNA gene sequences of the isolates from both the salt pans revealed a total 13 different genera viz. *Micromonospora*, *Nocardiopsis*, *Arthrobacter*, *Gordonia*, *Brevibacterium*, *Rhodococcus*, *Kitasatospora*, *Kocuria*, *Streptomyces*, *Actinomadura*, *Streptomonospora*, *Rhodococcus* and *Actinopolyspora* comprising of both halophilic and halotolerant members.

5. 1. 3. Biodiversity of hypersaline actinomycetes:

Actinomycetes reported from marine environments were earlier thought to have a terrestrial origin and gained entry into marine waters. Kerkar (1994) has reported a marine *Streptomyces* from the sediments of Coben's cove island of Andamans, Indian Ocean. Their occurrence from the near-shore and off-shore sediments of the coast of La Jolla, California, USA and salt requirement has been documented [Davé *et al.*, 2008]. The first marine *Kocuria* sp. nov. was reported from the Troitsa Bay of the Gulf of Peter the Great, East Siberian Sea which tolerated upto 15% NaCl concentration [Kim *et al.*, 2004]. Reports shows a frequent distribution of genus *Micromonospora* in the marine environments such as sea sand, coastal sediment etc. [Wu *et al.*, 2009 and Tanasupawat *et al.*, 2010]. Occurrence of actinomycetes in the hypersaline environment have been reported by Wu *et al.* (2009) stating the diversity of actinobacteria in saline sediments of Yunan and Xinjiang province, China comprising of nine suborders including *Streptomycineae* and *Micromonosporineae*, which are also reported from both our salt pans in the present study. *Streptomyces*, *Micromonospora*, *Nocardiopsis* has

been reported from salt pans sediments from the 2 different salt pans situated on the east coast of India [Nakade, 2012 and Jose *et al.*, 2012].

The halophilic isolates viz. SBSK-57 (*Streptomyces* sp.) and SBSK-115 (*Nocardiopsis* sp.) and SBSK-120 (*Kocuria* sp.) did not grow in presence of synthetic NaCl (Hi-Media) however grew when the media was supplemented with natural sea salt. Hamedi *et al.* (2013) have also reported that halophilic actinomycetes of marine ecosystems have a mixed requirement of different salts. SEM-EDS analysis of the natural salt from Ribandar saltern, has shown the presence of trace metals like molybdenum, magnesium, potassium and calcium with sodium as the dominant positive ion and chlorine as the negative ion [Kerkar and Fernandes, 2013]. These ions and nutrients together may probably contribute to the both halophilic and halotolerant actinomycetes growth from 32 to 300 psu (shown in the present study). The decrease in CFU counts with increase in the salinity could be because of the dominance of halotolerant isolates at lower salinity up to 50 psu. However at higher salinity of 75 and 100 psu, slight to moderate halophiles were abundant. The phylogenetic analysis also showed their close association with the type strains and closest relatives of respective genera reported from various marine ecobiomes viz. coastal sediments, marine sponges, deep sea etc. suggesting their marine nature.

5. 1. 4. Ubiquitous distribution of halophilic and halotolerant actinomycetes:

The isolates obtained from both the salt pans represented 58 different species and belonged to 13 different genera including one unidentified species from the genus

Actinopolyspora which could be a novel species. Among 58 species, there were 18 obligate halophilic members remaining 40 being halotolerants. Apart from marine salt pans or hypersaline environments, these isolates are reported from various environments viz. desert soil, deep oceanic sediments, mangroves, rhizosphere of various plants etc. The isolates which were found to be inhabitants of marine environment are: SBSK-170, 180 and 175 which were identified as *Streptomyces marinus*, *Streptomyces haliclonae* and *Streptomyces tateyamensis*, were isolated from the coastal marine sponge *Haliclona* sp. of Tateyama city, Japan [Khan *et al.*, 2010]; *Streptomyces nankiensis* and *Streptomyces oceanii* was previously isolated from the deep sea sediment of South China sea [Tian *et al.*, 2012] and Northern South China Sea [Tian *et al.*, 2012]. *Streptomyces qinglaensis* was previously isolated from mangrove soil of Qinglan Harbour, Wenchang, Hainan, China [Hu *et al.*, 2012], strain SBSK-245 identified as *Actinomadura sediminis* Mangrove sediment of Dugong Creek, Little Andaman, India [He *et al.*, 2012], *Streptomonospora alba* (SBSK-708) Hypersaline sediment of salt lake at Xinjiang province, China, *Streptomonospora salina* (SBSK-155) Salt lake sediment of Xinjiang province, China and *Streptomonospora halophila* (SBSK-250) Hypersaline sediment of Xinjiang province, China [Cui *et al.* (2001), Li *et al.* (2003) and Cai *et al.* (2008)], *Nocardiopsis halophila* (SBSK-160) was isolated from a saline soil sample in Iraq [Al-Tai *et al.*, 1994]. *Nocardiopsis salina* (SBSK-551) was isolated from a soil sample collected from a hypersaline habitat in Xinjiang Province, China [Li *et al.*, 2004] and *Nocardiopsis halotolerans* (SBSK-119) was isolated previously from

salt marsh soil of desert area in Al-Khiran, Kuwait [Al-Zarban *et al.*, 2002]; *Kocuria indica* was previously isolated from Marine environment of Chorao Island, Goa, India [Dastager *et al.*, 2014]; *Brevibacterium album* and *Brevibacterium oceanii* have been isolated from the saline soil of Xinjiang province of north-west China [Tang *et al.*, 2008] and deep oceanic sediment of Chagos Trench in Indian Ocean [Bhadra *et al.*, 2008]; *Streptomyces sparsus* was isolated from saline and alkaline soil sample collected from Qinghai Province, China [Jiang *et al.*, 2011]. *Streptomyces klenkii* was isolated from the coastal sediment of southern Black Sea coast, Turkey [Veyisoglu and Sahin, 2015]. Isolation of all these strains from the marine salt pans suggest that these eco-niche works as reservoir of diverse marine actinomycetes.

During monsoon seasons a large number of terrestrial and fresh water microbial strains gain entry into the salt pans with the monsoon water run-off. We have recovered a significant number of these ‘migrated strains’ in our study. Few of them are as follows: *Kocuria palustris* and *Kocuria rhizophila* was previously isolated from rhizoplane of narrow-leaved cattail (*Lypha angustifolia*) Hungary [Kovács *et al.*, 1999]. *Kocuria marina* was isolated from the marine sediment of Troitsa Bay of East Siberian Sea [Kim *et al.*, 2004]; *Kocuria salsicia* was previously isolated from the Salt fermented seafood in Korea [Yun *et al.*, 2011]; SBSK-520 as *Actinomadura namibiensis* soil from Namib Desert, Namibia [Wink *et al.*, 2003], SBSK-560 as *Actinomadura catellatispora* Seawage, Zhanjiang City, Canton Province, southern China [Lu *et al.*, 2003] and SBSK-701 as

Actinomadura chokoriensis Sandy soil of Chokoria, Cox's Bazar, Bangladesh [Ara *et al.*, 2008].

5. 1. 5. Description of extremely halophilic Strain SBSKN₅ (*Actinopolyspora* sp.):

The genus *Actinopolyspora*, was first proposed by Gochnauer *et al.* (1975) belonging to the suborder *Acintoplysporineae* (Zhi *et al.*, 2009) as obligate and extremely halophilic group of actinomycetes [Gochnauer *et al.* (1975) and Zhi *et al.* (2009)]. First validly described halophilic actinomycete species *Actinopolyspora halophila* belonged to this genera. In the present study, SBSKN₅ an extremely halophilic isolate which was isolated on R2A agar at 250 psu salinity from Ribandar salt pan during pre-monsoon season belonged to this genera. This filamentous actinomycete didn't resemble any of the conventionally reported actinomycete in its microscopic features. The isolate was found even to grow at salt saturation (300 psu) attached to the salt crystals. SBSKN₅ didn't show any positive result in BIOLOG microplate assay for carbohydrate utilization but grew well when the basal salt solution of ISP-4 medium was supplemented with carbon sources (one source at a time) at 250 psu salt concentration. This could be explained with the isolate being 'extreme border line halophile' and has an obligate requirement of 250 psu salt concentration for growth. The isolate didn't grow in other 6 ISP media, suggesting it to belong to 'rare actinomycete' genera. SBSKN₅ also didn't grow in presence of only synthetic NaCl (25%) and had a requirement of 'mixed salt concentration' (24% NaCl + 1% KCl + 0.5% MgCl₂)

in optimized broth media (1/2X nutrient broth and inorganic salt starch broth), indicating it to be exclusive inhabitant of salt pan.

Molecular characterization by 16S rRNA gene sequencing and phylogenetic analysis showed strain SBSKN₅ showed maximum similarity (97%) with *Actinopolyspora algeriensis* strain H19^T but their colony characters, microscopic morphology and other physiological characters were found to be completely different. Strain SBSKN⁵ grows as light pink coloured sporulating colonies with off-white substrate mycelia where as *Actinopolyspora algeriensis* strain H19^T was found to produce yellowish white colour colonies. *Actinopolyspora algeriensis* strain H19^T was found to produce substrate mycelia which fragments into non-motile rods and aerial mycelia forms flexious spore chains [Meklat *et al.*, 2012] whereas strain SBSKN⁵ produces branched substrate mycelia and sporangiophores are formed from which rounds spores originate either singly or as multiples as described earlier. *Actinopolyspora algeriensis* strain H19^T grows optimally on ISP-2 medium and in a salinity range of 70-300 psu. Strain SBSKN₅ didn't show any growth in any of the 7 ISP media tested but showed optimum growth in R2A and 1/2X nutrient broth with 250 – 300 psu salt concentration. The other three strains which were similar in 16S rRNA gene sequencing are *Actinopolyspora alba*, *Actinopolyspora erythrea*, *Actinoployspora xinjiangensis* but they grow at 10-15% salt concentration [Guan *et al.* (2010) and Tang *et al.* (2011)] and morphologically varying from isolate SBSKN₅. Only similarity with these strains and SBSKN₅ were all four grew well on Inorganic salt starch agar (ISP-4) and their optimal growth temperature was 37°C. Carbohydrate utilization

pattern of SBSKN₅ also varied significantly with these strains. Hence, it is suggested that strain SBSKN₅ could be a ‘novel species’ under the genera *Actinopolyspora*.

5. 1. 6 Seasonal variation and assessment of biodiversity in both the salt pans:

The distribution of actinomycetes species not only varied in both sampling sites (Ribandar and Agarvado) but there was significant difference in the number of various species during pre and post-monsoon seasons in the same salt pan. The actinomycete species which exist in the pre-monsoon are comparable to the genera present in the post monsoon where the same genus and species show an overlap in both the seasons. However, in the post monsoon the statistical analysis of the diversity indices viz. Shannon index (H)', Simpson's diversity index and 'SHE' show the biodiversity to be much higher in the post monsoon as compared to the pre-monsoon season. This is ascertained from the data of the post monsoon season where the number of species are higher in numbers in the post monsoon season. We have sampled the Ribandar salt pan which is in close vicinity to the mangrove ecosystem and these species may have originated from the mangrove ecosystem since they have been reported to be indigenous to mangrove marshes. On the other hand typical soil actinomycetes *viz.* *Kocuria palustris*, *Streptomyces specialis*, *Micromonospora aurantiaca*, may have been transported from the rhizospheric soil which becomes turbulent and loose during the monsoon season, resulting in the desiccation of the rhizospheric bacteria into the overlying water, which gain entry into the salt pan ecosystem. This can be supported by the

organic carbon measurements which are high during the post-monsoon season. This carbon influx may be a resultant of the entry of the flooding water from the estuaries into the salt pans, when the bunds (which normally act as barriers) are kept open during the heavy rain fall.

Ribandar is in the influence of the anthropogenic factor specially the estuarine water is influenced by an influx of municipal treated water and fringed by multiple restaurants along the estuary and casino boats anchored within the estuary. These factors contribute to the increased organic carbon concentration in Ribandar (7.5 to 8.2%) as compared to Agarvado (4.2 to 6%) which gradually settles in to the salt pan sediment. Thus Ribandar salt pan has higher number of microbial load and less diverse in its actinomycetes population. The available labile organic carbon might be promoting the growth of ‘fast growing’ species which overshadows the slow growing indigenous actinomycete population.

The present study represent a culture dependent approach and comprehensive assessment of the biodiversity of the hypersaline actinomycetes in two different salt pans of Goa. Phenotypic, metabolic and molecular characterization revealed the community structure of actinomycetes in these salt pans and also the variation in the species compositions during the pre and post-monsoon seasons. This study is the first of its kind carried out to explore the biodiversity of these ‘unique actinomycetes’ in the salt pans of Goa, being a coastal thalassohaline ecosystem with salt dependent (NaCl) and variant microbes.

5. 1. PART-II: BIOPROSPECTING

5. 2. 1. Anti-microbial compounds from hypersaline actinomycetes:

In the discovery of novel bioactive molecules, especially antimicrobial compounds, natural products remain as the most promising source since combinatorial chemistry and fragment based drug designing contribute very little to counter and reverse the spread of antibiotic resistant pathogens [Goodfellow and Fiedler, 2010]. Bull and Stach (2007) explained that with the urgent need of antibiotics, indigenous marine actinomycetes are the most promising source with their unrivalled capacity of producing diverse metabolites as exploitable natural products. Among the diverse extremophilic niches, marine salterns are unique microbial habitats, with varying salinity (30 – 300 psu) which harbour a diverse range of hypersaline microbes [Kerkar and Lokabharathi, 2007]. Actinomycetes are predominant members of marine microbial communities and are also known to adapt to harsh environmental conditions. In the present study, we isolated 380 halotolerant and 120 halophilic actinomycetes and explored their anti-microbial potential against 15 pathogenic bacteria and 7 phyto & human pathogenic fungi. Our present study shows the potential of halophilic and halotolerant actinomycetes communities to produce anti-bacterial and anti-fungal metabolites. We report 55 *Streptomyces* spp. and 77 rare actinomycetes of 10 different genera viz. *Micromonospora*, *Nocardiopsis*, *Kocuria*, *Actinomadura*, *Brevibacterium*, *Gordonia*, *Kitasatospora*, *Micrococcus*, *Rhodococcus* and *Streptomonospora* producing anti-microbial compound(s) against human and phyto pathogens. This study is the first of its kind to show such a broad range of hypersaline

actinomycete genera producing anti-microbial metabolites. These results suggest that synthesis of anti-microbial compounds are not restricted to a unique species or genera. The genus *Streptomyces*, being the major producer of anti-bacterial metabolites and are the most frequently found species in saline soils. Production of antimicrobials from saline environments has been earlier reported. Reports from salterns from India where a *Streptomyces* sp. from saline soils of Ennore salt pan, Chennai, shows activity against dermatophytes [Lakshmi pathy and Kannabiran, 2009] and Dhanasekaran *et al.* (2005) reported a *Saccharomonospora* sp. and two *Streptomyces* spp. producing anti-bacterial metabolites. Very recently, Jose and Jevakumar (2015) have shown a rare actinomycete *Actinoalloteicus* sp. from an inland solar saltern adjacent to Sambhar lake, Jaipur Rajasthan to produce anti-microbial metabolites against human pathogenic bacteria and fungi.

5. 2. 2. Salinity affecting the production of anti-microbial compounds:

Salt concentration is known to have a significant effect on the production of secondary metabolites as reported by Magarvey *et al.* (2004). It is expected that this metabolic shuffling is due to the adaptation to this environment hence in saline habitats, it could be possible to find novel bioactive metabolites as anti-microbial compounds even from the known indigenous species. To support this Okami *et al.* (1976), has referred to a *Streptomyces griseus* (known to produce streptomycin) isolated from the marine sediment which didn't show production of streptomycin in normal media. The same strain produced a novel ionophoric

polyether when grown in a medium containing high salt and a low concentration of organic nutrients.

In our study no antibiotic production was recorded below 20 psu in the halophilic spp. whereas the antibiotic production by the halotolerant and halophilic actinomycetes was maximum at 35 psu and above (Fig. 3 and Fig. 4). Salinity of 35 psu was found to be the optimum salinity for growth for the halotolerant isolates and but the minimum salinity required for growth and antibiotic production for halophilic spp. These halophilic isolates are slight to moderate halophiles. The production of antibacterial compounds increased in presence of sea water, a common trend with all the halotolerant isolates. This suggests that the sea salt from the salterns could be a stimulating factor for the production of anti-bacterial metabolites. The production of anti-microbial compounds by the halotolerant isolates at 35 psu (the salinity of sea water), could also be a stringent requirement for the survival of these species in this microbial niche. We report a halotolerant *Micromonospora* sp. strain SBSK- 356 producing an antibiotic against *Vibrio cholerae* in presence of sea water. The presence of *Nocardiopsis* species from hypersaline environments such as saline soils and salt marsh soils have been reported. This is the first report of a halophilic *Nocardiposis* sp. strain SBSK-115) from a hypersaline environment producing an antimicrobial compound against *Staphylococcus citreus*. The genus *Kocuria* is present in the marine environment. Reports on its antibacterial production is limited, except for a report on *Kocuria* sp. from sponges producing antibacterial compound against methicillin resistant *Staphylococcus aureus*, identified to be ‘Kocurin’.

Halophilic *Kocuria* sp. from salterns, inhibiting *Staphylococcus aureus* is reported for the first time, suggesting this genera as a resource for bioactive compounds. Several *Streptomyces* spp. both halophilic (SBSK-57) and halotolerant (SBSK-3, SBSK-8 and SBSK-150) from the salterns, produced anti-bacterial metabolite(s) against various Gram-negative bacteria viz. *Escherichia coli*, *Proteus vulgaris* and *Salmonella typhimurium*. The phylogenetic analysis, shows that the halotolerant *Streptomyces* strains are taxonomically related to their terrestrial counterparts. Hence, this is suggestive that the saline environment triggers anti-microbial compound production. Imada et al., (2010) has also shown that NaCl strains growing at 5% and 12% belonging to the terrestrial actinomycetes being from the genera *Micromonospora* and *Streptomyces* respectively, produced anti-microbial compounds only in presence of sea water in the media. The anti-microbial compound inhibited *Bacillus subtilis* and *Candida albicans* [48].

In the present study the final candidate strain SBSK-364 was found to be *Micromonospora marina* which showed 100% similarity with previously reported strains JSM1-1 and JSM1-3 which produced a broad spectrum anti-bacterial and anti-fungal metabolite(s) at 35 psu (sea water salinity) in R2A broth medium generally referred to be a minimal media. This observation supports the previous report by Okami *et al.*, (1976) as described above.

5. 2. 3. Anti-microbial compounds from marine *Micromonospora* species:

From the past few decades, secondary metabolites from the genus *Micromonospora* have been of considerable interest with regards to the bioprospecting for novel antibiotics, anti-cancer compounds, enzyme inhibitors

etc. The first report of an anti-bacterial metabolite from the genus *Micromonospora* was published by Waksman *et al.* (1946) against Gram positive bacteria viz. *Bacillus subtilis*, *Sarcina lutea*, *Staphylococcus aureus* and *Bacillus mycoides*. The compound was termed as “Micromonosporin” and it was found to be an extracellular peptide, attached to a carbohydrate moiety. Various reports describe the genus *Micromonospora* as a prolific producer of novel macrolide, aminoglycoside, enediyne and oligosaccharide antibiotics against various Gram positive and Gram negative bacterial pathogens. In a recent review, citing the diversity of rare actinomycetes and their contribution for marine natural product discovery, Subramani and Aalbersberg (2013) highlighted members of the family *Micromonoporaceae* as producer of major chemically diverse compounds. Marine strains of the genus *Micromonospora* are of major focus of today’s research since they are rarely explored compared to *Streptomyces* and also a prominent source of novel secondary metabolites. Charan *et al.* (2004) for the first time showed a marine *Micromonospora* sp. as a producer of novel anti-microbial alkaloid ‘Diazepinomicin’. In a very recent study, Carlson *et al.* (2013) have reported a macrolide antibiotic from a marine *Micromonospora* sp. with potential chemo-preventive activity as an inducer of phase II enzymes and exhibited anti-cancer activity against Hepa 1c1c7 cells. Imada *et al.* (2010) have reported *Micromonospora* strains from open ocean,bays and the deep sea to be tolerant to NaCl concentrations up to 5% and in presence of sea water they produced anti-microbial compounds.

The candidate strain SBSK-364 in the present study was found to be similar to *Micromonospora marina* confirmed by microscopic, biochemical and molecular characterization. *Micromonospora marina* was first discovered by Tanasupawat *et al.* (2010) from the sea sand collected from the Hua-Hin sea shore of Thailand and till date there are no reports on the production of anti-microbial compounds by the strain. The anti-microbial metabolite of the strain SBSK-364 which was designated as ‘SBSKC1’ was found to be a broad spectrum anti-microbial compound against wide range of human and phyto pathogens. ‘SBSKC1’ didn’t get extracted in any solvents which was employed in sequential solvent extraction (petroleum ether> hexane> diethyl ether> chloroform> ethyl acetate> butanol) hence it was purified using HP20-Dia ion resin followed by methanol extraction from the aqueous phase of the solvent extracted culture supernatant. SBSKC1 was found to be soluble only in water and methanol. This explains the failure of solvent extraction for this particular compound. MIC of SBSKC1 for the bacterial pathogens ranged from 1-2 $\mu\text{g/mL}$ and for fungi 1.5-5 $\mu\text{g/mL}$. Chemical characterization by TLC & FTIR revealed it to be a ‘peptide’. Liquid chromatography/Mass spectrometry-Electron Spray Ionization (LC/MS-ESI) analysis and bioactivity profiling, showed the compound to be a ‘synergistic mixture of peptides’, each with distinct structures. Till date, this is the first report of such synergistic mixture of peptides with broad spectrum anti-microbial activity from a hypersaline *Micromonospora marina* strain. ESI-QTOF tandem mass spectrometry characterization suggested these two new peptides with m/z 1362 (Peptide-1) and m/z 1066 (Peptide-2) as $[\text{M}+\text{H}]^+$. The amino acid sequences

of both the peptides were proposed using tandem experiments and it was observed that there is a sequential loss of amino acids indicating the presence of a linear peptide rather than a cyclic peptide. Q-ToF MS/MS (targeted) further ascertained the structures of the peptides viz. Peptide-1 with amino acid sequence (from N-terminal) as H-Asn-Pro-Leu-Leu-Asn-Ser-Lys-Ser-Tyr-Asp-Ser-Lys-OH and Peptide-2 as H-Ile-Asp-Gly-Asp-Leu-Cys-Ser-Phe-Gly-His-OH, which suggests them to be ‘novel anti-microbial peptides’ with a scope of being established as a ‘marine drug’ with remarkable applications. We proposed the new antibiotic to be **‘Micromonosporopeptide’**.

The present study on the exploration of anti-microbial compounds of hypersaline actinomycetes from the marine salt pans of Goa, India, litanies various anti-microbial compound producing genera among the *Streptomyces* as well as rare actinomycetes. Various strains also prevalent in terrestrial ecosystems didn’t produce anti-microbial metabolites unless cultivated in a saline environment. Hence, marine salt pans not only act as a unique extreme environment harboring diverse hypersaline microbes, but is a ‘metabolic reservoir’ of unexplored compounds. The observations and results shows the immense potential for the discovery of novel anti-microbial compounds from the hypersaline strains of rare genera of actinomycetes, to counter act the probable evolving drug resistant pathogens in the years to come.

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SUMMARY:

- The present study explores the diversity of culturable hypersaline actinomycetes in two marine salt pans of Goa viz. Ribandar and Agarvado; one being anthropogenically affected whereas the other one relatively pristine and also located in geographically distant region.
- Sample collections were done for a period of three years (2011, 2012 and 2013) during pre-monsoon (April-May) and post-monsoon (November-December) seasons from the salt crystallizer ponds.
- Analysis of the physico-chemical and microbiological parameters revealed that the sediments of Ribandar contain more organic content (TOC), soil biomass carbon (SMBC) and has higher number of heterotrophic bacterial counts as compared to Agarvado salt pan.
- A total of 500 isolates, halophilic (120) and halotolerant (380), morphologically variant actinomycetes were isolated from sediment samples of Ribandar and Agarvado saltpans on 13 different isolation media with 8 different gradients of salinity (32 to 300 psu).
- Among the 13 different media employed, R2A agar was found to be the most suitable medium for the isolation of hypersaline actinomycetes followed by Starch-casein agar, Inorganic salt-starch agar and Yeast-malt extract agar.

- The number of halotolerant actinomycetes were higher in lower salinities (32-75 psu) whereas with increase in salinity (100-300 psu), the numbers decreased. Halophilic actinomycetes were isolated at 100-300 psu.
- Actinomycetes were grouped by classical taxonomy, metabolic and molecular characterization into 13 different clusters belonging to 13 different genera and one as unidentified species of genus *Actinopolyspora*.
- Actinomycete population from Ribandar salt pan was found to belong to 9 different genera viz. *Micromonospora*, *Nocardiopsis*, *Rhodococcus*, *Kitasatospora*, *Kocuria*, *Streptomyces*, *Actinomadura*, *Streptomonospora*, *Micrococcus* whereas Agarvado salt pan was found to harbor three genera viz. *Arthrobacter*, *Brevibacterium* and *Gordonia*.
- Irrespective of the salt pans, members of genera *Nocardiopsis*, *Streptomonospora* was found to be exclusively halophilic whereas genera *Streptomyces*, *Kocuria*, *Kitasatospora* and *Micromonospora* comprised of halotolerant members.
- Extreme borderline halophilic isolate SBSKN₅ (from unidentified cluster) of Ribandar salt pan which grew solely in presence of a minimum concentration of 250 psu NaCl, was found to be phenotypically and metabolically different from the other members of genus *Actinopolyspora*. Molecular characterization by 16S rRNA gene sequencing indicated lower similarity ($\leq 97\%$) with the reported type strains, hence holds promise for being a novel species under the genus *Actinopolyspora*.

- Statistical analysis of species distribution and diversity by Simpson and Shannon diversity index revealed that in spite of higher actinomycete counts in Ribandar salt pan, the community structure of Agarvado salt pan was more diverse.
- Compared to the pre-monsoon, in post-monsoon more number of halotolerant species variants were predominant, suggesting the influx of the terrestrial strains in the salt pans with the monsoon run off.
- Actinomycetes (26%) were found to be positive for the production of antagonistic metabolites against the tested pathogens; among the positive isolates 42% belonged to *Streptomyces* and remaining 58% belonged to 10 different rare actinomycete genera.
- Sea water salinity (35 psu) was found to be a stimulant for the production of anti-microbial compounds in halotolerant strains.
- We report a halophilic *Kocuria* sp. (SBSK-120), a *Nocardiopsis* sp. (SBSK-115) and a halotolerant *Micromonospora* sp. (SBSK-356) producing anti-bacterial compound(s) against *Staphylococcus aureus*, *Staphylococcus citreus* and *Vibrio cholerae* respectively, which is a first of its kind till date.
- Compound ‘SBSKC1’ extracted from cell free metabolite preparation of *Micromonospora marina* strain SBSK-364 of Agarvado salt pan, showed broad spectrum anti-microbial activity against both 9 pathogenic bacteria and 5 different fungi.
- Purification and characterization through TLC, LC/MS and Q-ToF ESI MS/MS data proved the compound ‘SBSKC1’ to be a ‘synergistic mixture of two peptides’ with

temperature stability up to 60°C, pH stability 5 to 10 with MIC ranging from 1-2 µg/mL for bacteria and for fungi 1.5-5 µg/mL.

- Based on detailed structural research, it was found that these anti-microbial peptides have not been reported earlier. The producer organism *Micromonospora marina* have been discovered very recently (2010) and this is the first report of the anti-microbial peptides from the same organism.
- Our study reveals for the first time an antibiotic produced by a hypersaline strain of *Micromonospora marina* which is a ‘synergistic mixture of peptides’.
- We propose the name of this antibiotic to be ‘Micromonosporopeptide’ with two peptides; Peptide-1 with amino acid sequence (from N-terminal) as H-Asn-Pro-Leu-Leu-Asn-Ser-Lys-Ser-Tyr-Asp-Ser-Lys-OH and Peptide-2 as H-Ile-Asp-Gly-Asp-Leu-Cys-Ser-Phe-Gly-His-OH.

CONCLUSION

Marine salt pans of Ribandar and Agarvado (Goa) harbor a diverse community of novel and rare hypersaline actinomycete species which are affected with seasonal variations, organic matter content as well as anthropogenic activity. Strain SBSKN₅ an extremely halophilic isolate from Ribandar salt pan, being a phenotypic and metabolic variant could be a ‘novel species’ under the genus *Actinopolyspora*. Hypersaline actinomycetes are a prolific resource of bioactive secondary metabolites with an immense potential to be developed as anti-microbial compounds where sea water acts as a stimulant for the production of anti-microbial compounds. *Micromonospora marina* strain SBSK-364 a halotolerant isolate from Agarvado produces a stable and broad spectrum peptide antibiotic, ‘Micromonosporopeptide’ which is being reported for the first time which could have promising applications in Biotechnology as a new “marine drug”.

Manuscripts published:

1. **Ballav S**, Kerkar S, Thomas S, Augustine N (2015) **Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites.** *J Biosci Bioeng.* 119(3): 323-330. (International, peer reviewed, Impact Factor: 1.9)
2. **Ballav S**, Dastagar SG, Kerkar S (2012) **Biotechnological significance of Actinobacterial research in India.** *Recent Res Sci Technol,* 4(4):31-39. (International, peer reviewed)
3. Viveka S, Shama P, Nagaraja GK, **Ballav S**, Kerkar S (2015) **Design, synthesis of some new pyrazolyl-pyrazolines as potential anti-inflammatory, analgesic and antibacterial agents” (2015)** *European J Med Chem.* 101:442-451. (International, peer reviewed, Impact Factor: 3.2)

Manuscripts in pipeline:

4. **Ballav S**, Kerkar S, Damare S, Majik MM, Shouche Y (2016) **Novel antimicrobial peptides from a hypersaline strain of *Micromonospora marina* from a marine saltern of Agarvado, Goa, India.** (Under review)
5. Fernandes MS, **Ballav S**, Kerkar S, Kamat N (2016) **Mushroom tyrosinase inhibitor from a saltpan *Kitasatospora* sp. in preventing the enzymatic browning of *Agaricus bisporus*: A bench-top trial.** (Under review)

6. Kerkar S, **Ballav S**, Kappagunta C, Verma R and Kadam H (2016) **Characterization of anti-microbial metabolites of a hypersaline *Bacillus* sp. strain BGUMS27 against methicillin resistant *Staphylococcus aureus* (MRSA) and infectious fungi.**
7. **Shuvankar Ballav**, Namrata Singh, Savita Kerkar (2016) **Bioremediation of lead (Pb) and iron (Fe) by hypersaline actinomycetes of a marine saltern of Ribandar, Goa, India.**

Paper in International Conferences proceeding:

- “**Phylogenetic diversity of hypersaline actinomycetes in the Ribandar & Agarvado saltern, Goa, India and characterization of their antimicrobial metabolites**” (2016) Shuvankar Ballav and Savita Kerkar, Proceedings of International Seminar on “New Frontiers in Microbiology and Applied Biology” organized by Department of Microbiology, St. Xavier’s College, Mapusa, Goa on 7th – 8th January, 2016.

Abstracts published in various International and National Conferences:

- I. “**Hypersaline sediment actinomycetes as potential bioremediators of Lead (Pb) and Iron (Fe)**” by **Shuvankar Ballav**, Namrata Singh and Savita Kerkar at the **55th Annual Conference of Association of Microbiologists in India (AMI), “National Conference on Empowering Mankind with Microbial Technologies” (AMI-EMMT-2014)**, organized by AMI Coimbatore Unit & Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore – 641003, India held on 12th – 14th November, 2014 (**First prize as Best Poster Award**).

- II. “Marine salterns of Goa: A hypersaline niche for diverse actinobacteria with anti-microbial potential” by **Shuvankar Ballav** and Savita Kerkar at **53rd Annual AMI International Conference** on “*Microbial World: Recent Innovation & future trends*” organized by KIIT University, Bhuvaneshwar, Odisha, India held on 22 – 25th November, 2012.
- III. “Salt pan microbes: Potential factories of diverse metabolites” by **Shuvankar Ballav**, Tonima Kamat, Flory Pereira, Savita Kerkar at **52nd Annual Conference of Association of Microbiologists of India (AMI)**, “*International Conference on Microbial Biotechnology for Sustainable Development*” organized by Panjab University, Chandigarh, India held on 3rd – 6th November, 2011.
- IV. “Biodiversity of hypersaline actinomycetes from Agarvado & Ribandar saltern, Goa as producers of anti-microbial metabolites” by **Shuvankar Ballav** and Savita Kerkar at “*National Seminar on Life and Life processes: sustainable development*” organized by Dept. of Zoology, Goa University held on 19th – 21st February, 2015.
- V. “Hypersaline actinomycetes: an unique microbial community as producers of a plethora of anti-microbial compounds from marine salterns” by **Shuvankar Ballav** and Savita Kerkar at **National Symposium on “Biotechnology in India: a Panoramic View”** organized by Department of Biotechnology, Goa University, India held on 25th – 26th February, 2014.